



Development of an Intelligent Framework for Early Diagnosis of Cardiovascular Disease and Diabetes Mellitus Using Handheld Spectroscopy Techniques and Fingernails

Megan Wilson



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Abstract

Aim: Cardiovascular diseases and diabetes mellitus represent leading contributors to morbidity and mortality, imposing substantial strain on healthcare systems across the globe. These burdens are particularly detrimental in low- and middle-income countries, where access to advanced diagnostic technologies and training is limited. Timely detection of CVDs and DM is imperative for effective clinical intervention and disease prevention. However, conventional diagnostic techniques are often invasive, resource intensive and dependent on user knowledge. Therefore, this research aimed to develop and evaluate an intelligent, non-invasive framework using fingernails as an alternative biological matrix, combined with vibrational spectroscopic techniques and machine learning for the detection of cardiovascular diseases and/or diabetes mellitus.

Methods: Two quantitative studies, including a systematic review and structured questionnaire were conducted. The first examined current trends in diagnostic practices, such as diagnostic technique and biomarkers. The second created an understanding of disease-related factors and contextualised spectral data. The detection of disease was assessed through the physical and biochemical analysis of fingernails and was conducted using a variety of vibrational spectroscopic techniques including infrared, near-infrared and Raman spectroscopy. Moreover, the impact of disease in terms of elemental composition and tissue damage to the fingernail plate were evaluated through scanning transmission electron microscopy and energy dispersive X-ray spectrometry, respectively.

Data analysis: Quantitative data obtained from the systemic review of literature and participants' data from questionnaires were analysed using Microsoft Excel version 2019 and SPSS version 29.0.2.0, where descriptive statistics and logistic regression were applied. Machine learning algorithms including correlation in wavenumber space, principal component analysis and self-organising maps, as well as classification models, were applied to explore patterns in the spectroscopic data using MATLAB version R2024a.

Findings: A systematic review of the existing literature revealed frequent use of advanced analytical techniques and biomarkers for the detection of cardiovascular diseases or diabetes mellitus. Evidence from the systematic review, supported the notion of exploring alternative techniques and biological matrices for rapid and cost-

effective analysis in low- and middle-income countries. The results of the questionnaires, paired with logistic regression, demonstrated the relationship between the manifestation of cardiovascular diseases and diabetes mellitus with patient- (age, biological sex and smoking habits) and clinical-related (comorbidities) risk factors. The chosen vibrational spectroscopic techniques highlighted the shared biochemical fingerprint of fingernails in the absence or presence of disease. However, the spectral activity of endogenous constituents (amino acids, lipids and proteins) varied dependent on factors such as age, biological sex, ethnicity and disease presence. Moreover, the spectroscopic techniques allowed for identification of biomarkers including cytochrome C and glucose. Disease manifestation was also visualised in the form of a rough topographic fingernail plate and indicated the presence of tissue damage attributed to poor circulation and/or hyperglycaemia.

Conclusions: This work established a proof-of-concept intelligent framework capable of identifying disease-related biochemical and structural patterns. By utilising a non-invasive sampling method, which requires minimal sample preparation and storage, this approach has the potential to significantly reduce per-test diagnostic costs and decrease reliance on conventional laboratory-based blood analysis. The integration of machine learning further enables automated data processing and classification, thus reducing clinician workload and shortening turnaround times. Factors that are beneficial for large scale and over-subscribed institutes such as the National Health Service. Moreover, traditional diagnostic pathways are often dependent on costly laboratory infrastructure and highly trained personnel, both of which are limited or inaccessible in low-resource settings. In contrast, vibrational spectroscopic techniques offer rapid and reagent-free analysis, which can be implemented using handheld devices. However, due to an imbalance of data, with limited disease representation, misclassification of machine learning and classification models were observed. Therefore, future work will focus on expanding and diversifying the datasets with larger-scale studies to encompass a greater number of cases, as well as participants with diverse backgrounds and characteristics. In addition, further research will include the application of diffuse reflectance infrared Fourier transform spectroscopy for deeper penetration of fingernails and application of additional Raman lasers for optimised instrumental parameters and standard operating procedures.

Declaration

I declare that no material contained in this thesis has been used in any other submission of an academic award.

This thesis is dedicated wholeheartedly to my beloved family, who throughout this journey have been my greatest source of inspiration, have provided me with the strength to carry on, who continually provided their moral, emotional and financial support. I could not have done this without you.

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List of Abbreviations

-2LL: -2 log likelihood

¹H-MRS: Proton magnetic resonance spectroscopy

¹H-NMR: Proton nuclear magnetic resonance

3HB: 3-beta-hydroxybutyrate

A2M: Alpha-2-macroglobulin

AAA: Abdominal aortic aneurysm

AcAc: Acetoacetate

ADAM15: A disintegrin and metalloproteinase 15

AGEs: Advanced glycation end-products

Al: Aluminium

AIDS: Acquired immunodeficiency syndrome

AMI: Acute myocardial infarction

ANOVA: Analysis of Variance

ANP: Atrial natriuretic peptides

ApoB: Apolipoprotein B

ATP: Adenosine triphosphate

ATR-FTIR: Attenuated total reflectance-Fourier transform infrared

Au: Gold

AUC: Area under the curve

BaF: Barium fluoride

BEH: Ethylene bridged hybrid

BME: Black and minority ethnic

BMI: Body mass index

BNP: B-type natriuretic peptide

Br: Bromine

Ca: Calcium

CABG: Coronary artery bypass grafting

CACS: Coronary artery calcium score

CAD: Coronary artery disease

CASP: Critical Appraisal Skills Programme

CBD: Complete blood count

CBRN: Chemical, biological, radiological and nuclear

CD163: Cluster of differentiation 163

CD5: Cluster of differentiation 5

CD56: Cluster of differentiation 56

CeB₆: Cerium hexaboride

CCT: Coronary computed tomography

CCTA: Coronary computed tomography angiography

CFR: Coronary flow reserve

CHD: Coronary heart disease

CKD: Chronic kidney disease

Cl: Chlorine

CMR: Cardiac magnetic resonance

COX4: Cytochrome C-oxidase

CP: Cysteine-proteases

CRP: C-reactive proteins

CT: Computed tomography

Cu: Copper

CVDs: Cardiovascular diseases

CWS: Correlation in wavenumber space

D1: Derivatives one

D2: Derivatives two

DGLA: Dihomo-gamma-linolenic acid

DKA: Diabetic ketoacidosis

DM: Diabetes mellitus

DPA: Docosapentaenoic acid

DSP: Desmoplakin

DT: Decision trees

ECGs: Electrocardiograms

ECM: Extracellular matrix
EDX: Energy dispersive X-ray
ELISA: Enzyme-linked immunosorbent assay
ER: Estrogen receptor
FABP4: Fatty acid-binding protein 4
Fe: Iron
FEG: Free electron gas
FFR: Fractional flow reserve
FHS: Framingham Heart Study
FLI: Fluorescence imaging
FN: False negative
FNR: False negative rate
FP: False positive
FPG: Fasting plasma glucose
FPR: False positive rate
FT: Fourier-transform
FTIR: Fourier-transform infrared
GAD: Gaseous analytical detect
GC: Gas chromatography
GC-FID: Gas chromatography with flame ionisation detection
GC-MS: Gas chromatography-mass spectrometry
GDF15: Growth differentiation factor 15
Gp130: Glycoprotein 130
GSED: Gaseous secondary electron detector
Hb: Haemoglobin
HbA1C: Haemoglobin A1C
HDL: High-density lipids
HER2: Human epidermal growth factor receptor 2
HF: Heart failure
HFW: Horizontal width field

HIC: High-income countries

HIV: Human immunodeficiency virus

HPLC: High-performance liquid chromatography

HPLC-Qtof-MS/MS: High performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

IBS: Irritable bowel syndrome

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

ID: Infectious disease

iFR: Instantaneous wave-free ratio

IFs: Intermediate filaments

IHD: Ischemic heart disease

IL-6: Interleukin-6

IL-8: Interleukin-8

IR: Infrared

IQR: Interquartile range

K: Potassium

KAPs: Keratin associated proteins

KNN: K-nearest neighbour

LC-MS/MS: Liquid chromatography and tandem mass spectrometry

LDL: Low-density lipoproteins

LiTTaO₃: Lithium tantalate

LMICs: Low- and middle-income countries

LR: Logistic regression

MCCs: Multiple chronic conditions

MCP-1: Monocyte chemoattractant protein

MDC: Malmö Diet and Cancer Study

Mg: Magnesium

MGE: Male Gender Expressivity

ML: Machine learning

ML-PLS: Multilevel partial least squares

MLAs: Machine learning algorithms

MMP: Matrix metalloproteinase

MS: Mass spectrometry

MSC: Multiplicative scatter correlation

MRI: Magnetic resonance imaging

MVD: Coronary microvascular disease

N: Number of

NCD: Non-communicable disease

NIR: Near-infrared

NIRM: Near-infrared module

NOS: Newcastle-Ottawa Scale

NOTCH1: Neurogenic locus notch homolog protein 1

NR: Not reported

OCT: Optical coherence tomography

OGTT: Oral glucose tolerance test

OPLS-DA: Orthogonal partial least squares discriminant analysis

OR: Odds ratios

PAD: Peripheral arterial disease

PAI: Photoacoustic imaging

PAI-1: Plasminogen activator inhibitor-1

PbS: Lead sulphide

PC: Principal component

PCA: Principal component analysis

PEA: Palmitoylethanolamide

PHE: Perihematoma edema

PI: Principal investigator

PIRAMID: Pioglitazone Influence on triglyceride Accumulation in the Myocardium in Diabetes

PKU: Phenylketonuria

PNF: Proximal nail fold

QoL: Quality of life

R: Reflectance
RAGE: Receptors of advanced glycation end products
ROC: Receiver operating characteristics
ROS: Reactive oxygen species
RSD: Relative standard deviation
SAT: Subcutaneous fat
SCGs: Sustainable development goals.
SEM: Scanning electron microscopy
Si: Silicon
SMBG: Self-monitoring blood glucose
SN: Study number
S/N: Signal-to-noise ratio
SNV: Standard normal variate
SOMs: Self-organising maps
SORS: Spatially offset Raman spectroscopy
STD: Standard deviation
STE: Speckle tracking echocardiography
STEM: Scanning transmission electron microscopy
SVM: Support vector machine
T1DM: Type 1 diabetes mellitus
T2DM: Type 2 diabetes mellitus
T: Transmittance
TAA: Thoracic aortic aneurysm
tCys: Total cysteine
TDI: Tissue doppler imaging
TIA: Transient ischaemic attacks
TIMP: Tissue inhibitor of metalloproteinase
TN: True negative
TNF- α : Tumour necrosis factor α
TP: True positive

uf-GC E-nose: Ultrafast gas chromatography electronic-noise

UHPLC: Ultrahigh-performance liquid chromatography

UHPLC/Q-Orbitrap HRMS: Ultrahigh-performance liquid chromatography-quadrupole-Orbitrap high-resolution mass spectrometry

UPLC: Ultra performance liquid chromatography

LaB₆: Lanthanum hexaboride

vCD: Low voltage high contract detector

VLDL: Very low-density lipoproteins

WHO: World health organisation

Zn: Zinc

Chapter one: Introduction

1.1. Cardiovascular diseases (CVDs)

For the last three decades, cardiovascular diseases (CVDs) have remained the leading cause of mortality globally (Cesare *et al.*, 2024; Vaduganathan *et al.*, 2022). In 2021 alone, 20.5 million deaths were attributed to CVDs and contributed to a third of all deaths globally (Lindstrom *et al.*, 2022). CVD-related deaths are classified as the leading cause of premature mortality among non-communicable diseases (NCDs) (Casare *et al.*, 2024). Ischemic heart disease (IHD) specifically, is the leading cause of premature death in 146 countries for males and 98 for females (Cesare *et al.*, 2024; NCD Countdown 2030 Collaborators, 2020). The complexity of the global picture is intensified by varying levels of inequalities, which impact the prevalence and severity of CVDs. It is however noted, that low- and middle-income countries (LMICs) have higher rates of CVD cases and related premature mortality in comparison to high-income countries (HICs) (Bowry *et al.*, 2015). The World Health Organisation (WHO) figures suggest that while three quarters of cardiovascular (CV) deaths take place in LMICs, affecting both females and males, these deaths occur prematurely in individuals of working-age (16 – 84 years old) (WHO, 2025).

The upsurge of CVDs poses a significant burden on healthcare systems in LMICs, creating critical challenges for national health systems. Therefore, a more integrated approach is required for detection, prevention and management of CVDs (Wurie and Cappuccio, 2022).

CVDs are a group of disorders that impact the heart and blood vessels (WHO, 2025). They are commonly associated with a build-up of fatty deposits, called atheroma, inside the arteries and an increased risk of blood clots. While they primarily impair the heart's functioning, damage to arteries within the brain, eyes and kidneys can also occur (American Diabetes Association, 2010; NHS, 2025).

1.2. Diagnosis of CVDs

The diagnostic process for CVDs is highly dependent on the specific condition. Therefore, while there is no 'gold standard' approach for the diagnosis of CVDs,

several are routinely applied. The diagnostic method of choice is tailored based on the individual patient's clinical presentation, including their description of key symptoms. Diagnostic tests utilised for the detection of CVDs, include cardiac magnetic resonance (CMR), cardiac catheterisation, coronary computed tomography angiography (CCTA), echocardiograms, electrocardiograms (ECGs) and optical imaging technology.

1.2.1. CMR

CMR has been described as a comprehensive imaging modality, that is able to assess cardiovascular morphology, coronary heart disease (CHD), flow quantification, myocardial perfusion, tissue characterisation and ventricular function (Wang *et al.*, 2024). Through specialised magnetic resonance imaging (MRI) technology, CMR can utilise strong magnetic fields and radio waves to produce high-quality images of the heart. As a result, CMR is routinely employed for the assessment of heart function. Nonetheless, the use of this technique does not come without difficulty and often faces challenges attributed to cardiac and respiratory motions and resulting artefacts (Tseng, Su and Tseng, 2016). The presence of motion artefacts can render images unusable. Therefore, while non-invasive, this technique requires synchronous cardiac and respiratory gating or breath-holding techniques (Graaf *et al.*, 2014; Wang *et al.*, 2024).

1.2.2. Cardiac catheterisation

Cardiac catheterisation is an invasive diagnostic technique used to retrieve key information regarding the heart's function and structure (NHS, 2022). This technique also requires the use of an X-ray in the form of a coronary angiography/arteriography, which results in coronary angiograms. The coronary angiogram allows the safe guidance of the catheter into the heart via a cannula, which is inserted into a peripheral artery or vein (Glowny and Resnic, 2012). As a result, provides detailed information about the severity of atherosclerotic lesions, valvular abnormalities and intracardiac pressures, factors that non-invasive modalities cannot reliably detect (Tavakol *et al.*, 2012). There are several catheter-based methods for the diagnosis of CVDs including coronary flow reserve (CFR), fractional flow reserve (FFR), instantaneous wave-free

ratio (iFR) and quantitative flow ratio (QPR). While this technique is invasive, it is frequently utilised for evaluating CHD and cardiac hemodynamic (Shoaib *et al.*, 2022). Nonetheless, as an invasive procedure, cardiac catheterization carries inherent risks caused by vascular access complications such as bleeding, haematoma and pseudoaneurysms (Tavakol *et al.*, 2012). In severe cases, stroke or myocardial infarction may occur. Although, it is worth noting that the risk of such events is associated with patient-related factors such as age and comorbidities (Patel *et al.*, 2024).

1.2.3.CCTA

Initially, coronary computed tomography (CCT) quantified coronary artery calcification, which was detected via computed tomography (CT) images and quantified to generate a coronary artery calcium score (CACS) (Channon *et al.*, 2022). The CACS acts as a surrogate marker for detection and severity of CHD. CCTA has now transformed the assessment of CHD, allowing for a non-invasive detection of the coronary lumen, stenoses and plaque, three-dimensionally. Images can be collected using a low X-ray exposure in short acquisition times. Moreover, the high sensitivity of this approach in detecting significant coronary stenosis, is particularly valuable for excluding coronary artery disease (CAD) patients with low-to-intermediate pretest probability and in turn reducing unnecessary invasive procedures (Ollendorf *et al.*, 2011). Nonetheless, the presence of heavy coronary calcification can produce motion artefacts, which often lead to degraded image quality and overestimation of stenosis severity (Le Roy *et al.*, 2019). Although, it is worth noting that previous research has looked at removing motion artefacts from CCTA images through deep learning methods including pixel-to-pixel generative adversarial networks (Ren *et al.*, 2022).

1.2.4.Echography

Echography utilises ultrasound technology with frequencies greater than 20,000 Hz, for the visualisation of the heart's structure and function (Kadappu and Thomas, 2015). Conventional modalities of echocardiography include doppler, M-mode and two-dimensional echocardiography. Compared to other imaging modalities such as cardiac

MRI and CT, echocardiography allows for portable and cost-effective analysis, with the development of handheld and point-of-care ultrasound devices. However, image quality is highly dependent on user-knowledge, as well as patient-related factors such as obesity and lung disease presence (Senior *et al.*, 2015). As a result, echocardiography is subject to inter-observer variability and image acquisition and analysis, factors that limit its scalability in low-resource settings or at oversubscribed institutes

1.2.5. ECGs

The ECG signal allows the heart's electrophysiological activity to be captured. While it is widely applied to clinical medicine, ECGs provide imperative information for the detection and treatment of several CVDs (Hinai *et al.*, 2021). Portable and wearable ECG devices now enable continuous cardiac monitoring within hospital and community environment, facilitating remote patient management (Perez *et al.*, 2019). Moreover, such devices allow for improved early detection of intermittent arrhythmias through consistent longitudinal monitoring of cardiac electrical function over time (Beard, Celse and Labrunee, 2025). Despite the aforementioned strengths, ECGs have notable limitations. For example, ECGs do not provide direct information regarding cardiac structure or coronary anatomy. Consequently, ECG findings may appear normal in patients with significant coronary artery spasms, especially during asymptomatic or early disease stages (Newby *et al.*, 2018). The limited sensitivity of ECGs is also accompanied with baseline variability, electrode placement/operator technique, which introduces diagnostic uncertainty.

1.2.6. Optical imaging technology

Optical imaging technology such as optical coherence tomography (OCT), photoacoustic imaging (PAI) and fluorescence imaging (FLI) have been utilised to investigate cardiac morphology, blood flow dynamics and tissue oxygenation (Pang *et al.*, 2024). The aforementioned techniques allow for high spatial resolution, real-time imaging capabilities and in some cases molecular-level characterisation (Pang *et al.*, 2024). Through capturing cross-sectional and three-dimensional images, OCT allows

for high-resolution of atherosclerotic plaque, as well as acting as a guide for percutaneous coronary interventions (PCIs) and evaluating stent implantation outcomes (Fujimoto *et al.*, 2000). This technique is analogous to ultrasound but utilises near-infrared (NIR) light rather than sound and measures backscattering of light once it has struck the sample (Aumann *et al.*, 2019; Fujimoto *et al.*, 2000; Rocholz *et al.*, 2019). As a result, high spatial resolution is obtained and allows the characterisation of plaque, microcalcifications and thrombus formation (Tearney *et al.*, 2012; Prati *et al.*, 2017). Nonetheless, OCT imaging is an invasive procedure, which requires intracoronary catheter insertion and can expose the patient to procedural risks such as vascular injury (Castaldi *et al.*, 2025; Prati *et al.*, 2017).

1.3. Causes and risk factors of CVDs

As a multifactorial disease, the manifestation and severity of CVDs is influenced by several modifiable and non-modifiable risk factors (Berry *et al.*, 2012; Capewell *et al.*, 2010; Mathers and Loncar, 2006). Modifiable risk factors include a lack of physical activity, an unhealthy diet, obesity and smoking habits. In addition, non-modifiable risk factors include age, biological sex, ethnicity, family history of disease, diabetes mellitus (DM) and/or renal disease (Adhikary *et al.*, 2022; Canoy *et al.*, 2014; Chow *et al.*, 2010; Ordúñez, 2011; Roth *et al.*, 2015).

1.4. DM

DM is a NCD characterised by chronic hyperglycaemia, which is caused by a lack of insulin secretion, impaired insulin action or in some cases both (Antar *et al.*, 2023). As an anabolic hormone, insulin plays a key role in the metabolism of carbohydrates, lipids and proteins (Poznyak *et al.*, 2020). Metabolic abnormalities related to DM impact tissues including adipose tissue, skeletal muscles and the liver and is primarily attributed to insulin resistance. Without sufficient treatment, uncontrolled DM can undergo further complications such as coma, confusion and in severe cases, diabetic ketoacidosis (DKA) (Poznyak *et al.*, 2020). There are two main types of DM, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).

T1DM is characterised by T-cell-mediated destruction of pancreatic β -cells, which results in the absolute deficiency of insulin synthesis and secretion (Akil *et al.*, 2021; Haller, Atkinson and Schatz, 2005; Diabetes UK, 2025a). In contrast, T2DM is characterised by heterogeneous progressive loss of islet β -cell insulin secretion, which often occurs after the presence of insulin resistance and metabolic syndrome (Antar *et al.*, 2023). T2DM makes up the vast majority of diabetic cases (96%) and can seriously threaten health without total clear cognition on pathogenesis (Lu *et al.*, 2024). While T1DM is often only accompanied by hyperglycaemia, T2DM is one of many components of metabolic syndrome, consisting of a group of health problems that increase the risk of developing T2DM and/or CVDs and forms a cluster of metabolic dysregulations such as atherogenic dyslipidaemia, central obesity, insulin resistance and hypertension (Fahed *et al.*, 2022).

1.5. Diagnosis of DM

Diagnosis of DM is frequently made through the measurement of haemoglobin A1c (HbA1c) plasma glucose levels or fasting plasma glucose (FPG) (Antar *et al.*, 2023). Capturing these values allows a correlation to be formed between the HbA1c or FPG level and retinopathy and in turn helps to set cut-off values for glucose and HbA1c to diagnose DM (Nakagami *et al.*, 2007). The cut-off values for the diagnosis of DM vary depending on the diagnostic test utilised (Table 1.1).

Table 1.1. Diagnostic cut-off points for the detection of DM (Antar *et al.*, 2023; Diabetes UK, 2025b; Genuth *et al.*, 2003).

Test	Healthy	Prediabetic	Diabetic
FPG	100 mg/dL (5.6 mmol/L)	100 – 125 mg/dL (5.6 – 6.9 mmol/L)	≥ 126 mg/dL (≥ 7.0 mmol/L)
HbA1c	$<5.7\%$	5.7 – 6.4%	$\geq 6.5\%$
OGTT	<140 mg/dL (7.8 mmol/L)	140 – 199 mg/dL (7.8 – 11.0 mmol/L)	≥ 200 mg/dL (11.1 mmol/L)

FPG: Fasting plasma glucose; HbA1c: glycated haemoglobin; OGTT: Oral glucose tolerance test.

FPG testing is widely utilised due to its low-cost and standardised procedure, however, FPG reflects glycaemic status at a single time point. As a result, this biochemical test is highly susceptible to short-term biological variability influenced by dietary

behaviours, stress and/or sickness (Kasujja *et al.*, 2025). Furthermore, the requirement for overnight fasting reduces patient compliance rates (Selvin *et al.*, 2011). HbA1c however offers the significant advantage of reflecting average blood glucose over a two- to three-month period. Therefore, provides a measure of long-term glycaemic exposure without the need for fasting (Sherwani *et al.*, 2016). Nonetheless, it has been noted that HbA1c measurements have been influenced by non-glycaemic factors including anaemia and renal disease, resulting in diagnostic misclassifications (Selvin *et al.*, 2011; Herman *et al.*, 2012). Considered the most sensitive diagnostic tool for DM, OGTT is frequently utilised for detection of impaired glucose tolerance and early-stage DM. However, OGTT is time-consuming and requires multiple blood samples (Bogdanet *et al.*, 2020). Therefore, has often been paired with poor patient tolerability and reproducibility (Bonora and Tuomilehto, 2011).

1.6. Causes and risk factors of DM

Similarly to CVDs, reported causes and risk factors of DM include age (Amir, Suhl and Alexander, 2020; Arya, 2023; Fong *et al.*, 2021; Gassasse *et al.*, 2018; Junker *et al.*, 2021; Urquhart, 2002; Xia *et al.*, 2021), biological sex (Yan *et al.*, 2023), high blood pressure (Brownlee, 2005; Chen *et al.*, 2022; Tsimihodimos *et al.*, 2018), family history of DM and other diseases (Alharithy *et al.*, 2018; Gopalakrishnan and Geetha, 2017; Hemminkki *et al.*, 2009; Liu *et al.*, 2020; Nagarathna *et al.*, 2020; Ndeti *et al.*, 2024; Papazafiropoulos *et al.*, 2017; Sheu *et al.*, 1999; Wang *et al.*, 2015) and obesity (Klein *et al.*, 2022; Manolopoulos, Karpe and Frayn, 2010).

1.7. Spectroscopy for disease diagnosis

As powerful and non-invasive analytical techniques, vibrational spectroscopy has previously been used for disease diagnosis (Baker *et al.*, 2018; Byrne *et al.*, 2015; Gaba *et al.*, 2022; Matousek and Stone, 2012; Nicolson *et al.*, 2021; Ralbovsky and Lednew, 2020; Stone *et al.*, 2004; Stone *et al.*, 2007; Stone and Matousek, 2008). Through the interaction of light, vibrational spectroscopy collects a signature/fingerprint, of the molecular bonds present within a sample. The collected fingerprint is characteristic of the sample's chemical structure and/or physical

properties and represents changes that occur in presence of diseases. In cases of complex biological samples such as blood, fingernails, saliva or urine, the collected spectroscopic signature comprises functional groups, corresponding to key endogenous constituents such as amino acids, carbohydrates, lipids and proteins.

Unlike, labelled techniques such as fluorescence, label free spectroscopy can identify signatures of subcellular phenomena, without assuming prior knowledge of specific biomarkers (Chaudhary *et al.*, 2022). Therefore, can be utilised in the detection of novel biomarkers, which are representative of cellular events (Farhane *et al.*, 2015). Therefore, vibrational spectroscopic techniques are now used for disease detection purposes (Barnas *et al.*, 2020; Naseer, Ali and Qazi, 2021; Roy *et al.*, 2017; Sbroscia *et al.*, 2020). The application of vibrational spectroscopy has shown great success in the detection of autoimmune diseases including ankylosing spondylitis, rheumatoid arthritis and osteoarthritis (Wu *et al.*, 2023), cancer (Liu *et al.*, 2022; Old *et al.*, 2014), COVID-19 (Zhao *et al.*, 2023) and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Paraskevaidi, Martin-Hirsch and Martin, 2018) and tuberculosis (Baron *et al.*, 2017).

1.7.1. Handheld spectroscopy

Handheld spectroscopy became popular because it allowed users to measure samples in the field. This is advantageous in clinical settings whereby rapid results have life-changing effects (Kranenburg *et al.*, 2020a; Kraneburg *et al.*, 2020b; Synytsya *et al.*, 2024). In addition, handheld spectrometers allow the use of spectral libraries for on-spot identification of samples in the field (Crocombe, 2020). However, in extreme environmental conditions, which are often encountered in field settings relevant to LMICs implementation, handheld devices' performance and reliability can be compromised. For example, environmental variables such as moisture can influence measurement quality and requires advanced modelling as compensation (Mayr *et al.*, 2021). There is also some argument to suggest that handheld spectrometers are less robust to extreme temperatures, which in turn causes issues with calibration stability and optical alignment (Yan *et al.*, 2023). Previous work also identified that the negative relationship between bright or dusty conditions and

effectiveness of handheld Raman devices (Deidda *et al.*, 2019). However, a dark cloth can be applied to Raman devices to limit the influence of light (Cletus *et al.*, 2013).

1.7.2. Attenuated total reflectance-Fourier transform infrared spectroscopy

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy is widely employed for the identification of biological matrices such as blood, saliva, semen, sweat and urine (Gozdzialski *et al.*, 2023; Mistek and Lednev, 2018). Previous research has utilised ATR-FTIR spectroscopy for detecting diseases such as acute myeloid leukaemia (Delrue *et al.*, 2023), Alzheimer's disease (Hahn *et al.*, 2010), anaemia (Delrue *et al.*, 2023), leukaemia, thalassemia, lymphoma and myelodysplastic syndrome (Delrue *et al.*, 2023), as well as cancer related to the brain (Sala *et al.*, 2020), breast (Depciuch *et al.*, 2016), colorectal (Dong *et al.*, 2014), lungs (Lewis *et al.*, 2010) and prostates (Mackanos and Contag, 2009).

ATR-FTIR spectroscopy refers to the study of absorption, reflection, scattering or transmission of IR radiation and occurs over the spectral range of 4,000-400 cm^{-1} . This range captures a variety of vibrational frequencies and groups (Table 1.2.) (Jee, 2021).

Table 1.2. Commonly reported ATR-FTIR vibrations and frequencies observed in the IR region (Jee, 2021).

Wavenumber (cm^{-1})	Vibration
3600 – 2500	O-H stretching
3400	N-H stretching
3000	C-H aromatic stretching
2900	C-H aliphatic stretching
1800 – 1650	C=O stretching
1740	Ester R-O-C=O
1715	Ketone C=O
1705	Carboxylic acid HO-C=O
1650	Amide $\text{H}_2\text{N-C=O}$
1300 – 1000	C-O stretching
1800 – 400	Forest of vibrations (fingerprint region)

To determine the energy associated with electromagnetic radiation, Planck's equation is referred to and suggests that photon energy is directly proportional to frequency (Equation 1.1).

Equation 1.1. Planck's equation (Jee, 2021).

$$E = h\nu = hc\bar{\nu}$$

$$E = \frac{hc}{\lambda}$$

Where, E is the energy,

h is representative of Planck's constant,

ν is the frequency,

c is the speed of light 3×10^8 m/s,

and λ is the wavelength of light (Jee, 2021).

When at room temperature, molecules are commonly found at their ground electronic and lowest vibrational state. ATR-FTIR radiation, with the appropriate energy, causes resonant absorption to occur and excites the molecule to a higher vibrational state. The vibrational transitions that arise, produce an absorption spectrum, characteristic of the sample of interest. Factors such as the number of absorption features, their associated wavenumber, and their strength (intensity) and sharpness, are characteristic to the sample and are employed for interpretation/identification purposes (Delrue *et al.*, 2023; Jee, 2021).

When excited, molecules undergo one of two vibrations, those being: stretching vibrations (changes in bond length) and bending vibrations (changes in bond angle) (Mokari, Guo and Bocklitz, 2023). Traditionally, IR spectra were reported as a plot of percentage transmittance ($T\%$) against wavenumber (ν) (Jee, 2021). However, spectra are now being reported as a plot of absorbance against wavenumber. The absorption of light by a molecule is referred to as the transmission of light. The ratio between transmitted intensity of light to the incident intensity (light impeding on

sample), is known as transmittance (Jee, 2021). To quantify the absorption of light, Beer's Law is employed (Equation 1.2).

Equation 1.2. Beer's Law.

$$A = \log \left(\frac{I_o}{I_t} \right) = cl$$

Where, A refers to the absorbance,

c is the molar concentration (mol/L),

l is representative of the path length (m) of the sample (Jee, 2021).

To calculate the absorbance, the log of the inverse of transmittance is utilised (Equation 1.3).

Equation 1.3. Absorbance.

$$A = \log \left(\frac{I_o}{I_t} \right) = \log \left(\frac{1}{T} \right) = -\log T = 2 - \log T\%$$

Where, A refers to the absorbance,

l is representative of the path length (m) of the sample,

T is the transmittance,

and $T\%$ is the transmittance percentage (Jee, 2021).

For this work, the Agilent 4500a ATR-FTIR spectrometer and the Agilent Cary 630 ATR-FTIR spectrometer were utilised. As ATR-FTIR spectrometers, the aforementioned devices utilise interferometers and samples are presented next to the detector to ensure heat effects produced by the IR source are reduced (Jee, 2021). The spectrometer locks into the mirror oscillation frequency and is coupled with the

signal filtering (associated with Fourier transform). The optics and detectors, then determine the preferred sample position (Figure 1.1) (Jee, 2021).

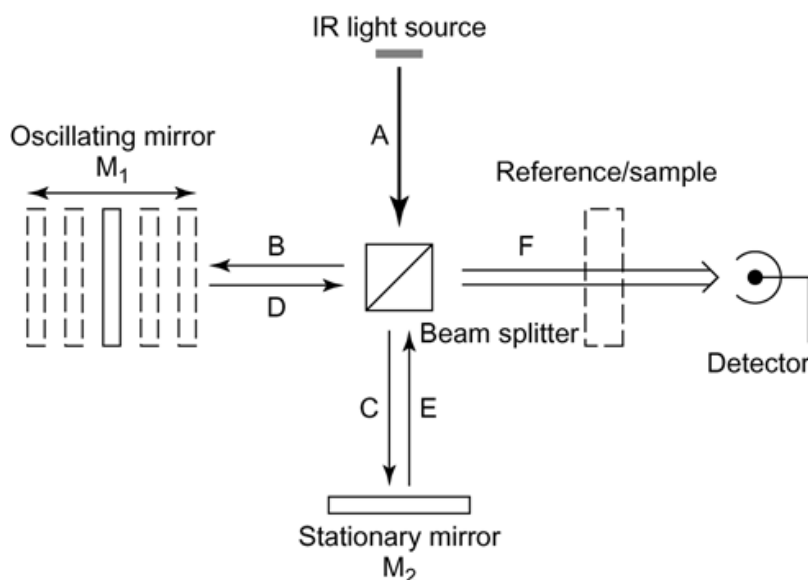


Figure 1.1. Layout of ATR-FTIR spectrometer (Jee, 2021). Figure used with permission from The Pharmaceutical Press. Copyright © 2021 Pharmaceutical Press, UK. All rights reserved.

In cases where mirror M_1 has been set to oscillate along the optic axis, the distance travelled by beam $(B + D)$ varies, while the distance travelled by $(C + E)$ remains unchanged. When distances $(B + D)$ and $(C + E)$ are identical, D and E are in phase and recombination is fully constructive (Jee, 2021). When mirror M_1 moves towards the splitter beam, beam D arrives before beam E . Consequently, recombination is not fully constructive and beam F 's intensity is reduced and phase changed. Overtime, mirror M_1 's movements cause beam D to become a half-wave ahead of beam E . As a result, the recombination is fully destructive and beam F 's intensity becomes zero. Finally, the movement of M_1 is reversed past the oscillation mid-point.

During the movement of the mirror in a single mirror oscillation period, the intensity of a single wavelength of light varies. The produced signal is subject to the mathematical procedure referred to as a Fourier transform. This procedure involves extracting the light intensity versus wavelength (wavenumber) information (Jee, 2021). When no

reference or sample is present in the beam pathway, the measurement taken is the background or I_0 spectrum. This measurement is stored and utilised in the subsequent transmittance/absorbance measurements (Jee, 2021). The interferometer allows for greater light collection and throughput in comparison to dispersive instruments. Hence, all wavelengths are observed within a single scan simultaneously. Moreover, the interferometer ensures lower noise and greater sensitivity.

As the ATR-FTIR light hits the sample of interest, it is reflected. Reflection occurs in the sense of a mirror reflection, whereby the angle of incidence equals the angle of reflectance and is often referred to as specular reflectance (Jee, 2021). When applying ATR, if a light arrives at an appropriate angle to the boundary between two media/materials and with appropriate refractive indices (n_1 and n_2), it can be reflected to the first medium. This reflection is referred to as internal reflectance. Internal reflectance occurs if the light beam has sampled the second medium. The evanescent wave (light impeding on the medium) provides information regarding the sample's absorption properties. The reflected beam has a reduced (attenuated) intensity, which is referred to as the ATR and provides the intensity versus wavenumber characteristics (Jee, 2021).

1.7.3. NIR spectroscopy

NIR spectroscopy captures overtones and combination bands found in the NIR region ($12,500 - 4,000 \text{ cm}^{-1}$) (Ozaki, Awa and Ishikawa, 2025). As an electronic and vibrational spectroscopic technique, it is unique in comparison to IR and Raman spectroscopy. Characteristic overtones and combination bands are forbidden transitions under the harmonic oscillator approximation, which suggests that due to equal spacing, transitions occur at the same frequency (Ozaki, Awa and Ishikawa, 2025). However, bands originating from electronic transitions or arising from vibrational overtones and combination modes are characteristically weak. The combination of weak transitions and NIR's ability to penetrate several millimetres into solid samples, allows for valuable non-destructive analysis, which can be made *in situ* (Ozaki, Awa and Ishikawa, 2025; Pasquini, 2018; Vitorino *et al.*, 2023).

The NIR region is split into three, those being: Region I ($12,500 - 8,500 \text{ cm}^{-1}$), Region II ($8,500 - 5,500 \text{ cm}^{-1}$) and Region III ($5,500 - 4,000 \text{ cm}^{-1}$) (Ozaki, Awa and Ishikawa,

2025). Bands observed in Region I are derived from a combination of electronic transitions, higher overtones (third overtone) and combination modes. Bands that fall under this region are particularly weak, hence Region I is characterised by its high level of transparency. Bands seen in Region II arise from first and second overtones, stretching vibrations and combination modes. Finally, Region III shows the lowest level of permeability due to the occurrence of relatively intense combination bands here and the first overtone (Ozaki, Awa and Ishikawa, 2025). Within the NIR region, water is absorbed between the regions of $7,000 - 7,425$ and $5,000 - 5,600 \text{ cm}^{-1}$ (Choquette *et al.*, 2006).

The potential energy curve of a simple diatomic molecule can be explained through Figure 1.2. The potential energy curve of a simple diatomic molecule ($v = 0 \rightarrow v = 1$) is seen as $v = 0 \rightarrow v = 2$ for the first overtone and $0 \rightarrow v = 3$ for the second overtone (Ozaki, Awa and Ishikawa, 2025).

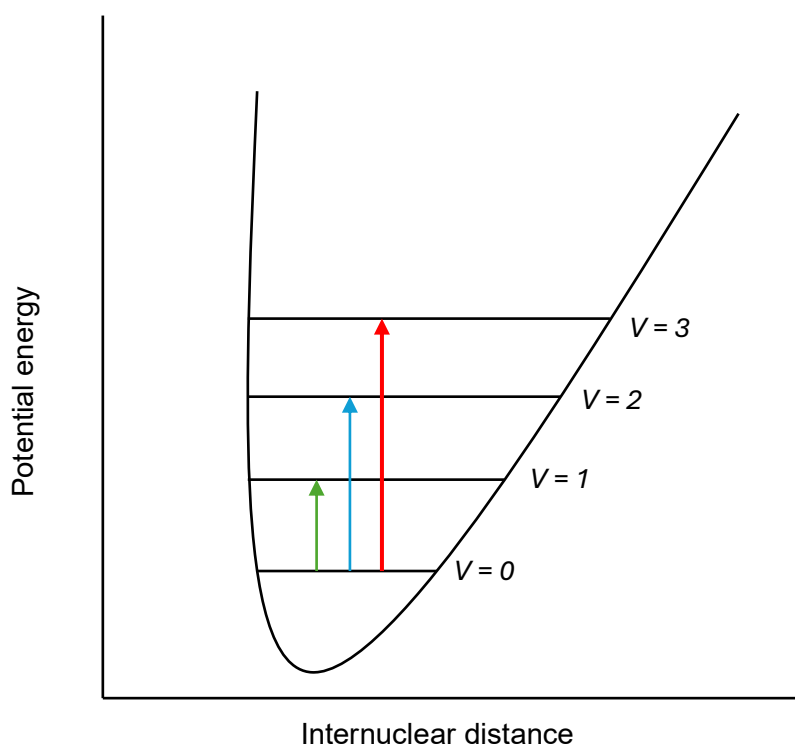


Figure 1.2. Potential energy curve for a simple diatomic molecule overtone. Figure adapted from Ozaki, Awa and Ishikawa (2025) with permission from The Pharmaceutical Press. Copyright © 2025 Pharmaceutical Press, UK. All rights reserved.

The allowed energy levels for an anharmonic oscillator can be calculated through the following equation:

Equation 1.4. Anharmonic oscillator energy levels.

$$E \approx (v + 0.5)hf - (v + 0.5)^2 hfx$$

Where, h is Planck's constant,

v is the vibrational quantum number,

f is the equilibrium frequency of oscillation,

x is the anharmonicity constant (Ozaki, Awa and Ishikawa, 2025).

Through the application of Equation 1.4, it is possible to determine energy differences between energy levels of vibrational quantum numbers, v and $v + 1$. Equation 1.5. also demonstrates that higher values of v and x dictate a smaller level of energy ($v \rightarrow v+1$). The vibrational quantum number can vary in size (0, 1, 2...). For an anharmonic oscillator, the selection rule is as followed: $\Delta v = \pm 1, \pm 2, \pm 3 \dots$ therefore transitions including $v_0 \rightarrow 1, v_0 \rightarrow 2, v_0 \rightarrow 3, v_1 \rightarrow 2$ and so on, can take place (Blinder, 2021). The energy transitions that occur are referred to as the fundamental frequency of vibration, first overtone, second overtone, etc., and are collectively known as fundamentals and overtones (Pirutin *et al.*, 2023). Due to the anharmonicity constant being relatively small ($x < 0.05$), the spectroscopic transitions that occur are seen at low frequencies such as $f, 2f$ and $3f$ (Ozaki, Awa and Ishikawa, 2025).

Equation 1.5. Energy differences between energy levels of vibrational quantum numbers v and $v+1$.

$$\Delta E_{(v \rightarrow v+1)} = h - 2hf x(v + 1)$$

Where, h is Planck's constant,

v is the vibrational quantum number,

f is the equilibrium frequency of oscillation,

x is the anharmonicity constant (Ozaki, Awa and Ishikawa, 2025).

Polyatomic molecules may undergo several simultaneous changes in energy at two or more vibrational modes, the sum of which will be between the individual frequencies (Ozaki, Awa and Ishikawa, 2025). Consequently, weak bands referred to as combination bands arise and occur when two vibrations involving a common atom, or bands connected via multiple chemical bonds are present (Beć, Grabska and Huck, 2022). As transition probabilities for overtones and combination bands are low (10^{-1} - 10^{-4}) in comparison to fundamental frequencies, levels of absorbance are weak (Ozaki, Awa and Ishikawa, 2025). Hence, the name forbidden transitions, as their probability of occurrence is low. Furthermore, the presence of weak absorbances can be attributed to higher-order overtones and combination bands appearing in the shorter wavelength region (Ozaki, Awa and Ishikawa, 2025). Hence, the shorter the wavelength, the weaker the NIR band becomes. As a result, it is common for NIR bands to be broad and many overlap with other bands in proximity. Therefore, the application of pre-processing and chemometrics to NIR spectra is not uncommon. In many cases, pre-processing algorithms such as multiplicative scatter correlation (MSC) and derivative one (D1) or derivative two (D2) are employed and allow separation of NIR bands (Rinnan, Berg and Engelsen, 2009). Moreover, NIR spectra are visualised through chemometrics and machine learning algorithms (MLAs) such as principal component analysis (PCA) and self-organising maps (SOMs).

NIR spectra can be collected by transmittance (T) or reflectance (R). This work employed the PerkinElmer Spectrum Two N FT-NIR Spectrometer (PerkinElmer, Waltham, MA), which was equipped with a near-infrared module (NIRM). The NIRM allowed for the collection of diffusion reflectance spectra non-destructively, which permitted the tangent use of complimentary techniques such as Raman and IR spectroscopy. Diffuse

reflected radiation refers to the radiation which has penetrated the sample's particles and has undergone multiple reflections within the sample itself (Myrick and English, 2023). After various characteristic absorptions have occurred, the radiation will then re-emerge (Figure 1.3).

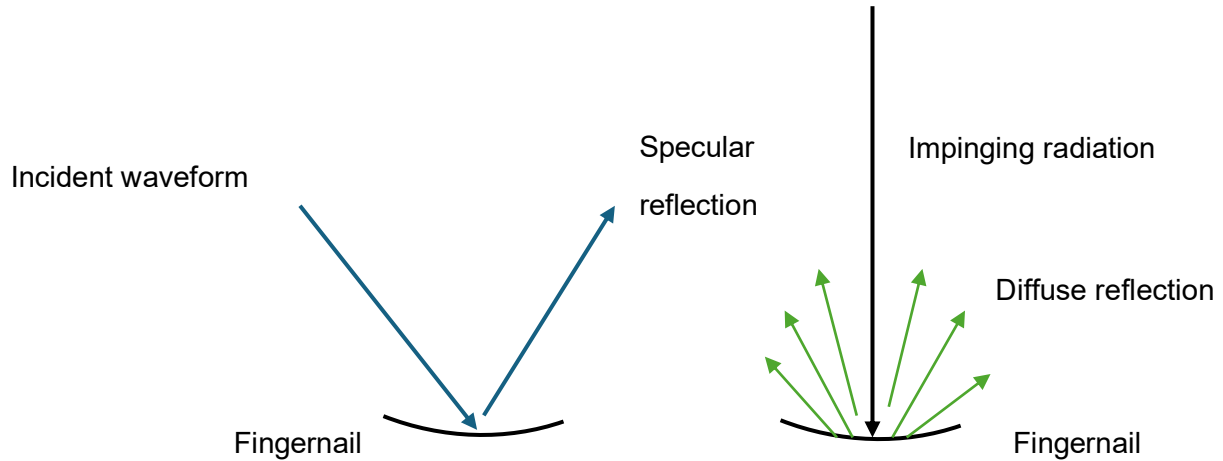


Figure 1.3. Mode of measurement of specular versus diffuse reflectance.

In the case of reflectance NIR spectroscopy, the intensity of the light reflected by the sample is measured (Hapke, 2017). Reflectance is calculated through the ratio of the intensity of radiation reflected by the sample itself (I_r) to the intensity of the radiation impinging on it (I_0) (Equation 1.6) (Ozaki, Awa and Ishikawa, 2025).

Equation 1.6. Calculation of reflectance.

$$R = I_r / I_0$$

Where, R is reflectance,

I_r is the intensity of radiation,

I_0 is the intensity of the impinging radiation (Ozaki, Awa and Ishikawa, 2025).

Dahm and Dahm (2021) described the theory of diffuse reflectance, suggesting that the radiation pathway is dependent on several factors including particle size/shape and sample composition. Thus, emitted radiation provides useful information regarding the sample's physical state. As the exact path taken by the radiation is difficult to determine, there is currently no satisfactory theory for the calculation of diffuse reflectance (Dahm and Dahm, 2021). Nonetheless, the Kubelka-Munk theory provides an approximation for the measurement of NIR reflectance (Equation 1.7.) (Ozaki, Awa and Ishikawa, 2025).

Equation 1.7. The Kubelka-Munk theory for the approximation of the NIR reflectance measurements.

$$f(R_a) = \frac{(1 - R_a)}{2R_a} = \frac{k}{s}$$

Where, $f(R_a)$ is the Kubelka-Munk function,

R_a is the absolute reflectance,

k is the sample's absorption coefficient,

s is the scattering coefficient (Ozaki, Awa and Ishikawa, 2025).

As defined by the Beer-Lambert law, the absorption coefficient is equal to absorptivity and then multiplied by the concentration (Ozaki, Awa and Ishikawa, 2025) Therefore, this function is related to concentration through the following equation (Equation 1.8).

Equation 1.8. Association between concentration and the Kulbelka-Munk theory.

$$f(R_a) = \frac{\varepsilon C}{S}$$

Where, $f(R_a)$ is the Kulbelka-Munk function,

ε is the absorption coefficient equal to absorptivity,

C is concentration,

s is the scattering coefficient (Ozaki, Awa and Ishikawa, 2025).

At a microvessel level, variation in the NIR absorbing components can be used to differentiate between healthy and unhealthy tissues (Vitorino *et al.*, 2023). Over recent years, NIR spectroscopy has shown the successful detection of Alzheimer's disease (Hock *et al.*, 1996; Paraskevaïdi *et al.*, 2018), bowel disease (Behdad, Massudi and Pakdel, 2024), DM (Pichler *et al.*, 2004; Mohammadi *et al.*, 2015; Monteyne *et al.*, 2018; Scheuermann-Freestone, 2003) and pulmonary disease (Jensen *et al.*, 2002) using cerebral blood and tissue oxygenation, blood plasma, saliva, blood flow, blood glucose levels, cardiac high-energy phosphate metabolites and cerebral oxygenation, respectively.

1.7.4. Raman spectroscopy

Raman spectroscopy is a powerful analytical technique for analysing compounds in biological matrices such as blood, fingernails, saliva and urine (Atkins *et al.*, 2017; Hardy *et al.*, 2022; Kourkoumelis *et al.*, 2018; Moreira *et al.*, 2017). The potential of this vibrational spectroscopic technique arises from its ability to detect biochemical changes at a molecular level.

Governed by Planck's equation, Raman spectroscopy is based on light scattering, which occurs when photons are excited by a monochromatic light and collide with the sample. Monochromatic radiation is either absorbed, transmitted or scattered. Scattered radiation refers to numerous photons scattering at the same frequency as

the incident radiation frequency and is referred to as elastic or Rayleigh scattering. Scattered radiation passes into the spectrometer, allowing the detection of a strong Rayleigh line at an unmodified frequency (Jones *et al.*, 2019) Thus, the energy of the scattered photon is equal to the incident photon.

Irradiation of a molecule via monochromatic radiation and the arising transitions can be observed in Figure 1.4. When radiation is of a sufficient energy, the absorption process that occurs is representative of an electronic transition ($S_0 \rightarrow S_1$). Radiation from a less energetic source means that molecules are promoted to the virtual state and are representative of a distortion of the electron distribution of a covalent bond present within the molecule (Assi, 2023). From the virtual state, the molecule will then relax to its original ground electronic state through emitting a photon at the same frequency as the incident radiation and represents Rayleigh/elastic scattering (Assi, 2023).

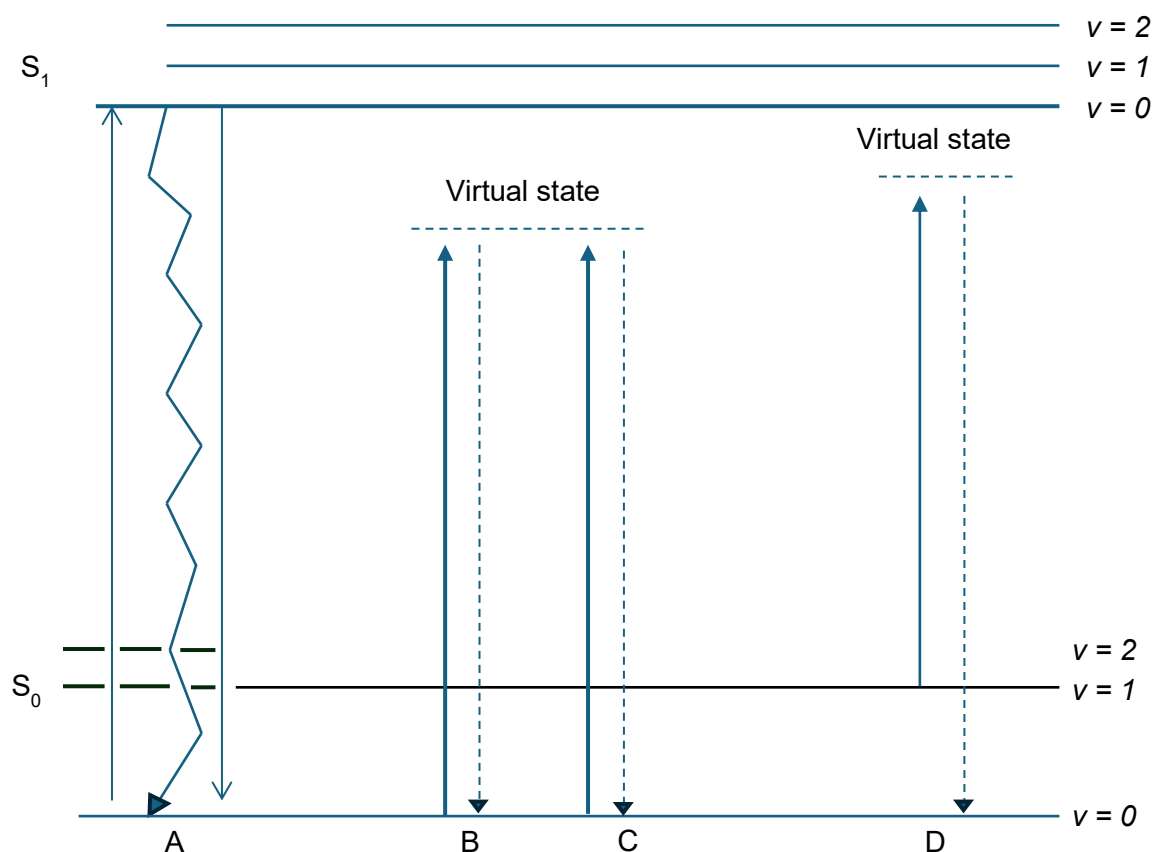


Figure 1.4. Jablonski energy level diagram illustrating transitions. Solid lines are representative of absorption processes and dotted lines of scattering processes. A, electronic transition with non-radiative decay (heat) or radiative decay (fluorescence);

B represents Rayleigh scattering; C, shows Stokes Raman transition and D anti-Stokes Raman transitions. S_0 is the singlet ground state, S_1 is the lowest singlet excited state and v is the vibrational energy levels within each electronic state (Assi, 2023). Figure used with permission from The Pharmaceutical Press. Copyright © 2023 Pharmaceutical Press, UK. All rights reserved.

In contrast, during collisions a small amount of photons exchange energy with the sample and this is referred to as an inelastic collision, occurring in one of two forms. Anti-Stokes Raman scattering occurs when the photon gives energy to the sample and the energy is reduced to $h(\nu_0 - \nu)$ with longer wavelengths (Assi, 2023). While Stokes Raman scattering arises when the photon takes energy from the sample and the energy is increased to $h(\nu_0 + \nu)$ with shorter wavelengths (Jones *et al.*, 2019). Hence, the scattered photon's energy is lower than the incident photons. The shift (ν) or Raman shift, and its intensity, form a characteristic Raman spectrum of the sample. Stokes Raman scattering is the consequence of the interaction between photons and the sample during ground state, while Anti-Stokes Raman scattering is the result of the interaction between photons and the sample in the excited state (Assi, 2023). Anti-Stokes lines are often less intense than Stokes lines because these transitions arise from higher vibrational energy levels, which contain fewer molecules. This is described by the Boltzmann distribution (Assi, 2023; Russel, 1996; Yeturu, 2020).

As a surface technique, conventional Raman spectroscopy limits the collection of spectral data from inside the sample. Spatially offset Raman spectroscopy (SORS) addresses this limitation by allowing for deeper sample penetration down to millimetre levels (Assi, 2023) SORS allows Raman spectra to be collected from regions that are spatially offset by varying degrees from the point of laser excitement (Figure 1.5). Thus, the arrayed spectra contain different relative Raman contributions, reflective of both the surface and subsurface layers. As the collection point moves away from the probe launch, the contribution the spectrum collects from the top layer diminishes at a faster rate than from the deeper layers. This is the result of deeper generated Raman photons migrating laterally before being emitted. This method is based on the variation of Raman signal's relative content from different layers. As the spatial offset originates from the random properties of the photon migration effect, the migrating photons undergo a 'random walk' within the sample. The collected Raman signal therefore

contains a relatively large signal contribution from the top layer. This is the result of the highest probe photon density being located at the top layer of the sample. Through increasing the sample depth, the intensity diminishes quickly as the intensity of light becomes progressively diluted via the photon diffusion process. Scattered Raman light from the deeper layers, undergoes further dilution, hence the intensity of these photons is low. Furthermore, the photons generated within the deeper layers of the sample, are less likely to migrate to the point of incidence for the probe beam. While photons generated at the surface or near the point of incidence are more likely to return to the probe beam. By collecting Raman light from a laterally offset point from the probe beam incidence, the probe light intensity becomes more equally distributed along the sample's depth.

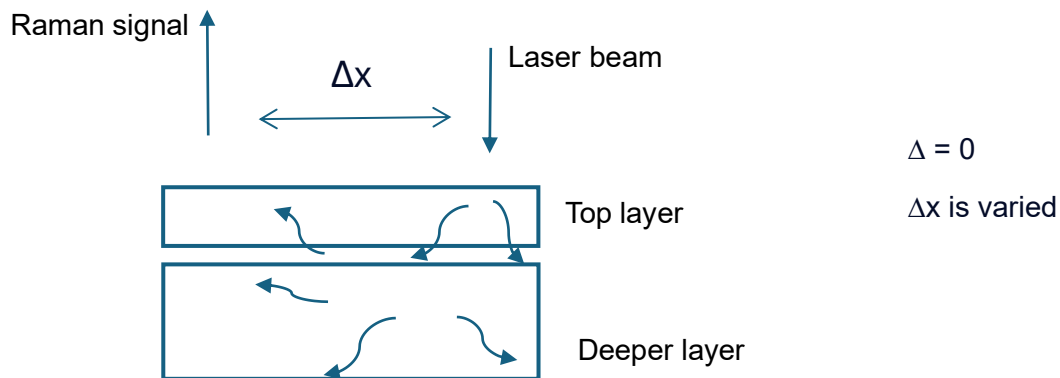


Figure 1.5. Principle of SORS (Matousek et al., 2005).

The resulting Raman spectra contains both the Raman contributions of the packaging and the content, which are then separated via a scale subtraction (Matousek *et al.*, 2005). Hence, as a depth-profiling technique, SORS grants the user the ability to chemically analyse subsurface layers of a range of turbid semi-opaque and powdered materials (Parker, 2017).

1.7.5. Chemometrics and Machine Learning

While the application of chemometrics is relatively novel, it has already had a great impact on the field of spectroscopy (Dang and Marini, 2019; Geladi, 2003). As a result, chemometrics software is now often integrated with spectroscopic laboratories and process instrumentation. The emergence of chemometrics can be attributed to two factors: (1) instrumentation giving multivariate responses during sample analysis and (2) increasing availability of computers (Geladi, 2003). Moreover, traditional univariate statistics are no longer sufficient in the description and modelling of chemical experiments.

Chemometrics refer to the use of mathematical and statistical methods to extract relevant chemical information and correlate quality parameters or physical properties with analytical data. The combination of spectroscopy with exploratory data analysis, classification and regression methods allows for effective, high-performing, rapid and non-destructive analysis (Biancolillo and Marini, 2018). The application of chemometrics allows redundant information to be reduced from the data matrix, allowing for greater data stability, understanding and the removal of noise or less useful information (Geladi, 2003).

Chemometrics can be supervised, whereby data analysis is performed on labelled training data, or unsupervised, with data analysis being performed on data without prior class labelling (Lima and Barbosa, 2019). Examples of supervised approaches include decision trees (DT), logistic regression (LR), random forest (RF) and support vector machine (SVM) (Abraham and Kellogg, 2021). While unsupervised approaches comprise the likes of correlation in wavenumber space (CWS), PCA and SOMs (Galindo-Prieto *et al.*, 2025).

Supervised and unsupervised chemometrics can be further separated into linear and non-linear approaches (Allegrini and Olivieri, 2022). Linear methods include hierarchical cluster analysis (HCA), PCA, principal component regression (PCR) and partial least squares regression (PLS) (Scatigno *et al.*, 2020). Whereas, non-linear methods include artificial neural networks (ANN), SVM and SOMs (Rocha, Prado and Blonder, 2020). Linear datasets are visualised as straight lines with small residuals (errors) demonstrating random behaviour (Rocha, Prado and Blonder, 2020). In contrast, non-linear data refers to data that shows curvature, saturation or complex

shapes, with residuals demonstrating a greater variance or pattern (Chesnaye *et al.*, 2024). Successful application of spectroscopy paired with chemometrics has been shown in the fields of aquaphotomics, forensics and pharmaceuticals (Biancolillo and Marini, 2018; Sauzier, Bronswijk and Lewis, 2021; Tsenkova *et al.*, 2018).

CWS matches the correlation coefficient (r) value of a test spectrum (A) against a reference spectrum (B) through the calculation of the momentum product between spectra A and B (Equation 1.9) (Assi *et al.*, 2020).

Equation 1.9. Correlation coefficient based on momentum product.

$$r_p = \frac{\sum(A_i - \bar{A})(B_i - \bar{B})}{\sqrt{\sum(A_i - \bar{A})^2 \sum(B_i - \bar{B})^2}}$$

Where, r_p is the correlation coefficient value corresponding to the momentum product,

A_i is the test spectrum of A,

B_i is the reference spectrum B and is the average spectrum of B (Brereton, 2006).

R values of -1 demonstrate completely dissimilar spectra, whereas +1 r values represent identical spectra. Sample noise and light interference often means that obtaining an r value of +1 is difficult, therefore a threshold of ≥ 0.95 is utilised as a match (Brereton, 2006).

PCA is often utilised to analyse multidimensional data, through the reduction of the original matrix's dimensionality into two subspaces (scores and loading) (Equation 1.10) (Jolliffe and Cadima, 2016). Scores demonstrate the distribution of spectra and allow the identification of clusters and patterns (Camargo, 2022). Loadings highlight significant spectral bands, which provide insight into the fingernail's biochemical properties (Vieira, 2012). PCs are a linear combination of the original data and are calculated/ordered based on size (Jolliffe and Cadima, 2016). The first dimension (PC1) provides explainability for the highest variance, the second dimension (PC2) for

the second highest variance, the third dimension (PC3) explains the third highest variance and so on... (Camargo, 2022).

Equation 1.10. PCA equation.

$$X = T . P + q$$

Where, X is the original data matrix ($m \times P$),

T is representative of the scores ($m \times n$),

P represents the loading ($n \times p$) and is representative of the residuals ($m \times p$) (Jolliffe and Cadima, 2016).

PCA models are assessed in terms of type I and II errors. Type I errors (false negatives) occur when scores of the same characteristics are not clustered together. While type II errors (false positives) occur when one score is clustered within another set of scores with different characteristics (Assi *et al.*, 2020). PCA is routinely paired with vibrational spectroscopic methods and has shown success in the detection of Alzheimer's disease and tuberculosis, as well as cancers including lung, oral and prostate cancer (Ghosh *et al.*, 2019; Medipally *et al.*, 2020; Ullah *et al.*, 2020; Yang *et al.*, 2021).

SOMs compliment PCA, with their ability to work with non-linear data in lower dimensions (Kohonen and Somervuo, 2002; Sacco *et al.*, 2017). For example, SOMs were paired with PCA alongside other MLAs such as fuzzy support vector machine for the diagnosis of CHD (Nilashi *et al.*, 2020) Moreover, SOMs can detect spectral features without awareness of spectral class/membership (Assi *et al.*, 2023). Composed of organised neurons, SOMs produce a similarity map of the input data. Therefore, neurons are connected to their neighbours through topological connections and each neuron i has a d -dimensional weight vector (Equation 1.11) (Chaudhary, Bhatia and Ahlakwat, 2014; Yang and Palaniswami, 1992).

Equation 1.11. Weight vector.

$$W = W_{i1}, W_{i2} \dots W_{id}$$

Where, W is the weight,

and i is 1, 2,... (Chaudhary, Bhatia and Ahlakwat, 2014).

The conventional SOM learning algorithm can be explained through three steps. The first step includes initialising the weight vectors w_i 's of the $m \times n$ neurons. An input vector $x(t)$ is then randomly selected and inputted to all neurons at the same time and in parallel. Finally, the winning neuron is identified (Equation 1.12).

Equation 1.12. Identification of the winning neuron.

$$c = \arg(\min_{1 \leq i \leq mn} \{\|w_i(t) - x(t)\|\})$$

Where, c is the winning neuron,

$\|\cdot\|$ is the Euclidean distance measure,

$x(t)$ and $w_i(t)$ are the input and weight vector of the neuron i at iteration (Chaudhary, Bhatia and Ahlakwat, 2014).

The weight vector of the neurons is then updated (Equation 1.13).

Equation 1.13. Updating of weight vector.

$$w_i(t + 1) = w_i(t) + h_{c,i}(t)[x(t) - w_i(t)]$$

Where, $h_{c,i}(t)$ is Gaussian neighbourhood function (Chaudhary, Bhatia and Ahlakwat, 2014).

The Gaussian neighbourhood is then calculated (Equation 1.14).

Equation 1.14. Gaussian neighbourhood.

$$h_{c,i}(t) = a(t) \cdot \exp \left(-\frac{\|r_c - r_i\|^2}{2\sigma^2(t)} \right)$$

Where, r is the coordinate position of the neuron located on the map,

$a(t)$ is the learning rate,

$\sigma(t)$ is the width of neighbour radius (Chaudhary, Bhatia and Ahlakwat, 2014).

The $a(t)$ and $\sigma(t)$ both decrease monotonically using equations 1.15 and 1.16, respectively.

Equation 1.15. Monotonical decrease of $a(t)$.

$$a(t) = a(0) \left(\frac{a(T)}{a(0)} \right)^{t/T}$$

Where, T is the training length,
and $a(t)$ is the learning rate (Chaudhary, Bhatia and Ahlakwat, 2014).

Equation 1.16. Monotonical decrease of $\sigma(t)$.

$$\sigma(t) = \sigma(0) \left(\frac{\sigma(T)}{\sigma(0)} \right)^{t/T}$$

Where, T is the training length,
and $\sigma(t)$ is the learning rate (Chaudhary, Bhatia and Ahlakwat, 2014).

Pre-processing algorithms can improve the quality and interpretation of spectra. Common algorithms include MSC, standard normal variate (SNV), D1 and D2. MSC is commonly employed to minimise light-scattering effects (Choi *et al.*, 2021). Through the production of a new spectrum, a linear combination of the original spectrum, the offset of scattered light is corrected (Equation 1.17).

Equation 1.17. Multivariate scatter correlation equation (Varmuza and Filzmoser, 2016).

$$y_{\text{MSC},i} = \frac{(y_i - a)}{b}$$

Where, $y_{\text{MSC},i}$ is the corrected spectrum value,

y_i is the original spectrum value,

a is the intercept of the line,

and b is the slope of the line.

In this respect, each spectrum is adjusted to fit the mean spectrum as closely as possible. Thus, values a and b are calculated via the mean spectrum and stored so that the same transformation can be carried out to test sample spectra.

SNV is another transformation applied to spectroscopic data. Normalisation of spectra is performed via subtracting each spectrum by its own mean and dividing by its own standard deviation (Equation 1.18) (Barnes, Dhanoa and Lister, 1989).

Equation 1.18. Standard normal variate transformation (Barnes, Dhanoa and Lister, 1989).

$$Z_i = \frac{(y_i - \bar{y})}{s}$$

Where, $\bar{y}$ is the mean spectral value over complete spectrum,

s is the standard deviation of the spectral values over the complete spectrum,

y_i is the spectral value at wavelength i .

Unlike, MSC transformations, which require mean spectra to be stored, SNV transformations ensure that corrected spectra possess a similar scale to the original spectra. Additionally, D1 or D2 can be applied to MSC or SNV treated data for further correction of offset and baseline. D1 removes a constant offset, while D2 removes the offset and a linear term (Rinnan, Berg and Engelsen, 2009). During this application, a second-order polynomial is fitted to the data by least square using 10 data points, five before and five after the point at which the derivative is required. This process is repeated across the whole spectrum, with one data point being moved each time. D2 removes the offset completely. Often in the form of a Savitzky-Golay filter, a polynomial is fitted in a symmetric window of the raw data and allows the identification of the derivative at the centre point i (Savitzky and Golay, 1964).

1.8. Scanning electron microscopy (SEM)

In practice, SEM is widely employed to observe the surface phenomena of materials. An electron beam of relatively low energy (few hundred eV to 50 kV) and a fine spot size (~ 5) is scanned across the sample's surface (Akhtar *et al.*, 2018; Kumar, 2013). During the penetration and passage of electrons, several interactions occur and result in the emission of electrons and photons from the sample. The most common interactions include elastic and inelastic scattering. Elastic scattering is the result of primary electrons deflecting upon contact with the sample's atomic nucleus or electrons of comparable energy (Mutalib *et al.*, 2017). As the deflection of scattered electrons occur at an angle of $> 90^\circ$, they are referred to as back scattered electrons, which are often employed for sample imaging. In contrast, inelastic scattering occurs when low energy secondary electrons are emitted from the sample after they have been bombarded with the primary beam electrons (Egerton, Hwang, Zhu, 2023).

Through the exposure of a low-energy electron beam, information regarding the sample's chemistry, composition, crystallographic information, morphology, orientation of grains and topography are collected (Akhtar *et al.*, 2018). Hence, SEM is a highly valuable tool for material and sample characterisation.

To determine the elemental composition of samples, energy dispersive X-ray (EDX) spectroscopy is often utilised within SEM (Mutalib *et al.*, 2017). During the collision with the electron beam in typical SEM, the sample interacts with the beam, to produce

characteristic X-rays, which are differentiated from the X-ray emission spectrum (Goldstein *et al.*, 2003). The X-ray is the result of the primary beam of electron interaction, with the sample atoms' nuclei (Mutalib *et al.*, 2017). The primary electron beam excites the electrons present within the atom's nucleus, causing them to eject from the nucleus and create an electron hole. An outer shell electron, of higher energy, will replace the missing ejected electron and in doing so releases the superfluous X-ray. The emitted X-ray consists of an X-ray continuum, which is generated by the deceleration of the electron and a characteristic X-ray. This characteristic X-ray is created as a result of the outer shell electron filling the electron hole within the nucleus shell (Mutalib *et al.*, 2017). Electronic transitions are characteristic of each chemical element, therefore are highly beneficial for material microstructural characterisation (Girão, Caputo and Ferro, 2017).

1.9. Fingernails

The use of fingernails as an alternative biological matrix is becoming increasingly recognised in fields such as forensic science, medical diagnostics and toxicology (Baumgartner, 2014; Lee and Lipner, 2022; Wilson *et al.*, 2023a; Wilson *et al.*, 2023b; Wilson *et al.*, 2022). As a biological matrix, fingernails offer several advantages related to structure, biochemical properties and practicality over alternative keratin-based samples such as hair. Composed primarily of keratin, fingernails and hair can incorporate biochemical information over extended growth periods (Baswan *et al.*, 2017). However, fingernails offer greater stability and reduced susceptibility to external contaminations. For example, hair is frequently exposed to cosmetic treatments including bleaching agents, dyes, shampoos and other styling products, which can alter its chemical composition and interfere with measurements (Baumgartner, 2014). Whereas, fingernails are less commonly subject to chemical processing, experiencing lower levels of environmental and/or cosmetic exposure. Therefore, their biochemical signature is well maintained. In addition, fingernails often exhibit a slower and more consistent growth rate in comparison to hair (Bali *et al.*, 2023). Thus, allow for a greater integration of systemic biochemical changes associated with disease presence.

Fingernails provide protection to the phalanges, enhance dexterity and facilitate scratching (Baswan *et al.*, 2017). They also possess the ability to accumulate

endogenous constituents, trace elements/heavy metals and xenobiotics, providing a retrospective record of compliance or exposure to drugs, as well as the presence of disease over extended periods of time (Morini *et al.*, 2012; Li *et al.*, 2025; Wilson *et al.*, 2023a; Wilson *et al.*, 2023b).

In forensic toxicology, fingernails comprise a non-invasive matrix for the detection of chronic drug use, offering an alternative to traditional biological matrices such as blood or urine (Cobo-Golpe, Castros-Ríos and Lendoiro, 2024). During fingernail growth, at the nail bed and germinal matrix, drugs are incorporated into the fingernail plate, allowing for 3-9 months retrospective reflection (Cappelle *et al.*, 2014; Shu *et al.*, 2015). The extended detection window of fingernails over previously utilised matrices is advantageous in several applications such as monitoring drug compliance rates, especially in drug rehabilitation programs, investigating prenatal drug exposure and post-mortem analyses. Fingernails have also been utilised as evidence of chronic drug exposure in cases of drug distribution (Wilson *et al.*, 2023a; Wilson *et al.*, 2023b; Wilson *et al.*, 2022). Beyond toxicology, fingernails can provide a multifaceted platform for the detection of disease by combining observable physical changes to the fingernails colour, shape and biochemical composition. As a non-invasive matrix, fingernails can be utilised for monitoring of chronic conditions.

Potential biological and external confounding variables can also influence the composition of fingernails and should be considered during spectral analysis and interpretation. Firstly, age not only influences fingernail growth rate but also affect compositions (Albucker *et al.*, 2024). Ageing has been closely associated with changes in keratin structure, as well as variations in thickness and lipid content (Lai-Cheong and McGrath, 2009). Collectively, the aforementioned factors impact the incorporation of endogenous constituents and creates inter-individual variability in fingernail spectra. Diet represents another important factor, as trace elements and minerals from the bloodstream are incorporated into the fingernails during growth (Li *et al.*, 2025). As a result, fingernail reflect long-term dietary patterns and overall nutritional status. Ethnicity can further shape fingernail composition through both genetic and cultural influences, including differences in diet. Previous studies have shown that the concentration of trace elements including calcium and magnesium varied across populations and reflected dietary habits (Sonntag *et al.*, 2025). Moreover, further work indicated that ethnic differences in fingernail morphology and

growth rate altered optical properties including spectral signals and scattering (Hakki *et al.*, 2023; Zhang *et al.*, 2012). Finally, cosmetic fingernail treatments such as nail polish or acrylic overlays, can introduce exogenous compounds and modify surface properties (López-López, Vaz and García-Ruiz, 2015). Factors that can interfere with measurements and alter the fingernail's spectral signatures.

1.9.1. Fingernail structure

The human fingernail unit is comprised of four main structures, those being: (1) the proximal nail fold (PNF), (2) fingernail matrix, (3) fingernail bed and (4) hyponychium (Figure 1.6). Collectively, the aforementioned structures form the fingernail plate, which is a flat, keratinised structure that sits on the upper surface of the fingers (Baswan *et al.*, 2017; De Berker and Baran, 2007; Haneke, 2017). Cells present in the fingernail plate are closely packed and lay perpendicular to the fingernail plate surface plane.

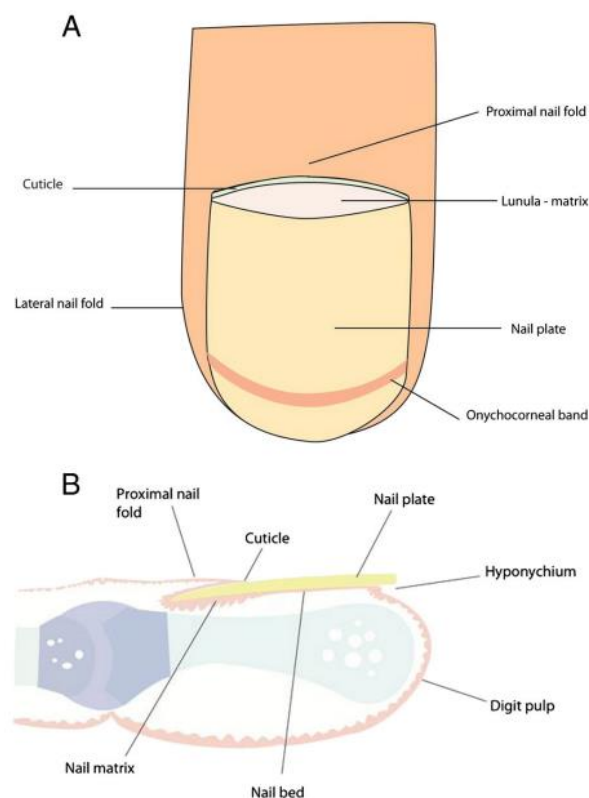


Figure 1.6. (a) Plane and (b) sagittal viewpoints of fingernail unit (De Berker, 2013). Used with permission from Elsevier. Copyright © 2013 Elsevier, Amsterdam. All rights reserved.

The wedge-shaped fold of skin that surrounds the dorsum of the finger is referred to as the PNF. This section of the fingernail covers approximately one-fourth of the total fingernail length. The lateral fold is an extension of the PNF and comprises two layers of epidermis. The first layer, the dorsal layer, forms the dorsum of the fingernail epidermis, while the ventral layer forms the eponychium (Baswan *et al.*, 2017). The outermost layer of epidermis, the stratum corneum of the eponychium, forms a thick keratinous rim, also known as the cuticle. The cuticle acts as a margin around the PNF and alongside the PNF and lateral folds, provides protection against penetration of toxins and water. In cases where the cuticle is disrupted, bacterial or fungal infections can occur (Dawber, De Berker and Baran, 2001).

Directly beneath the PNF lies a small section of highly proliferative epidermal tissue. This area is referred to as the fingernail matrix and is responsible for the production of the fingernail's plate (Baswan *et al.*, 2017). The epithelium of the fingernail matrix is split into three sections: (1) proximal, (2) medial and (3) distal (Haneke, 2017). The fingernail matrix is primarily covered by the PNF; however, the distal portion is located beneath the lunula. The pigmentation of the lunula varies race to race and is determined through the concentration of melanocytes within the fingernail matrix (Singal and Bisherwal, 2020). Without aesthetic intervention, the lunula is seen in the index and middle finger and thumb (Haneke, 2017).

On average, the fingernail plate is comprised of 196 cell layers, which are distributed into three tightly bound layers: the (1) dorsal, (2) intermediate and (3) ventral layers (De Berker, Mawhinney and Sviland, 1996). The ratio of thickness varies across the three layers in a ratio of 3:5:2 (Kobayashi *et al.*, 1999). As the outer layer, the dorsal acts as a primary barrier for fingernail permeation. Small variation in the endogenous constituent content has been noted between the highlighted layers. For example, sulfhydryl groups are predominant in the dorsal layer, whereas the activity of cystine is prevalent in the intermediate plate (Dawber, Berker and Baran, 2001). Cells within the dorsal plate, possess thickened plasma membranes and are enucleated, which is demonstrated via a lack of acid phosphate activity (Baswan *et al.*, 2017). On the other hand, the intermediate layer has some eosinophilic cytoplasm and nuclear remnants. The three layers of the fingernail plate are held together firmly via desmosome glue spots and interdigitations. Within the intermediate layer, keratin intermediate fibres or

filaments are aligned well and oriented in a transverse direction laterally. Therefore, are perpendicular to the axis of nail growth. In contrast, fibres in the dorsal and ventral layers are packed and overlapping, with no preferred orientation (Dawber, 1980; Farren, Shayler and Ennos, 2004; McKittrick *et al.*, 2012). The fingernail plate's strength lends itself to the close alignment of keratin fibres, as well as the high number of disulfide cross-links associated with high cysteine content (Gupchup and Zj, 1999).

The fingernail bed is a non-cornified epithelium surface and is devoid of granular layers and sebaceous glands (Haneke, 2017). The bed lies beneath the fingernail plate, extending from the fingernail matrix to the hyponychium. As this section of the fingernail contributes cell layers to the ventral layer of the fingernail plate, it is often referred to as the ventral matrix and is considered the transitional zone (Baswan *et al.*, 2017). Here, living cells are keratinised and integrated into the fingernail plate (keratinocytes). The pink colour associated with the fingernail bed is attributed to its rich vascular supply, alongside corpuscles and nerve endings, which are responsible for sensitivity to light, pressure and touch (Dawber, Berker and Baran, 2001).

Located at the end of the fingernail bed, the hyponychium provides an additional protective function in collaboration to the cuticle, lateral folds and PNF (Baswan *et al.*, 2017). Showing a broad granular layer, the hyponychium seals the nail and fingernail bed (Haneke, 2017).

1.9.2. Biological composition of fingernails

The biochemical composition of fingernails reflects a sophisticated integration of amino acids, lipids, proteins and trace elements. This composition grants the fingernails a hard and rigid structure, further supporting its protective mechanical role. Changes in the biochemical composition of fingernails can provide insight into nutritional status, presence of disease and toxic exposures.

1.9.2.1. *Amino acids in fingernails*

The biochemical composition of fingernails is centred primarily around hard α -keratin, in which is rich in cysteine, a sulphur-containing amino acid (Baswan *et al.*, 2017). Cysteine is imperative for the formation of disulfide bonds (S-S) between keratin strands. The covalent cross-links are imperative for maintaining the fingernail's

structural integrity. Other amino acids present within the fingernail include arginine, cystine, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, tryptophan and tyrosine (Brzózka and Kolodziejski, 2017).

1.9.2.2. *Lipids in fingernails*

In addition to amino acids, a small amount of lipids, primarily cholesterol, exist in the fingernail (Maeda and Iwashita, 2022). For example, the lipid content of the stratum corneum is approximately 10%, while in fingernails the lipid content can be as little as 0.1-1% (Baswan *et al.*, 2017). While it was previously suggested that the uptake or rate of water loss was influenced by the extraction of lipids, additional research has now indicated that the lipid pathway in the water's diffusion process across the fingernail plate is insignificant (Baden, 1970).

1.9.2.3. *Proteins in fingernails*

While there are three main classes of proteins (conjugated, fibrous and globular), the fingernail plate is comprised of a fibrous protein called scleroprotein. These proteins contain a high level of sulphur. Polypeptide chains form keratin and can exist in a curled helical conformation (α -keratin) or side-by-side pleated conformation (β -keratin) (Baswan *et al.*, 2017). In humans, α -keratins are the primary constituents of fingernails and hair, as well as the epidermal layer of the skin (McKittrick *et al.*, 2012). While in other mammals, β -keratin make up hooves, horns and quills. (McKittrick *et al.*, 2012). Two types of α -keratin exists, the first being classified as hard α -keratin, which is derived embryologically from epithelial cells and the second being soft α -keratin. Hard α -keratin is the main constituent for cornified mammalian tissues including fingernails and contains microfibrils, which are packed relatively ordered. In contrast, soft α -keratin is the main component of the epidermis and epithelial tissues, therefore possess loosely packed bundles of cytoplasmic microfibrils, which provide epithelial cells with mechanical resilience (Brzózka and Kolodziejski, 2017). A ratio of 80:20 is seen in fingernails for hard and soft α -keratin, respectively (Lynch *et al.*, 1986).

Keratin proteins are assembled as coils, whereby two right-handed helical polypeptide chains (monomers) are twisted into a left-handed coil (dimer) (Brzózka and Kolodziejski, 2017). The coils are then strengthened through the presence of disulphide cross-links between residues of cysteine. The dimers aggregate through

protofilaments and protofibrils, forming intermediate filaments (IFs) (Parry *et al.*, 2007; Wang *et al.*, 2016). IFs are low in sulphur, while keratin associated proteins (KAPs), found surrounding the α -keratin, are high in sulphur and glycine-tyrosine-proteins (Brzózka and Kolodziejski, 2017).

1.9.2.4. Trace elements in fingernails

Several minerals are found in the fingernails and include aluminium (Al), bromine (Br), calcium (Ca), chlorine (Cl), copper (Cu), iron (Fe), magnesium (Mg), potassium (K), silicon (Si) and zinc (Zn) (Brzózka and Kolodziejski, 2017). However, environmental exposure can influence the level at which trace elements occur (Barba *et al.*, 2010; Nowak and Chmielnicka, 2000; Sukumar, 2006). It has also been suggested that biological sex influences the elemental composition of fingernails. For example, fingernails taken from males showed lower levels of gold (Au), Bi and Si, but higher levels of Na, Mg and K than females (Rodushkin and Axelsson, 2000). Dittmar, Dindorf and Banerjee (2008) also suggested that fingernails taken from female participants demonstrated higher sulphur content than males and suggested a lower nitrogen/sulphur ratio. Nonetheless, it is worth noting that the increased sulphur activity does not mean a greater number of keratin disulphide bonds.

1.9.3. A window into systemic disease

Subtle fingernail abnormalities can provide insight into the presence of systemic diseases (Singal and Arora, 2015). Changes in colour, size, shape or texture of fingernails act as indicators for systemic disease and when successfully interpreted can guide diagnosis and management of disease (Gollins and Berker, 2021). Despite, often being overlooked in day-to-day practices, fingernails act as a window of disease for cardiac, gastrointestinal and hepatic, haematological, renal and respiratory disorders.

Generally, the manifestation of systemic illness is marked through transverse grooves in the fingernail plate. These grooves are referred to as Beau's lines and occur due to a temporary reduction in the fingernail matrix's activity (Gollins and Berker, 2021). In cases of prolonged systemic upset, complete division of the fingernail plate can occur. Nonetheless, fingernail shedding only follows when the fingernail is lost (onychomadesis) (Gollins and Berker, 2021).

1.9.3.1. *Cardiac disorders*

The presence of bacterial endocarditis, a serious infection of the heart's inner lining (endocardium), has previously been detected through the identification of splinter haemorrhages. Splinter haemorrhages, red, purple or brown in colour, appear as small, linear subungual structures at the distal third of the fingernail plate (Gollins and Berker, 2021). The appearance of such defects is attributed to damaged capillaries and platelet microthrombi within the fingernail bed. Nonetheless, splinter haemorrhages have also been associated with vasculitis and trauma to the fingernails (Fanning, Aronson and Osler, 1977; Yoshida and Taniguchi, 2021). Further signs of bacterial endocarditis include Osler's nodes and Janeway lesions. The former are described as tender red nodules, that develop in the finger pulp at the proximal and lateral fingernail folds, while the later are distal, non-tender nodules that affect the palmoplantar surfaces (Gollins and Berker, 2021).

The presence of congenital heart disease was noted through fingernail clubbing, which is defined as an increased angle present between the fingernail plate and PNF (Gollins and Becker, 2021). This angle is referred to as the Lovinbond's angle and first occurs due to a sponginess at the fingernail base. The soft texture allows movement of the fingernail plate and is proposed to be caused by fibrovascular hyperplasia. Pathophysiological mechanisms of clubbing include increased blood flow and proliferation of fibroblasts, secondary to vascular endothelial growth factor (Gollins and Becker, 2021).

1.9.3.2. *Haematological disorders*

Pale nail beds have been observed in patients with anaemia, however, classically koilonychia (spoon shaped nails) have been associated with iron deficiency (Figure 1.7) (Hogan and Jones, 1970). As a fingernail defect, koilonychia causes the fingernail

to concave, everting the distal and lateral edges and therefore giving the fingernails a spoon-like appearance (Gollins and Berker, 2021).



Figure 1.7. Example of koilonychia (Gollins and Berker, 2021).

1.9.3.3. Renal disorders

Half and half fingernails are characterised by proximal white ground-glass changes (Figure 1.8) (Gollins and Berker, 2021). Unlike Terry's nails, which are characterised by a white fingernail plate, half and half fingernails possess a distal pink band, >20% of the fingernail bed surface area. On occasion, half and half fingernails can also appear brown or red in colour (Gollins and Berker, 2021).

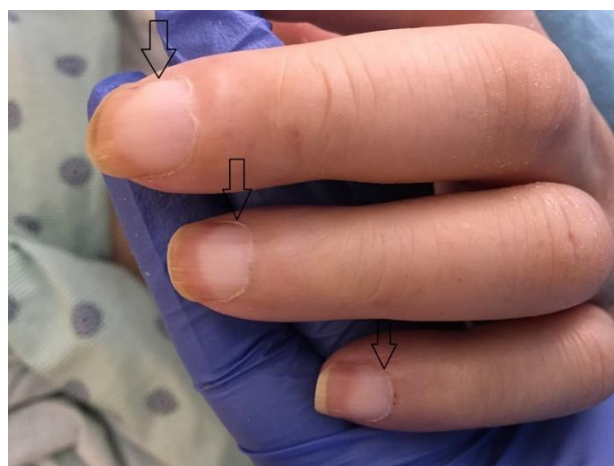


Figure 1.8. Half and half fingernails (Pitukweerakul and Pilla, 2016). Used with permission from Elsevier. Copyright © 2016 Elsevier, Amsterdam. All rights reserved.

1.10. Research rationale

As previously mentioned, the incidence of CVDs and DM has increased substantially in the last three decades, becoming some of the most prevalent and costly NCDs worldwide (Cesare *et al.*, 2024; Vaduganathan *et al.*, 2022). A close link exists between CVDs and DM, with CVDs being the most prevalent cause of morbidity and mortality in diabetic patients. Moreover, when diagnostic equipment is available such as the likes of CT scans and ECGs, interpretations are made based on limited user-knowledge or inaccurate automated computer responses. As a result, patients can be victims to diagnostic errors and develop further disease-related complications, which not only reduce the patient's quality of life (QoL), but create additional medical expenses linked to the administration of further treatment and therapies. Limited equipment and training also influence the collection and use of traditional biological matrices including blood, plasma, serum and urine. Although the aforementioned matrices are routinely applied for disease detection, they are intrusive, invasive and often require extensive sample-preparation prior to analysis. Therefore, their use can be limited. While there has been some use of fingernails as an alternative matrix, few studies have explored the use of fingernails for the detection of CVDs and/or DM. Moreover, a limited number of studies have explored the use of vibrational spectroscopic techniques for detection of CVDs and/or DM. Therefore, this research focussed on the application of fingernails as an alternative biological matrix for the detection of CVDs and/or DM through the application of novel vibrational spectroscopy techniques including IR, NIR and Raman spectroscopy.

1.11. Research questions

The research questions to be investigated included:

- What are the current techniques utilised within the literature for the detection of CVDs and/or DM?
- What types of biomarkers, molecules or other endogenous constituents indicate the presence of CVDs or DM?
- What risk factors, modifiable or non-modifiable in nature, increase a patient's risk of developing a CVD and/or DM?

- Are fingernails a feasible alternative biological matrix for the detection of CVDs and/or DM?
- Can vibrational spectroscopic techniques including IR, NIR and Raman spectroscopy provide insight into the participant's health or disease status?

1.12. Aims and objectives

1.12.1. Aims

The overall aim of this research was to develop an intelligent framework for the detection of CVDs and/or DM using vibrational spectroscopic techniques and fingernails as an alternative biological matrix.

1.12.2. Objectives

Specific objectives were set to fulfil the research aim and included:

- Investigating the current state of diagnosis for CVDs and/or DM in terms of disease detection techniques and biomarkers within the literature.
- Establishing relationships between patient- (sociodemographic and lifestyle) and clinical-related factors and the presence of disease.
- Constructing and validating ATR-FTIR, NIR and Raman spectroscopic libraries that consist of spectroscopic datasets of biomarkers and endogenous constituents encountered in fingernails of healthy, CVD, diabetic and CVD-diabetic participants.
- Optimising and validating spectroscopic methods for the characterisation of fingernails non-destructively in reference to sample measurement modes and instrumental parameters.
- Building anonymised inclusive spectroscopic datasets of fingernails for participants with CVDs and/or DM of different ages, biological sexes and ethnicities, incorporating patient- (sociodemographic and lifestyle) and clinical-related factors alongside spectroscopic signatures.

- Exploring patterns in the spectroscopic data of healthy and diseased participants, considering varying characteristics such as age, biological sex and ethnicity using MLAs and classification models.

Research questions and objectives were fulfilled via appropriate methods and discussed in corresponding chapters, as demonstrated by Table 1.3.

Table 1.3. Relationships between research questions, objectives and how the aforementioned were fulfilled in terms of methods and corresponding chapters.

Research questions	Objective(s)	Method(s)	Chapter(s)
What are the current techniques utilised within the literature for the detection of CVDs and/or DM?	Investigating the current state of diagnosis for CVDs and/or DM in terms of disease detection techniques and biomarkers within the literature.	Systematic review of existing literature.	Two
What types of biomarkers, molecules or other endogenous constituents indicate the presence of CVDs or DM?	Investigating the current state of diagnosis for CVDs and/or DM in terms of disease detection techniques and biomarkers within the literature.	Systematic review of existing literature.	Two
What risk factors, modifiable or non-modifiable in nature, increase a patient's risk of developing a CVD and/or DM?	Establishing relationships between patient- (sociodemographic and lifestyle) and clinical-related factors and the presence of disease. Building anonymised inclusive spectroscopic datasets of fingernails for participants with CVDs and/or DM of different ages, biological sexes and ethnicities, incorporating patient- (sociodemographic and lifestyle) and clinical-related factors alongside spectroscopic signatures.	Anonymous questionnaires answered by participants.	Three
Are fingernails a feasible alternative biological matrix for the detection of CVDs and/or DM?	Optimising and validating spectroscopic method for characterisation of fingernails non-destructively in reference to sample measurement modes and instrumental parameters.	Vibrational spectrometers including ATR-FTIR, NIR and Raman spectrometers. SEM equipped with specialised detectors such as the EDX and STEM detectors.	Four, five, six and seven

Can vibrational spectroscopic techniques including ATR-FTIR, NIR and Raman spectroscopy provide insight into the participant's health or disease status?	Constructing and validating ATR-FTIR, NIR and Raman spectroscopic libraries that consist of spectroscopic datasets of biomarkers and endogenous constituents encountered in fingernails of healthy, CVD, diabetic and CVD-diabetic participants.	Vibrational spectrometers including ATR-FTIR, NIR and Raman spectrometers.	Four, five and six
	Optimising and validating spectroscopic methods for characterisation of fingernails non-destructively in reference to sample measurement modes and instrumental parameters.	Patterns in the spectroscopic data were explored through MLAs including CWS, PCA and SOMs.	
	Exploring patterns in the spectroscopic data of healthy participants and participants with NCDs, considering varying characteristics such as age, biological sex and ethnicity using MLAs and classification models	Classification models such as bagged and boosted tree ensemble, cubic and quadratic SVM and SVM kernel were also applied to spectroscopic data for the prediction of disease.	
ATR-FTIR: Attenuated total reflectance-Fourier transform infrared; CVDs: Cardiovascular diseases; CWS: Correlation in wavenumber space; DM: Diabetes mellitus; EDX: Energy dispersive X-ray; MLAs: Machine learning algorithms; NIR: Near-infrared; PCA: Principal component analysis; SEM: Scanning electron microscopy; SOMs: Self-organising maps; STEM: Scanning transmission electron microscopy; SVM: Support vector machine.			

1.13. Key messages

- CVDs and DM contribute to an increase in morbidity, mortality and healthcare expenditure on a global scale.
- Diagnostic errors are the largest cause for malpractice claims, furthering the medical and economic burden seen in LMICs.
- Patients with CVDs are likely to develop DM and vice versa, due to their shared epidemiology and pathophysiological characteristics as comorbidities, as well as shared risk factors such as physical inactivity and smoking habits.
- Traditional disease detection techniques are often expensive and, in some cases, invasive and intrusive. They commonly rely on user-knowledge for use and interpretation, which in LMICs is restricted.
- It is essential that alternative disease detection techniques and alternative matrices are explored, ensuring that sensitivity is not comprised and patient's safety is of the utmost importance.

Chapter two: Identification of biomarkers used in the diagnosis of cardiovascular diseases and diabetic mellitus: a systematic review of quantitative studies

To diagnose and manage CVDs and DM, clinical practices should optimise diagnostic panels with a range of biomarkers. In such cases, non-invasive and non-intrusive biological matrices such as fingernails can improve diagnostic monitoring. Previous literature has focused mainly on traditional biological matrices such as blood, plasma and serum. This systematic review investigated studies that looked at the identification of diagnostic biomarkers for CVDs and DM. A total of 19 studies were obtained related to the identification of 78 CVD and 224 DM biomarkers. The primary biomarkers identified for CVDs were C-reactive proteins (CRP), growth differentiation factor 15 (GDF15) and neurogenic locus notch homolog protein 1 (NOTCH1). Whilst the primary biomarkers for DM were alpha-2-macroglobulin (A2M), glutamine, isoleucine, linoleic acid, myristoleic acid, palmitic acid, triglycerides, tyrosine and valine. For the identification of the aforementioned biomarkers both chromatographic and spectroscopic techniques were employed, with the most frequently recorded being liquid chromatography with tandem mass spectrometry (LC-MS/MS). Biomarker identification was carried out using a range of biological matrices including: blood, dried blood spots, fingernails, plasma, saliva, serum and urine. The findings demonstrated that a combination of biomarkers allowed for a superior diagnostic technique of CVDs and/or DM.

2.1. Introduction

In recent years, the WHO has seen an increased prevalence of CVDs and DM. Figures now estimate that 20.5 million and 2 million lives are lost annually to CVDs and DM, respectively (WHO, 2025; WHO, 2024).

Diagnosis of CVDs and DM is not straightforward and is often undertaken at a late stage or with difficulty. Furthermore, diagnosis of CVDs is particularly difficult as females display atypical symptoms such as abdominal pain, back/neck pain, dyspnoea, fatigue, indigestion, palpitations and nausea, which are often delayed in comparison to the onset of symptoms in males (Keteepe-Arachi and Sharma, 2017). Yet, males typically display symptoms of chest pains, which are often recognised by doctors as a symptom of CVDs (Gao *et al.*, 2019). When females do show indications of chest pain, their symptoms are often misdiagnosed as anxiety or stress-related issues. Misdiagnosis often leads to the development of angina and heart attacks (Ford and Berry, 2020). Similarly, DM is often misdiagnosed as initial symptoms are non-specific (Maahs *et al.*, 2010). Through misdiagnosis of CVDs and DM, patients are at risk for developing further adverse complications. The misdiagnosis of DM has shown an increased number of DKA cases, creating prolonged recovery, morphological and functional brain changes, which have been associated with adverse neurocognitive outcomes, longer hospital visits and increased health care expenses (Cameron *et al.*, 2014; Vanelli *et al.*, 2007; Garibaldi and Becker, 2016).

Prevention strategies for CVDs and DM include modifiable risk factors such as body mass index (BMI), diet, increased cholesterol, high blood pressure, lack of physical activity and smoking (Bellou *et al.*, 2018; Ruan *et al.*, 2018). Research has also identified a link between CVDs and DM. For example, sufferers of DM are more likely to develop a CVD and undergo a CV event (Leon and Maddox, 2015). In cases where patients do not display the aforementioned risk factors and/or display typical symptoms, diagnosis is difficult. Thus, it is crucial that diagnostic testing is utilised and explored across a wide variety of diagnostic biomarkers and metabolites.

Research has found that CVDs and DM were best managed when diagnosed early and in turn reducing disease-related morbidities and mortalities (Habetha, 2006). This systematic review, therefore, aimed to identify and review a range of biomarkers and metabolites used in diagnosis of CVDs and/or DM. The review objectives were:

- To identify what biomarkers/metabolites are monitored for the diagnosis of CVDs and DM.
- To establish the most common analytical techniques employed for the identification of biomarkers/metabolites in biological matrices.

- To assess the most commonly used matrix for the diagnosis of CVDs and DM.
- To determine frequently reported biomarkers/metabolites utilised for the diagnosis of CVDs and/or DM.

2.2. Methods

2.2.1. Search Strategy

Six databases were searched between January 2002 and January 2022 and were: British Nursing Index, Google Scholar, PubMed, Sage, Science Direct and Scopus. The search strategy first evaluated each paper's title, followed by abstract then full text. No language or publication status limits were applied to the search strategy.

The following search terms were used on all databases to collect papers: 'identification and biomarkers and cardiovascular diseases', 'identification and biomarkers and diabetes', 'identification and metabolites and cardiovascular diseases', 'identification and metabolites and diabetes', 'analysis and biomarkers and cardiovascular diseases', 'analysis and metabolites and cardiovascular diseases', 'analysis and metabolites and diabetes', 'detection and biomarkers and cardiovascular diseases', 'detection and biomarkers and diabetes', 'detection and metabolites and cardiovascular diseases' and 'detection and metabolites and diabetes.'

2.2.2. Inclusion Criteria

Studies were included into the systematic review if they looked at the identification and/or quantification of biomarkers and/or metabolites used in diagnosis of CVDs and DM. Research studies which looked at the up- and/or down-regulation of biomarkers and metabolites during the prognosis of the disease were also included.

2.2.3. Exclusion Criteria

Secondary sources of research such as reviews, metaanalyses and editorials were excluded. Furthermore, biomarkers and metabolites used for the diagnosis of CVDs and DM secondary complications such as diabetic nephropathy, retinopathy and gum

disease were excluded. This was attributed to this review looking at early disease diagnosis rather than its developments and further complications.

2.2.4. Ethical Approval

Due to the nature of this study, no ethical approval was required as no participants were recruited. Data used in this study was retrospective data extracted from previous studies with no identifiable information.

2.2.5. Quality Assessments

The quality of case control and cohort studies were determined using the Critical Appraisal Skills Programme (CASP) checklist and Newcastle-Ottawa Scale (NOS) quality tool (CASP, 2022; Wells *et al.*, 2021). This tool was comprised of three sections, those being: selection, comparability and exposure and provided the assessed studies with a star rating out of three. The higher the star rating, the higher quality the study was. The accepted papers received a score of two ($n = 1$) or three ($n = 18$) stars. The overall quality and validation of the systematic review was assessed through PROSPERO (National Institute for Health and Care Research, 2023).

2.2.6. Data Extraction

Data extraction was conducted by three reviewers (IA, MW and SA) and was carried out systematically including titles, abstracts and full articles. Reviewers selected one of three options, those being: include, exclude or unsure. Where a disagreement was encountered, it was resolved by a discussion. The final reviewer collated key information from the accepted studies, including: country in which the research took place in, study setting (laboratory, hospital or other clinical environment), population age (years), sample size (participants), biological matrix and reported biomarkers/metabolites (Table 2.1). Studies were organised based on their study type, including cohort (retrospective and prospective cohort) and case control (prospective case control) studies.

Table 2.1. Information extracted from published data during the data extraction procedure.

Sections	Sub-sections
Title	Aim of study
Study characteristics	Experimental settings, study design, sample size, population, instrumentation
Extraction procedure	Cleaning procedures, storage conditions, sample type
Experimental conditions	Diagnostic technique applied, parameters
Biomarkers	Nature of biomarker, concentration

2.2.7. Data Analysis

The summary statistics were reported for study setting, population age (years), sample size (patients) (median = 147, inter quartile range (IQR) = 85.5 – 497.3), biological matrix, diagnostic tool and reported biomarkers.

2.2.8. List of Definitions

Biomarkers are defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Strimbu and Tavel, 2010). Metabolites are endogenous constituents including amino acids, lipids, sugars and organic acids, which are routinely formed in the anabolism or catabolism process (Baghel *et al.*, 2021). CVDs are characterised by a group of disorders of the heart and blood vessels. ICD-11 codes of CVDs used included: arrhythmia (BC60-BC65), hypertension (BA00-BA04), CAD (BA80-BA86), heart failure (HF) (BD10-BD14), IHD (BA40-BA43) and coronary microvascular disease (MVD) (BB60-BB65) (WHO, 2025). DM is a group of chronic metabolic diseases, which are characterised by chronic hyperglycaemia and result from defects in insulin secretion and/or insulin action (Kharroubi and Darwish, 2015). A risk factor is defined as a condition or measurement associated with the probability of developing a disease or death (Nexøe, Halvorsen and Kristiansen, 2007).

2.3. Results

2.3.1. Study Extraction

3,640,260 articles were screened through the databases and 2,764 were obtained. Out of 2,764 papers, 2,574 were excluded based on their titles and considering the aforementioned inclusion and exclusion criteria. Of the remaining 190 studies, 37 were excluded after the abstracts had been evaluated. From the remaining 153 studies, 134 were found to be irrelevant. Consequently, the research resulted in 19 studies, which were then subject to quality assessments and tools. All of the 19 studies were included in the review (Figure 2.1).

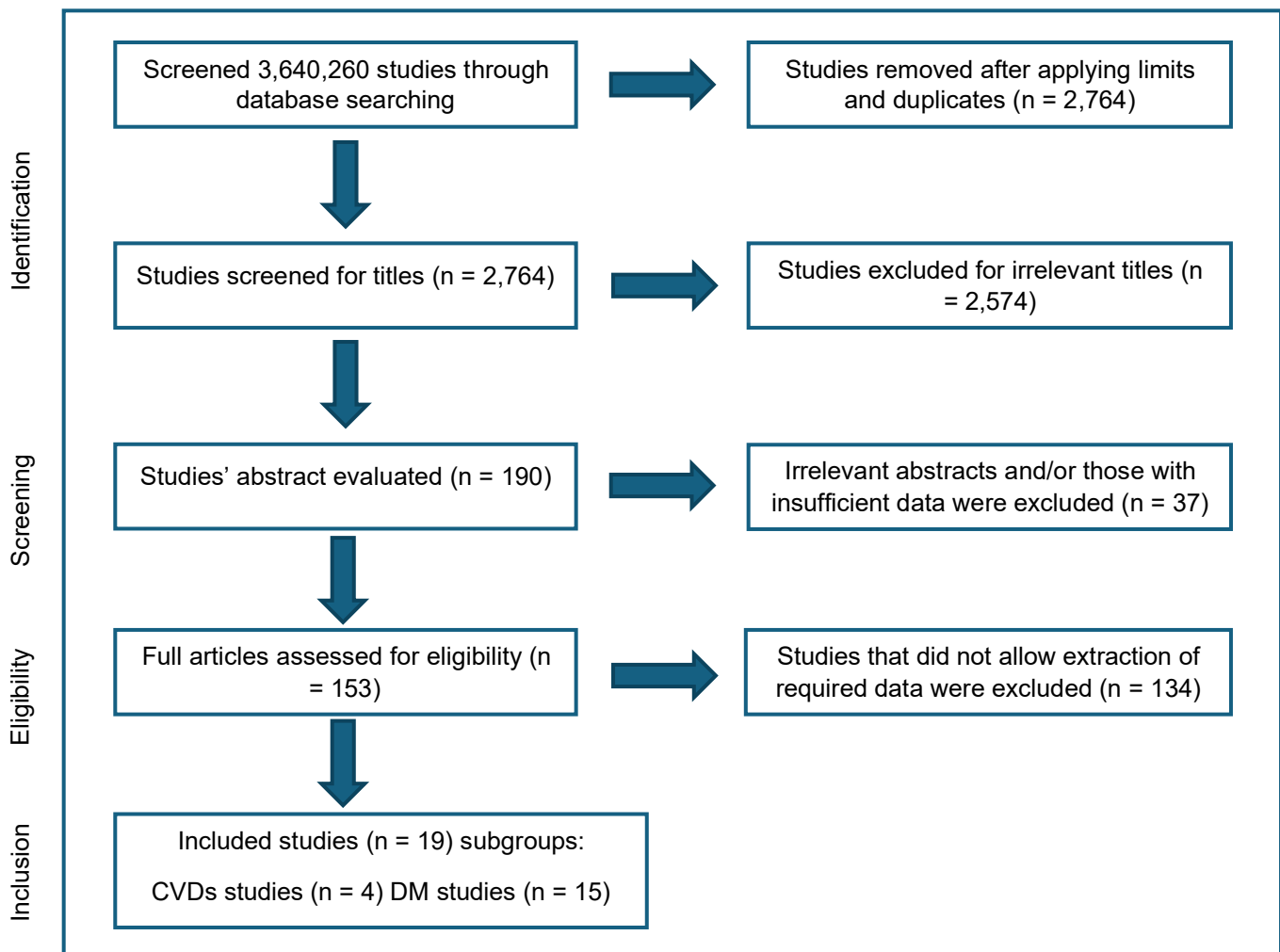


Figure 2.1. Data extraction and study selection workflow (Page et al., 2021).

CVDs: cardiovascular diseases; DM: diabetes mellitus; n: number of studies.

2.3.2. Study Characteristics

Characteristics of the included studies are listed in Table 2.2. Study publication dates ranged from 2008 to 2022. The included studies' sample size varied between 23-7,184, with a median range of 147 (IQR = 75-640). Out of the aforementioned studies, 12 (63%) studies were case control (Carter *et al.*, 2016; Li *et al.*, 2020; Lin *et al.*, 2020; Long *et al.*, 2020; Méndez-Rodríguez *et al.*, 2020; Monteyne *et al.*, 2018; Nogueira *et al.*, 2022; Scott *et al.*, 2010; Shetty, Kumari and Shetty, 2020; Shi *et al.*, 2018; Tariq *et al.*, 2022; Wang *et al.*, 2013), two (11%) cohort (Martínez *et al.*, 2019; Yap *et al.*, 2010), one (5%) retrospective cohort (Hatzenbuehler, McLaughlin and Slopen, 2013), two (11%) prospective cohort (Lau *et al.*, 2019; Rao *et al.*, 2009) and two (11%) prospective case control (Salih and Abduleef, 2020; Rodríguez-Calvo *et al.*, 2019). Where specified, studies were conducted in 12 countries across the globe, those being: US (Carter *et al.*, 2016; Hatzenbuehler, McLaughlin and Slopen, 2013; Lau *et al.*, 2019), Belgium (Monteyne *et al.*, 2018), Brazil (Nogueira *et al.*, 2022), China (Lau *et al.*, 2019; Li *et al.*, 2020; Lin *et al.*, 2020; Long *et al.*, 2020; Yap *et al.*, 2010), India (Shetty, Kumari and Shetty, 2020; Rao *et al.*, 2009), Iraq (Salih and Abduleef, 2020), Netherlands (Rodríguez-Calvo *et al.*, 2019), Mexico (Méndez-Rodríguez *et al.*, 2020), Pakistan (Tariq *et al.*, 2022), Spain (Martínez *et al.*, 2019), Sweden (Shi *et al.*, 2018; Wang *et al.*, 2013) and Turkey (Scott *et al.*, 2010). The study settings reported included: hospitals (n = 8, 42.1%) and laboratories (n = 11, 57.9%). When reported the duration of studies ranged between two months and five years (IQR = 11 months – 3 years, 3 months).

Table 2.2. Characteristics of studies that identified biomarkers and/or metabolites for the detection and diagnosis of CVDs and DM.

SN	Reference	Study type	Aim	Setting	Country	Sample size	Population and age (years old)	Biological matrix	Analysis technique	Analysis details
1	Rodríguez-Calvo <i>et al.</i> , 2019.	Prospective case control	To investigate the role of adipokine in regulating cardiac metabolism and myocardial neutral lipid content in patients with T2DM.	Recruited from the PIRAMID study	Netherlands	86	Adults aged 45-65	Blood	¹ H-MRS	FABP4 levels determined using a commercial ELISA.
2	Li <i>et al.</i> , 2020.	Case control	To develop a rapid and accurate diagnosis of pre-diabetes.	Department of Endocrinology, First Affiliated Hospital of Jinan University.	China	147	Adults aged 30-61	Blood	NIR spectroscopy	Spectra acquisition range was 400-2500 nm and the detectors were Si (400-1100 nm) and PbS (1100-2500 nm).
3	Hatzenbuehler, McLaughlin and Slopen, 2013.	Retrospective cohort	To determine whether sexual orientation disparities in biomarkers of early cardiovascular risk are present young adults.	Data from Wave IV of the National Longitudinal Study for Adolescent Health	US	520	Adults aged 24-32	Dried blood spots	Standardized enzyme immunoassay protocol	Quantification of HbA1c was carried out using the immunoturbidimetric method. Hb was quantified using a colourimetric method. The lowest concentration of HbA1c was 5.54 %
4	Monteyne <i>et al.</i> , 2018.	Case control	To explore if NIR spectroscopy could be used as a non-invasive diagnostic tool for assessing glycation in DM.	Department of Endocrinology of the Ghent University Hospital	Belgium	159	Adults aged 28-34	Fingernails	Fourier-transform NIR spectrometer	Spectrometer equipped with an InGaAs detector and a quartz halogen lamp.

5	Salih and Abdulateef, 2020.	Prospective case control	To assess the detection of C-peptide in hair and nail samples to find whether hair and nail C-peptide levels are different in T1DM compared with healthy individuals.	Diabetes Centre Hospital, Oxford English Centre and College of Medicine	Iraq	83	Adults aged 18-34	Fingernails	COBAS c111 (HITACHI) and electrochemiluminescence immunoassay method using a COBAS e411 analyser	Hair and fingernail samples cut into small pieces (1-2 mm) and incubated in methanol for 16 hours and at 50 °C. Quantified C-peptide (3.77 ng/ml), insulin (0.95 ng/ml), adiponectin (11.90 ng/ml), IL-6 (1.61 pg/ml), glucagon (90.77 pg/ml) and leptin (28.45 ng/ml) in patients with T2DM.
6	Lin <i>et al.</i> , 2020.	Case control	To identify metabolic pathways and core metabolites for prevention and treatment of disease.	Second Shaanxi Provincial People's Hospital and the First Affiliated Hospital of Xi'an Medical University	China	84	Adults aged 40-60	Plasma	GC-MS	Derivatised in two steps: Agilent 7890A GC and 5975C MS.
7	Carter <i>et al.</i> , 2016.	Case control	To identify plasma metabolites and protein biomarkers that, in combination with glucose, can better predict future T2DM compared with glucose alone.	Bavarian Red Cross Blood Transfusion Centre study for identification. Medicine Research Project in Wisconsin for validation	US	640	Adults aged 30-60	Plasma	GC-MS, LC-MS/MS and Multiplex ELISA	85 circulating protein biomarkers were ascertained from the study population using a discovery proteomic platform.

8	Lau <i>et al.</i> , 2019.	Prospective cohort	To investigate sex differences in circulating biomarkers representative of biological pathways implicated in the development of CVD among Framingham Heart Study participants.	FHS Offspring cohort	China	7184	Adults aged 35-63	Plasma	Modified enzyme-linked immunosorbent assay sandwich approach and Luminex xMAP platform	Fasting plasma glucose levels were measured using via a glucose oxidase method and HbA1c levels were measured by using an ion exchange resin method on the spectrophotometer.
9	Shetty, Kumari and Shetty, 2020.	Case control	To find an association between w-6 to w-3 fatty acid ratio and T2DM.	Justice K.S. Hegde Charitable Hospital, Deralakatte, Mangaluru	India	429	Adults aged 30-60	Plasma	GC-FID	Data were acquired using an ACQUITY UPLC coupled to a 5500 QTRAP triple quadrupole mass spectrometer.
10	Wang <i>et al.</i> , 2013.	Case control	To investigate 2-aminoadipic acid as a biomarker for diabetic risk.	FSH offspring and MDC cohort	Sweden	2261	Adults aged 45-65	Plasma	LC-MS/MS	Reverse-phase and hydrophilic interaction chromatography was applied to lipophilic and hydrophilic metabolites, using positive and negative electrospray ionisation.
11	Shi <i>et al.</i> , 2018.	Case control	To identify plasma metabolites that predict future T2DM, to investigate the changes in identified metabolites among individuals who later did or did not develop T2DM over time, and	Västerbotten Intervention Programme cohort	Sweden	1006	Adults aged 42- 58	Plasma	HPLC-qTOF-MS/MS	ATR-FTIR diamond crystal and heating function applied to dry sample.

			to assess the extent to which inclusion of predictive metabolites could improve risk prediction.							
12	Nogueira <i>et al.</i> , 2022.	Case control	To evaluate the feasibility of diagnosing DM and periodontitis through saliva samples based on their FT-IR spectra	University of Taubaté	Brazil	23	Adults aged 18+	Saliva	FTIR spectroscopy	Nominal resolution of 2 cm ⁻¹ , with a blank BaF window employed for background measurement. 256 scan signals were averaged for each film.
13	Scott <i>et al.</i> , 2010	Case control	To monitor DM-specific alterations to the molecular composition of saliva from subjects with DM and control subjects using this unique optical tool.	Department of Metabolic Diseases and Endocrinology at Ege University and School of Dentistry, Ege University	Turkey	62	Adults aged 30-61	Saliva	IR spectroscopy	Spectrum One FT-IR spectrometer at a nominal resolution of 2 cm ⁻¹ , with a blank BaF window employed for background measurement.
14	Rao <i>et al.</i> , 2009.	Prospective case control	To characterise the human salivary proteome in T2DM to identify potential biomarkers of DM.	Nizam's Institute of Medical Sciences	India	40	Adults aged 36-62	Saliva	LC-MS/MS	Injection volume of 5 µL for all samples, employing an ACQUITY BEH C18 column.
15	Long <i>et al.</i> , 2020.	Case control	To search for an integrated biomarker for early diagnosis by determining metabolites associated with the progression of T2DM.	Beijing Jiaotong University Community Health Centre	China	149	Adults aged 48-61	Serum	UHPLC/Q-Oribitrap HRMS	UHPLC system was used to separate the metabolites in serum with an injection volume of 5 µL for all samples, employing an ACQUITY BEH C18 column (100 mm ×

										2.1 mm, 1.7 μm; Waters) at a column temperature of 40 °C.
16	Tariq <i>et al.</i> , 2022.	Case control	To discover more sensitive potential protein biomarker candidates capable of predicting progression to hyperglycaemia before its onset (at pre-diabetes stage).	Dow University from January 2018 to February 2019	Pakistan	75	Adults aged 24-43	Serum	nano LC–MS/ MS	Orbitrap Fusion mass spectrometer.
17	Martínez <i>et al.</i> , 2019.	Cohort	To identify a molecule profile with added value to classical CV risk estimation.	Hospitl 12 de Octubre	Spain	81	Adults aged 30-50	Urine	High-pH reverse phase LC-MS/MS and ELISA	Analysed at 600 MHz using a Bruker DRX600 spectrometer operating in flow injection mode.
18	Méndez-Rodríguez et al., 2020.	Case control	To identify metabolites in urine through FGC eNose that can be used as biomarkers in the identification of T2DM patients.	Health Centres in León, Guanajuato	Mexico	41	Adults aged 20-55	Urine	uf-GC E-nose	Trap column: acclaim PepMap TM nano viper, C-18, 3 μm, 100 Å (0.75 μm × 2.0 cm) was used for a concentrated peptide with a flow rate of 15 μL/min.
19	Yap et al., 2010.	Cohort	To investigate population biomarkers that might relate to the difference in CVD risk.	INTERMAP cohort	China	767	Adults aged 40-59	Urine	¹ H NMR spectroscopy	Bruker DRX600 spectrometer operating in flow injection mode.

¹H-MRS: proton magnetic resonance spectroscopy; ¹H-NMR: proton nuclear magnetic resonance; ATR-FTIR: attenuated total reflectance-Fourier transform infrared; BaF: barium fluoride; BEH: ethylene bridged hybrid; CV: cardiovascular; CVD: cardiovascular disease; DM: diabetes mellitus; ELISA: enzyme-linked immunosorbent assay; FABP4: fatty acid-binding protein 4; FHS: Framingham Heart Study; FGC: Fast gas chromatography; FT: Fourier transform; FTIR: Fourier transform infrared; GC: gas chromatography; GC-

FID: gas chromatography with flame ionisation detection; GC-MS: gas chromatography mass spectrometry; Hb: haemoglobin; HbA1c: haemoglobin A1c; HPLC-qTOF-MS/MS: high pressure liquid chromatography coupled with quadrupole time-of-flight mass spectroscopy; IR: infrared; IL-6: Interleukin-6; LC-MS/MS: liquid chromatography with tandem mass spectroscopy; MDC: Malmö Diet and Cancer Study; MS: mass spectrometry; NIR: near-infrared; NR: not reported; PbS: lead sulphide; PIRAMID: Pioglitazone Influence on tRiglycer-ide Accumulation in the Myocardium in Diabetes; Si: Silicon; SN: study number; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; uf-GC E-nose: ultrafast gas chromatography electronic-noise; UHPLC: Ultrahigh-performance liquid chromatography; UHPLC/Q-Orbitrap HRMS: ultrahigh-performance liquid chromatography-quadrupole-Orbitrap high-resolution mass spectrometry; UPLC: ultra performance liquid chromatography.

2.3.3. Participants' Characteristics

Participants reported in the 19 studies were within the age range of 18 – 65 years old (Table 2.3). The sample population consisted of healthy controls, CVD patients and diabetic patients. Where current cardiac conditions were reported, the specific CVD diagnosis comprised CV death (n = 1, 33.3%), HF (n = 1, 33.3%) and hypertension (n = 1, 33.3%). DM patients received diagnosis of pre-diabetes, T1DM or T2DM. The median male:female ratio for case control studies was 82.5:79.5 (IQR = 70.25:51.5 – 334 – 253) and for prospective case control it was 59:19.5 (IQR = 51.5:9.75 – 66.5:29.25). Only one cohort study reported a male:female ratio (69:12). The included retrospective cohort study, one cohort study, one prospective case control and two case control studies did not disclose the number of male and female participants.

Table 2.3. Participant's characteristics including age, male:female ratio, population size and non-disease:disease ratio.

SN	Age range (years)	M:F (%:%)	Population size	ND/D (%:%)
1	45-65	74:0 (100:0)	86	12:74 (14:86)
2	30-61	67:80 (46:54)	147	53:94 (36:64)
3	24-32	NR	520	NR
4	28-34	80:79 (51:49)	159	107:52 (67:59)
5	18-34	44:39 (53:47)	83	42:41 (51:49)
6	40-60	84:0 (100:0)	84	42:42 (50:50)
7	30-60	366:274 (57:43)	640	523:117 (82:18)
8	35-63	3289:3895 (46:54)	7184	6737:447 (94:6)
9	30-60	241:188 (56:44)	429	216:213 (50.3:49.7)
10	45-65	1017:1244 (45:55)	2261	1911:350 (85:15)
11	42-58	443:563 (44:56)	1006	503:503 (50:50)
12	18-65	NR	23	2:21 (8:92)
13	30-61	24:38 (39:61)	62	23:39 (37:63)
14	36-62	NR	40	20:20 (50:50)
15	48-61	81:68 (54:46)	149	48:101 (32:68)
16	24-43	29:46 (39:61)	75	25:50 (33:67)
17	30-50	69:12 (85:15)	81	32:49 (40:60)
18	20-55	NR	41	20:21 (49:51)
19	40-59	NR	767	0:100 (0:100)

Data reported as n:n (%:%), M/F: male to female ratio; ND/D: non-diseased to diseased ratio; NR: not reported; SN: study number.

2.3.4. Biomarkers and Metabolites

Seven biological matrices were reported across both CVD and DM studies, and were: blood (Li *et al.*, 2020; Rodríguez-Calvo *et al.*, 2019), dried blood spots (Hatzenbuehler, McLaughlin and Slopen, 2013), fingernails (Monteyne *et al.*, 2018; Salih and Abdulateef, 2020), plasma (Carter *et al.*, 2016; Lau *et al.*, 2019; Lin *et al.*, 2020; Shetty, Kumari and Shetty, 2010; Shi *et al.*, 2018; Wang *et al.*, 2017), saliva (Nogueira *et al.*, 2022; Scott *et al.*, 2010; Rao *et al.*, 2009), serum (Long *et al.*, 2020; Tariq *et al.*, 2022) and urine (Martínez *et al.*, 2019; Méndez-Rodríguez *et al.*, 2020; Yap *et al.*, 2010).

Across both diseases, a total of 302 biomarkers were reported, seven of which occurred in both CVD and diabetic patients. Biomarkers identified in both CVD and diabetic patients included ADAM15, alanine, collagen, creatine, cystatin-C, glucose and leptin. The remaining 78 and 224 biomarkers were characteristic for the diagnosis of CVDs or DM, respectively, but could not be utilised for the diagnosis of both conditions.

2.3.4.1 Biomarkers and Metabolites Related to CVDs

A total of 78 biomarkers were identified in relation to CVDs diagnosis (Table 2.4). The most frequently reported biomarkers included C-reactive protein (CRP), growth differentiation factor 15 (GDF15) and neurogenic locus notch homolog protein 1 (NOTCH 1) (Hatzenbuehler, McLaughlin and Slopen, 2013; Lau *et al.*, 2019; Martínez *et al.*, 2019). The levels of CRP reported in dried blood spots (n = 1, 33,3%) and plasma (n = 2, 66.6%) samples increased during the onset of CVD and was closely related to biological sex. Research showed that the level of inflammatory markers such as CRP were significantly higher in females than males (Lau *et al.*, 2019). An increased amount of GDF15 positively correlated with CV risk in urine (130 – 150 pg/mL in cases versus 75 – 80 pg/mL in controls) (Martínez *et al.*, 2019). This level was further heightened in females (Lau *et al.*, 2019). NOTCH1 was also observed at higher concentrations in women (Lau *et al.*, 2019; Martínez *et al.*, 2019).

The identified biomarkers were prevalent during the early stages of CVD development, which is characterised as pre-HF. Individuals with a family history of HF and/or a related medical condition, are at a higher risk of developing HF at this stage. For example, individuals with cardiomyopathy, CAD, hypertension, IHD or PAD are more

at risk of developing HF (Hajar, 2017; Inglis *et al.*, 2013; Severino *et al.*, 2020; Velagaleti and Vasan, 2007; Zile and Nappi, 2000).

Table 2.4. Biomarkers and metabolites reported only once in the diagnosis of CVDs.

Matrix	Biomarker
Blood spots	ADAM15, CRP, glucose
Plasma	Adipsin, Alpha-1-acidic glycoprotein, Alpha-1-microglobulin, Angiopoietin-like protein 3, Apolipoprotein A1, Apolipoprotein B-100, Bikunin, Brain natriuretic peptide, Butyrylcholinesterase, CD163, CD5, CD56, Ceruloplasmin, Clusterin, Collagen, Contactin-1, CRP, CXC motif chemokine ligand 16, Cystatin C, Dephosphorylated uncarboxylated matrix Gla protein, Fibrillin-1, Fibroblast growth factor 23, GDF15, Hemopexin, Insulin growth factor binding protein 1, Insulin growth factor binding protein 2, Insulin growth factor binding protein 3, Insulin growth factor binding protein 7, Insulin-like growth factor, Intercellular adhesion molecule 1, Leptin, Leptin R, Matrix metalloproteinase-8, Matrix metalloproteinase-9, Melanoma cell adhesion molecule, Monocyte chemoattractant protein-1, Myeloperoxidase, Myoglobin, NOTCH1, Paraoxonase-1, Peripheral myelin protein 2, Plasminogen activator inhibitor-1, Platelet basic protein, Regenerating protein 1 alpha, Resistin, Serum amyloid A-1 protein, Soluble gp130, Soluble intercellular adhesion molecule-1, Soluble receptor for advanced glycation end products, Stromal cell-deprived factor 1, Tetranectin, Tissue inhibitor of metalloproteinase-1, Vascular endothelial growth factor
Urine	2-hydroxyisobutyrate, 4F2 cell surface antigen heavy chain, Adrenodoxin, Alanine, Betaine, Branched-chain amino acids, Creatine, Dimethylglycine, Eosinophil cationic protein, GDF15, Guanine deaminase, Hippurate, Lactate, Methyl guanine, N-acetyls neuraminic acid, N-acetyls of glycoprotein fragments, NOTCH1, Pentanoic/heptanoic acid, Phenylacetylglutamine, Proline, scyllo-inositol, Succinate, Trans-aconitate

ADAM15: A disintegrin and metalloproteinase 15; CRP: C-reactive protein; C163: Cluster of differentiation 163; CD5: Cluster of differentiation 5; CD56: Cluster of differentiation 56; GDF15: Growth differentiation factor 15; gp130: Glycoprotein 130; NOTCH1: Neurogenic locus notch homolog protein 1.

2.3.4.2. Biomarkers and Metabolites Related to DM

A total of 224 biomarkers were reported for diagnosis of DM (Table 2.5). Across the reported biomarkers, isoleucine was the most frequently reported ($n = 4$) and was identified in blood ($n = 1$, 25%), plasma ($n = 2$, 50%) and serum ($n = 1$, 25%) specimen. The level of isoleucine significantly increased (1.22-fold change) in diabetic patients in comparison to healthy controls (Lin *et al.*, 2020; Long *et al.*, 2020; Shi *et al.*, 2018; Wang *et al.*, 2013). Tyrosine was the second most frequently reported biomarker for DM ($n = 3$) and was identified in plasma ($n = 2$, 66.6%) and serum ($n = 1$, 33.3%) (Long *et al.*, 2020; Shi *et al.*, 2018; Wang *et al.*, 2013). During the onset of DM (type 1 and 2), tyrosine levels significantly increased (1.14-fold change) in comparison to healthy baseline levels. Linoleic acid was also reported in blood ($n = 1$, 50%) and plasma ($n = 1$, 50%) (Lin *et al.*, 2020; Shetty, Kumari and Shetty, 2020). The level of the aforementioned biomarker significantly increased in patients with T2DM (0.462% from 0.416%). Leucine was identified twice in plasma and positively correlated with

the presence of DM (Shi *et al.*, 2018; Wang *et al.*, 2013). Other amino acids commonly reported as biomarkers for DM are glutamine and valine, which were reported in plasma and serum (Lin *et al.*, 2020; Long *et al.*, 2020; Shi *et al.*, 2018).

Higher levels of fatty acids including myristoleic acid and palmitic acid were seen in serum samples taken from T2DM patients (Lin *et al.*, 2020; Shetty, Kumari and Shetty, 2020). Acetyl carnitine positively correlated with T2DM (Lin *et al.*, 2020; Long *et al.*, 2020). A key biomarker for DM diagnosis were triglycerides, which positively correlated with T2DM in blood (n = 1, 50%) and urine (n = 1, 50%) (Méndez-Rodríguez *et al.*, 2020; Rodríguez-Calvo *et al.*, 2019). Alpha-2-macroglobulin (A2M) levels were significantly up-regulated in pre-diabetes and T2DM (Rao *et al.*, 2009; Tariq *et al.*, 2022).

In contrast, the level of C-peptides found in fingernails decreased in T1DM patients in comparison to healthy controls. Research showed that this level further decreased after a DM diagnosis of > 7 years (Salih and Abdulateef, 2020). However, the level of C-peptides found in plasma increased in T2DM (Carter *et al.*, 2016). This suggest that C-peptides can act as a biomarker for the differentiation of DM types. The levels of glucose between T1DM and T2DM can also be used for DM classification.

Table 2.5. Reported biomarkers and metabolites used in the diagnosis of DM.

Matrix	Biomarker
Blood	9-OxoODE, 12-Hydroxyeicosatetraenoic acid, 13-Hydroxyeicosatetraenoic acid, 1-Oleoyl-L- α -lysophosphatidic acid, 1-Oleoyl-sn-glycero-3-phosphocholine, 1-Palmitoylglycerol, 1-Palmitoyl-sn-glycero-3-phosphocholine, 1-Stearoyl-2-oleoyl-sn-glycerol 3-, 1-Stearoyl-rac-glycerol, 2E-Eicosenoic acid, 3-Phenylpropanoic acid, 9-hydroxyoctadecadienoic acid, Acetylcarnitine, Alpha-Linolenic acid, Alpha-tocopherol, Betaine, Capric acid, Carnitine, Chenodeoxycholate, Cis-(6,9,12)-linolenic acid, Cis-9,10-Epoxy stearic acid, Cis-9-Palmitoleic acid, Dodecanoic acid, D-Proline, D-Quinovose, Dulcitol, Embelin, Erucamide, Fatty acid-binding protein, Glucose, Glutamine, Glycochenodeoxycholate, High-density lipoprotein, Hypoxanthine, Isoleucine, Leucine, L-Gulonic gamma-lactone, Linoleic acid, Low-density lipoprotein, L-Pipecolic acid, Myristic acid, Myristoleic acid, Norethindrone acetate, Palmitic acid, PEA, Phenol, Phenylalanine, Phosphocholine, Pyroglutamic acid, Sunitinib, Taurine, Triglycerides, Uridine, Water molecules without hydrogen bonds
Fingernails	C-peptides, Glycated proteins

Plasma	2-Hydroxyethanesulfonate, 2-Aminoadipic acid, 2-Hydroxybutyrate, 2-Methylbutyrylcarnitine, 3-hydroxyisovalerylcarnitine, ADAM15, Adiponectin, Alanine, α -linolenic acid, Arachidonic acid, Bile acid 386.2455 @8.21, Cholesteryl ester, Citrulline, C-peptide, Diacylglycerol (14:0/16:0)h, Diacylglycerol (14:0/18:1)h, Diacylglycerol (16:0/18:1)h, Diacylglycerol (16:1/16:1)h, Diacylglycerol 531.4484@13.29h, Diacylglycerol 571.4437@13.30h, DGLA, Dipalmitoyl phosphatidic acid, Docosahexaenoic acid, DPA, Eicosapentaenoic acid, Fatty acid 259.1608@7.63, Fatty acid 364.333@10.46, Glucagon, Glucosamine, Glucose, Glutamate, Glutamate derivate 316.0887@6.19, Insulin, Interleukin-6, Isoleucine, Leptin, Leucine, Lignoceric acid, Linoleic acid, Lysophospholipid (17:0), Lysophospholipid (18:1), Lysophospholipid (18:2), Lysophospholipid (20:1), Lysophospholipid (p-16:0), Mannose, Myristic acid, Myristoleic acid, N-Acetylglycine, Oleic acid, Palmitic acid, Palmitoleic acid, Phenylalanine, Phosphatidylcholine 583.3792@10.75, Phosphatidylcholine (15:1/18:2)h, Phosphatidylcholine (16:0/16:1)h, Phosphatidylcholine (17:0/18:2), Stearic acid, Tryptophan, Tyrosine, Valine, γ -linolenic acid
Saliva	Acidic leucine-rich nuclear phosphoprotein 32 family, Actin-like protein 2, Actin-related protein 2/3 complex subunit 5, Aldo-keto reductase family 1 member B10, Alpha-1-antitrypsin, Alpha-2-macroglobulin, Annexin A1, Annexin A2, Apolipoprotein B-100, Basic proline-rich peptide IB-8a, Calmodulin-like protein 5, Calnexin, Carbonic anhydrase 1, Carbonic anhydrase 2, Carbonic anhydrase 6, Cathepsin Z, Chitinase-3-like protein 1, Collagen, Complement component C6, Cystatin-A, Cystatin-C, DSP, Dimeric aldehyde dehydrogenase, Epidermal fatty acid-binding protein, Flavin reductase, Glucose, Glycogen, Glycogen phosphorylase, Grancalcin, Histone cluster 1, Inter-alpha (globulin) inhibitor H2, Isoform 2 of P60953 cell division control, Isoform 2 of P67936 Tropomyosin alpha-4 chain, Isoform 2, 3 and 4 of Chitotriosidase-1, Kallikrein-10, Kallikrein-13, Lamin A/C, Ly6/PLAUR domain-containing protein 3, Moesin, NADP-preferring, Neuroblast differentiation-associated protein AHNK, Nucleobindin-2, Peroxiredoxin-1, Peroxiredoxin-2, Peroxiredoxin-6, Phosphoglycerate kinase 1, Protein 42 homologue precursor, Protein plunc, Protein S100-A7, Purine nucleoside phosphorylase, Pyruvate kinase isozymes M1/M2, Retinol binding protein 4, Ribonuclease pancreatic, Serpin peptidase inhibitor clade B, Small proline-rich protein 2F, Sugar moieties, Transthyretin
Serum	1,3-p-menthadien-7-a, (Z)-3-Hexenyl hexanoate, 1,1,2-trichloroethane, 1,2-dichloroethane, 2-acetolactate, 2-ethyl-6-methylpyrazine, 2-hydroxy-2,4-pentadienoate, 2-methylbutyryl-carnitine, 2-propanol, 2-propenal, 3-methylbut-2-en-1-ol, 3-phenyllactic acid, Acetyl-L-carnitine, Alanine, Alpha-2-HS-glycoprotein, Alpha-2-macroglobulin, Alpha-selinene, Antithrombin-III, Apolipoprotein A2, Apolipoprotein A-IV, Arabinose, Arginine, Beta-himachalene, Bilirubin, Biliverdin hydrochloride, Butane-2,3-dione, Butanoic acid, Choline, Cis-1,2-dichloroethene, Complement factor B, Creatinine, Fenchol, Glutamic acid, Glutamine, Haemoglobin subunit beta, Hexadeca fluoroheptane, Immunoglobulin heavy variable 3-7, Immunoglobulin J chain, Indole-3-lactic acid, Inter-alpha-trypsin inhibitor heavy chain H4, Isoleucine, Lysine, Lysophospholipid (14:0), Lysophospholipid (15:0), Lysophospholipid (16:0), Lysophospholipid (16:1), Lysophospholipid (18:0), Lysophospholipid (20:1), Lysophospholipid (20:5), Methyl furfuryl disulfide, Methyl isobutyrate, Palmitoyl-carnitine, PHE, Phenylalanine, Proline, Propan-2-one, Propionyl-carnitine, Serum amyloid P-component, Triglycerides, Tyrosine, Uric acid, Valine, Vitronectin

DGLA: Dihomo-gamma-linolenic acid; DPA: Docosapentaenoic acid; DSP: Desmolaki; PEA: Palmitoylethanolamid; PHE: Perihematoma edema.

2.3.5. Identification Techniques

The techniques and instrumentation used for identification of CVDs and DM biomarkers varied in terms of biological matrix choice and extraction procedure, technique choice and method validation (Table 2.6).

For biological matrices, the extraction procedure varied depending on the matrix and included centrifugation (for blood and saliva) or washing for nails (Carter *et al.*, 2016; Lau *et al.*, 2019; Li *et al.*, 2020; Lin *et al.*, 2020; Rodríguez-Calvo *et al.*, 2019; Shetty,

Kumari and Shetty, 2020; Shi *et al.*, 2018; Tariq *et al.*, 2022; Wang *et al.*, 2013). Fingernail clippings were washed in 1 mL distilled water (Monteyne *et al.*, 2018) or 1.6 mL of methanol (Salih and Abdulateef, 2020). Storage of biological matrices was made between -20 °C or -80 °C for blood and saliva; whereas, fingernails were stored at room temperature.

In terms of technique, five common techniques were reported in the extracted studies and were LC-MS/MS, enzyme linked immunosorbent assays (ELISA), GC-MS, NIR and FTIR spectroscopy. LC-MS/MS was the most reported technique for analysis of biomarkers in plasma (n = 2, 40%), saliva (n = 1, 20%), serum (n = 1, 20%) and urine (n = 1, 20%) (Carter *et al.*, 2016; Martínez *et al.*, 2019; Tariq *et al.*, 2022; Rao *et al.*, 2009; Wang *et al.*, 2013). This was followed by ELISA that was used for plasma (n = 2, 66.6%) and urine (n = 1, 33.3%) samples (Carter *et al.*, 2016; Lau *et al.*, 2019; Martínez *et al.*, 2019). The third technique was GC-MS that was used for plasma (n = 2, 100%) specimens (Carter *et al.*, 2016; Lin *et al.*, 2020). Moreover, several spectroscopic techniques including NIR and FTIR spectroscopy were used for detecting biomarkers in blood (n = 1, 25%), fingernails (n = 1, 25%) and saliva (n = 2, 50) (Li *et al.*, 2020; Monteyne *et al.*, 2018; Nogueira *et al.*, 2022; Scott *et al.*, 2010).

Method validation of techniques was carried out in eight studies (42.1%) and the choice of the parameters depended on the technique used (Carter *et al.*, 2016; Hatzenbuehler, McLaughlin and Slopen, 2013; Li *et al.*, 2020; Nogueira *et al.*, 2022; Scott *et al.*, 2010; Shi *et al.*, 2018; Rodríguez-Calvo *et al.*, 2019; Yap *et al.*, 2010). For instance, Li *et al.*, (2020) validated a NIR spectroscopic method with SVM by calculating the performance metrics of the SVM model applied to the NIR spectra of diabetic patients. In this respect, metrics used were accuracy (97.22%), specificity (95.66%) and sensitivity (100%). Carter *et al.*, (2016) validated a metabolite panel for the early diagnosis of T2DM. For this study, validation was carried out using two independent study populations, the first being for biomarker identification (discovery group) and the second for confirmatory testing (validation group). This research also utilised an analysis of variance (ANOVA) test and Bayesian network classifier, with a 10-fold cross validation, to compare the plasma proteins' concentration between matched diabetic cases and healthy controls. Moreover, Nogueira *et al.*, (2022) built saliva classification models for the diagnosis of DM and periodontitis. This work employed a 5-fold cross-validation method for the classification of performance

metrics. Research by Scott *et al.*, (2010) employed a cross-validation method and linear discriminant analysis for determining sensitivity, specificity, positive and negative predictive values for classification of healthy versus diabetic saliva. Moreover, this study utilised two-thirds of its sample for a training set, while the remaining one-third was used as the test set to diagnose DM through IR spectroscopy. For the diagnosis of DM in plasma, Shi *et al.*, (2018) also incorporated a cross-validation framework. Specifically, this work developed a multilevel partial least squares (ML-PLS) classification algorithm, which was incorporated into a double cross-validation framework. This work also avoided overfitting through a 60:40 split of 10,000 samples into prediction and validation sets. Yap *et al.*, (2010) carried out a similar validation procedure when applying urine to proton nuclear magnetic resonance (^1H -NMR) spectroscopy for the detection of CVDs. To determine the discriminatory power of metabolites found in urine, orthogonal partial least squares discriminant analysis (OPLS-DA) was applied, which was assessed using a Q^2Y statistic and a 7-fold cross-validation. Rodríguez-Calvo *et al.*, (2019) validated the relationship between circulating fatty-acid binding proteins and T2DM using a murine model of insulin resistance. While Hatzenbuehler, Slopen and McLaughlin (2013) validated the relationship between CRP and CV risk, through the comparison of plasma and dried blood spot concentrations.

Table 2.6. Diagnostic techniques employed for the identification of CVDs and DM biomarkers.

SN	Instrument	Biological Matrix	Extraction	Procedure	Validation
1	¹ H-MRS	Blood	Lipids were extracted in methanol 0.1% formic acid. After sample fragmentation by vortexing, immersion in liquid nitrogen and ultrasonication, dichloromethane and water were added sequentially. Samples were incubated and centrifuged. The organic phase (lipidic) was collected for drying under a stream of nitrogen. Lipid pellets were resuspended in methanol: toluene for LC/MS analysis.	Myocardial ¹ H-MRS spectra were obtained from the interventricular septum. Spectroscopic data acquisition was double triggered with ECG triggering and respiratory navigator echoes. Water-suppressed spectra were acquired to measure myocardial triglyceride content.	Validated data using a murine model of insulin resistance induced by a high-fat diet.
2	NIR Spectroscopy	Blood	147 peripheral blood samples were preserved (about 1mL), kept in an anticoagulant tube and stored in a -20 °C refrigerator.	1 mL sample portions were placed in a quartz cuvette (optical path of 1 mm), and spectra were recorded at a wavelength increment of 2 nm in the range of 400 – 2,500 nm. The laboratory temperature was 24 ± 1 °C and the relative humidity was 41%.	The sample set was divided into a training set and a prediction set at a ratio of 3:1 by using a random selection method and repeating sampling 10 times.
3	Standardised enzyme immunoassay protocol	Dried blood spots	Blood spot samples were obtained using a finger prick and were submitted for laboratory analysis of high-sensitivity CRP (mg/L) and HbA1c (%), reflecting average blood glucose. Trained interviewers collected blood spots on standardised filter paper using a sterile disposable lancet. Blood spots were dried overnight and then frozen.	CRP was assayed from blood spots using a highly sensitive standardised enzyme immunoassay protocol.	Validation study compared CRP concentrations of 87 pairs of plasma and dried blood spot samples.
4	Fourier-transform NIR spectrometer	Fingernails	Fingernail clippings of healthy subjects and of patients with diabetes mellitus were collected in Eppendorf tubes and washed by adding 1 mL of distilled water. The tubes were placed in a sonication bath. Clippings were removed from Eppendorf tubes and dried in an incubator for 12 hours.	Nine sets of clippings were pulverized with a dental drill. Three groups of either three nail powders and three nail clippings were incubated with 1 mL of sodium chloride solution, 0.278 mol/L glucose solution and 0.556 mol/L glucose solution. Complete fingernails were incubated with 0.833 mol/L glucose. After four weeks, samples were centrifuged and supernatant removed. Powders were washed using 1 mL of distilled water.	NR

5	COBAS c111 (HITACHI) and electrochemiluminescence immunoassay method using a COBAS e411 analyser	Fingernails	Nails samples were cut into small pieces and weighed. Samples were placed into coded glass tubes with 1.6 mL of methanol and incubated using a shaking incubator. Samples were centrifuged and 1 mL of supernatant was added. Supernatant was left to dry and a mixture of phosphate buffer saline solution was added.	Samples were analysed by the electrochemiluminescence immunoassay method, using a COBAS e411 analyser.	NR
6	GC-MS	Plasma	Venous blood samples were collected into tubes coated in the morning after overnight fasting. Samples were immediately chilled and centrifuged at 12,000 g for 15 minutes and at room temperature. Plasma samples were collected and stored at - 80 °C until further analysis.	Plasma samples of 30 µL were mixed with a single-phase mixture and after centrifugation, Supernatant was aliquot into a tube and dried via a vacuum lyophilizer. Derivatisation was carried out before application to the GC-MS system.	NR
7	GC-MS, LC-MS-/MS and Multiplex ELISA	Plasma	Fasting blood samples were collected and centrifuged. The plasma layer was divided into aliquots and stored at -80 °C.	Proteins were removed from plasma samples by precipitation with acetonitrile. Polar and non-polar plasma fractions were separated for both GC-MS and LC-MS/MS analyses by adding water and a mixture of ethanol and dichloromethane followed by centrifugation.	Matched controls formed the validation group. A validated algorithm for identifying patients with T2DM from electronic medical record data was used to determine a T2DM diagnosis in the validation group.
8	Modified ELISA sandwich approach and Luminex xMAP platform	Plasma	Fasting blood samples were collected, centrifuged and stored at -80 °C.	A Modified ELISA sandwich approach and Luminex xMAP platform was utilised to create 17 distinct multiplex panels and were based on factors including dilution rate, cross-reactivity, the time when the target was added to the assay list, and standard Luminex assays.	NR
9	GC-FID	Plasma	2 mL of fasting blood sample was collected from the subjects for biochemical estimations and fatty acid methyl ester preparation and analysis.	Tubes of 100 mL of plasma were thawed at room temperature and was then added to triheptadecanoin. Isoctane phase (1 mL) was injected onto capillary column GC-FID to determine the FA distribution. FA levels were determined in the presence of an internal standard by using gas chromatography with flame-ionization detection.	NR

10	LC-MS/MS	Plasma	Discovery analyses were performed on individuals from the FHS Offspring Study. Metabolite profiling was performed on samples from 1,937 attendees who were free of diabetes at baseline. Fasting plasma samples were obtained from 5,305 subjects in the MDC Cardiovascular Cohort.	Data were acquired using an ACQUITY UPLC coupled to a 5500 QTRAP triple quadrupole mass spectrometer. Samples were prepared by the addition of 120 µl of extraction solution (80% methanol) plus the internal standards inosine-15N4, thymine-d4, and glycocholate) to 30 µl of plasma. The samples were centrifuged and the supernatants were injected directly.	NR
11	HPLC-qTOF-MS/MS	Plasma	Fasting plasma samples were sourced from a biobank. Samples chosen if their donor had later developed T2DM after a median of seven years.	Fasting heparin plasma samples were de-proteinised and analysed by HPLC-qTOF-MS/MS. Reverse-phase and hydrophilic interaction chromatography were applied, using both positive and negative electrospray ionisation modes.	Incorporated standard ML-PLS into a repeated double cross-validation framework with unbiased variable selection.
12	FTIR spectroscopy	Saliva	Samples were homogenised in vortex equipment, transferred to a centrifuge microtube by using a calibrated pipette and stored in a freezer at -80 °C.	Samples were pipetted without additives on the crystal of the spectrometer and dried for 1–3 min. Samples were analysed by the FTIR spectral fingerprint region between 600 and–1800 cm ⁻¹ .	Classification performance metrics were obtained by using 5-fold cross-validation.
13	IR spectroscopy	Saliva	Unstimulated whole saliva samples were obtained by expectoration into polypropylene tubes prior to clinical measurements. The saliva samples were weighed and then immediately frozen at -40°C. The samples were then lyophilised and stored at -20°C.	Duplicate 50 µl saliva aliquots were dried at 25 Torr on 13 mm BaF windows. IR spectra were recorded using The Perkin Elmer Spectrum One FTIR spectrometer	Linear discriminant analysis was used to partition the saliva samples into disease and non-disease into a validation set. Sensitivity, specificity and positive and negative predictive values were determined for the classification process through cross-validation.
14	LC-MS/MS	Saliva	Specimens were immediately frozen and stored at -80 °C. Saliva samples (10 mL) were centrifuged. The supernatants were transferred to 4-mL Ultrafree 5K membrane concentrators and spun.	1 mg portions of samples were reduced, alkylated and digested with trypsin, and the resulting peptides were separated with strong cation-exchange (SCX) chromatography. 9 µL of each was analysed by LC-MS/MS.	NR
15	UHPLC/Q-Orbitrap HRMS	Serum	Serum samples were thawed and vortex. 30 µL of serum was mixed with 90 µL of a methanol solution (containing L-2-chlorophenylalanine and ketoprofen). After vortexing, the mixture was centrifuged to precipitate the protein. The 80-µL supernatant was transferred to an autosampler vial.	Nitrogen was used for spray stabilization and damping gas in the C-trap. Precursor ions were fragmented at 20, 40 and 60 eV collision energy, with the AGC target set as 1.0e5 and a maximum injection time of 50 ms, with the isolation window set as 1.0 m/z unit at a resolution of 17,500 in ddMS2 mode.	NR

16	Nano LC-MS/MS	Serum	Serum was separated from whole blood via a serum separator blood collecting tubes. Samples clotted at 37 °C for 30 minutes and were then separated by centrifugation at 10,000-12,000 for 15 minutes. 2 µL of protease inhibitor per 100 µL of serum was added to deplete protease activity against the proteins. Samples were divided into aliquots of 200 µL and stored at -80 °C.	Proteins (100 µg) were digested using trypsin and the resulting peptides were desalted using C-18 zip tips. Analysis of 10-50 fmol aliquots were analysed using a nano LC-MS/MC with maXis II platform.	NR
17	High-pH reverse phase LC-MS/MS and ELISA	Urine	Urine samples were collected in sterile containers, centrifuged and stored at -80 °C until analysis.	Urine proteome from 30 subjects was quantitatively analysed by isobaric labelling. Urine proteins were subject to tryptic digestion and the resulting peptides were TMT-labelled, desalted and fractionated by high-pH reverse phase chromatography. The fractions were analysed by LC-MS/MS on an Orbitrap Fusion mass spectrometer.	NR
18	uf-GC E-nose	Urine	Urine samples were taken from the first-morning micturition in 100 mL sterile bottles. Urine was then stored at -20 °C until analysis.	Analysis was carried out using a non-targeted metabolomics approach through an uf-GC E-nose detector, which was equipped with two columns of different polarities. At a volume of 500 µL, duplicates were made and incubated in the equipment for 900 s at 40°C with a 500 rpm of agitation. Samples were then volatilized by headspace for further analysis.	NR
19	¹ H-NMR spectroscopy	Urine	Each participant provided two timed 24-h urine collections. Borate preservative was added to the urine specimen bottles prior to collection. Urine volume was measured and aliquots were obtained and stored at -20 °C.	500 µL of urine were mixed with 250 µL of phosphate buffer and 75 µL of the sodium 3-trimethylsilyl-(2,2,3,3-2H ₄)-1-propionate (TSP) in D ₂ O. The resulting solution was then transferred into a 96-well plate and left to stand for 10 min before centrifuging. The urine specimens were analysed by ¹ H NMR spectroscopy at 600 MHz	Discriminatory ability of the models was assessed by Q ² Y statistic, that is, the percent variance of the NMR data explained by geographic location (north-south), using 7-fold cross-validation.

¹H-MRS: proton magnetic resonance spectroscopy; ¹H-NMR: proton nuclear magnetic resonance; ELISA: enzyme-linked immunosorbent assay; FA: fatty-acid; FTIR: Fourier-transform infrared; GC-FID: gas chromatography with flame ionisation detection; GC-MS: gas chromatography mass spectrometry; HPLC-qTOF-MS/MS: high pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; IR: infrared; LC-MS/MS: liquid chromatography with tandem mass spectrometry; NIR: near-infrared; NR: not reported; SN: study number; T2DM: Type 2 diabetes mellitus; uf-GC E-nose: ultrafast gas chromatography electronic-noise; UHPLC/Q-Orbitrap HRMS: ultrahigh-performance liquid chromatography-quadrupole-Orbitrap high-resolution mass spectrometry.

2.4. Discussion

The systematic review identified key biomarkers for diagnosing CVDs and/or DM. Biomarkers such as CRP, GDF15 and NOTCH1 were frequently utilised for diagnosing CVDs. While biomarkers such as isoleucine, tyrosine, linoleic acid, glutamine, valine, myristoleic acid, palmitic acid, triglycerides and A2M were commonly used for DM diagnosis. Furthermore, C-peptides demonstrated the capability of differentiating between T1DM and T2DM. The aforementioned biomarkers were sourced from a range of biological matrices including blood, dried blood spots, fingernails, plasma, saliva, serum and urine. Therefore, clinical practices should consider employing a combination of matrices during the diagnosis process.

Previous systematic reviews assessed biomarkers taken from traditional matrices such as blood, plasma and/or serum (Bahbah *et al.*, 2021; Kaufman and Lamster, 2002; Hochholzer *et al.*, 2010; Lyngbakken *et al.*, 2019). Hochholzer *et al.* (2010) and Lyngbakken *et al.* (2019) reviews did not consider the benefits of alternative matrices such as fingernails and saliva. Moreover, Bahbah *et al.* (2021) and Kaufman and Lamster (2002) reviews focused on a single biological matrix that limited the number of biomarkers identified (Bahbah *et al.*, 2021; Kaufman and Lamster, 2002).

With regards to biological matrices, plasma was the most reported matrix (n = 5, 26.3%) (Carter *et al.*, 2016; Lau *et al.*, 2019; Shetty, Kumari and Shetty, 2020; Shi *et al.*, 2018; Wang *et al.*, 2013). This can be attributed to plasma glucose and HbA1c levels' monitoring being the preferred method for the diagnosis of T2DM (Inzucchi *et al.*, 2012). Furthermore, the circulating nature of plasma means that the matrix reflects inflammation, stress and damage around the body for an indication of disease prognosis. Similarly, blood (n = 3, 15.8%) and serum (n = 3, 15.8%) are also reflective of the inflammation caused in the prognosis of CVDs and DM. Serum is particularly useful for disease diagnosis due to its richness in proteins and metabolites (Yu *et al.*, 2011).

The upregulation of inflammatory markers such as CRP are key indications of CVDs; however, clinical practices must consider variation between male and female patients. Previous work showed that biological sex played a significant role in the regulation of CVD biomarkers (Li *et al.*, 2009). For example, Lakoski *et al.*, (2006) identified that

females have a substantially higher median CRP level in comparison to males (2.56 versus 1.43 mg/L). Moreover, when risk factors such as BMI were accounted for, females were still at a higher risk of developing CVDs (Lakoski *et al.*, 2006). Research also suggested that males possessed higher levels of CD56 and myoglobin; whereas, adiponectin and leptin were higher in females (Kautzky-Willer, Harreiter and Pacini, 2016; Lau *et al.*, 2019). Increased plasma leptin is strongly associated with subcutaneous fat and the development of T2DM in males. Nonetheless, an inverse correlation between plasma adiponectin levels and insulin sensitivity was seen in obese and diabetic patients, with this often being pronounced in female patients (Li *et al.*, 2009). Variation in biomarker regulation has been attributed to sex differences observed in risk factor/behavioural habits including high blood pressure, hypercholesterolaemia, smoking and obesity (Woodward, 2019). Commonly CVDs are perceived as a 'man's problem', with females experiencing atypical symptoms, which are often misdiagnosed as stress or anxiety. Furthermore, the misconception that females are 'protected' against CVDs based on their exposure to endogenous oestrogens means that females are more likely to encounter diagnostic errors (late diagnosis/misdiagnosis) and further life-threatening complications. Biological sex also plays a role in metabolic disorders such as DM. Research demonstrated that DM prevalence is higher in males in comparison to females and this is related to their higher risk of developing obesity, insulin resistance and hyperglycaemia than females (Tramunt *et al.*, 2019). Based on this information, it is imperative that clinical practices acknowledge variations in biomarker concentration and how this is influenced by biological sex.

Regarding techniques for detecting biomarkers, the review identified that LC-MS/MS was an extremely desirable technique for the diagnosis of CVDs and DM. The incorporation of tandem MS/MS ensured high sensitivity and specificity for a range of biomarkers that vary in terms of quantity and nature (Pitt, 2009). ELISA was another frequently used diagnostic technique, and this can be attributed to its ability to specifically detect a wide range of target analytes (Hosseini *et al.*, 2018). This ability could be further improved through an ELISA sandwich approach, which is more sensitive and specific than conventional ELISA (Lau *et al.*, 2019; Shah and Maghsoudlou, 2016). The frequent use of GC-MS for clinical practices could be explained by its high resolution, which made it ideal for resolving complex biological

samples such as plasma (Kałużna-Czaplińska, 2011). Moreover, clinical practices should consider employing non-invasive and non-destructive techniques such as IR and NIR spectroscopy (Blanco and Villarroy, 2002).

2.5. Strengths and Limitation

A key strength of the systematic review was the quality of included studies and the type of data extracted. For data extraction, two independent reviewers systematically reviewed published data. Data were then verified by a third reviewer and this ensured that bias was avoided. Moreover, the application of quality tools including PROSPERO and CASP checklist, ensured that the overall quality of the systematic review was maintained and validated. However, limitations were still encountered in the review. Firstly, the reviewed studies varied in design, technique and inclusion criteria. Therefore, it was extremely difficult to make comparative conclusions between the published data. For example, a range of analytical techniques were used to assess similar outcomes. The lack of a standardised approach for biomarker identification created heterogeneity within the results. The studies' variable sample size further contributed to the study's heterogeneity. Studies with smaller sample size (< 40) were more likely to see a lack statistically significant outcomes. Hence, generalisability of findings was not possible.

2.6. Conclusions

To maximise diagnostic potential, panels should include a range of biomarkers varying in concentration and nature e.g. amino acids, enzymes, fatty acids, hormones and proteins. However, biological sex played an imperative role in the regulation of CVDs and DM biomarkers and should be acknowledged during diagnosis. Analytical techniques employed for the detection of biomarkers included numerous chromatographic and spectroscopic techniques, although LC-MS/MS was most frequently reported due to its high accuracy, sensitivity and specificity. Nonetheless, clinical practices should look at employing rapid and non-destructive techniques such as IR and NIR spectroscopy and working with non-invasive biological matrices like fingernails and saliva for improved diagnostic monitoring.

2.7. Key messages

- CRP, GDF15 and NOTCH1 showed the ability to frequently predict CVDs.
- Isoleucine, followed by tyrosine and linoleic acid, were frequently utilised for the diagnosis of DM.
- Biological sex played a significant role in the regulation of CVDs and DM biomarkers.
- LC-MS/MS showed accurate diagnosis but was costly and time-consuming. IR and NIR were non-destructive and economic on the other hand.
- Clinical practices should employ novel biological matrices for optimised diagnostic abilities such as fingernails.

Chapter three: Investigating risk factors of cardiovascular diseases and diabetes mellitus from the perspective of the public.

This chapter investigated the relationship between risk factors and CVDs/DM. Risk factors were investigated through an anonymous questionnaire survey. The questionnaire was completed by the participants independently and was divided into three parts, related to the participants' sociodemographic, lifestyle and clinical characteristics. A total of 126 questionnaires were completed by participants of varying age, ethnicity, educational levels and occupation. Diseases reported included atrial fibrillation (AF), CAD, cardiomyopathy, heart disease, hypertension, left ventricular hypertrophy and DM, alongside comorbidities such as hypercholesterolaemia. To understand the relationships between risk factors and CVDs/DM, LR models were utilised. Age demonstrated the highest predictive capabilities for the prediction of CVDs, suggesting that individuals aged ≥ 40 years old were more likely to develop a CVD than those aged ≤ 39 years old. This was followed by family history of CVDs, religion (as a coping strategy) and DM. In contrast, CVDs also acted as a risk factor for DM, followed by age, family history of DM, number of meals and coexistence of other diseases.

3.1. Introduction

The presence of clinical and lifestyle risk factors increase the susceptibility of individuals to CVDs and/or DM (Bays *et al.*, 2021; Gaba *et al.*, 2022; Ismail, Materwala and Kaabi, 2021; Kong *et al.*, 2021). Modifiable risk factors refer to behaviours and habits that can be changed (Alwan *et al.*, 2024). Common modifiable risk factors related to CVDs and DM included: alcohol/tobacco consumption, inadequate physical activity, inappropriate diet, lipid profile and obesity (Hachesu, Feli and Sakhvidi, 2017; Mohamed, 2022; Krishnappa *et al.*, 2021; Razaei *et al.*, 2022; Rezaianzadeh *et al.*, 2023).

Semi-modifiable risk factors are defined as comorbidities that through pharmacological and/or lifestyle intervention can be modified (Johansson *et al.*, 2021). For example, work by Bazmandegan *et al.*, (2023) mentioned the relationship between CVDs and DM, with diabetic patients having a 10% higher risk of developing a CVD than non-diabetics. Moreover, DM has been linked with a 75% increase in the mortality rate of CV patients (Canto *et al.*, 2019). In contrast, non-modifiable risk factors are factors that cannot be adjusted and include age, biological sex, ethnicity, family history of disease and genetics (Das *et al.*, 2024; Shakoori *et al.*, 2020).

To understand trends in the prevalence and management of disease, it is first imperative to develop the knowledge of risk factors and how they make an individual more susceptible to disease (Adu *et al.*, 2019; Barlow *et al.*, 2002; Clark *et al.*, 1991). Addressing such risk factors allows for the understanding and dissemination of interventions. Rippe (2018) suggested that the adaption of daily actions and habits could act as a powerful tool for management of disease severity and prevalence. Regular physical activity, sound nutrition, weight management and non-smoking, positively influenced the risk and severity of CVDs (Rippe, 2018). Similarly, improved diabetic outcomes have been associated with healthier dietary choices, regular exercise regimens, reducing alcohol consumption and non-smoking (Hackett *et al.*, 2018).

To advise patients of healthier life-style actions and habits, it is necessary to understand the relationship between risk factors and disease presence. CVD and DM risk factors were separated into two groups: patient- and clinical-related factors. Patient-related factors included sociodemographic and lifestyle factors. Through the application of anonymous questionnaires, the aforementioned risk factors and their relation to CVDs/DM were investigated in this chapter. To determine the presence or absence of such relationships, collected participants' data (found in Appendix B1) was used in LR models. The objectives of this work were set as followed:

- To identify risk factors from the selected target population of healthy controls and CVD/diabetic cases.
- To determine the predictor values of risk factors for future cases of CVDs and/or DM.

- To explore the relationship between modifiable risk factors including diet, exercise and smoking habits for the dissemination of impactful health strategies and intervention.
- To investigate the relationships between a variety of comorbidities and diseases in relation to CVDs and/or DM through the application of LR.

3.2. Methods

Structured questionnaires were completed by participants to understand the relationships between risk factors and CVDs/DM (Appendix A6). The independence granted by questionnaires allowed participants the time and opportunity to contemplate questions and reflect on answers without the presence of an interviewer/researcher. Moreover, the anonymity of questionnaires ensured that responders could answer honestly with no fear of stigmatisation. Hence, the questionnaire data was a highly valid and represented the target population of CVD and diabetic cases (Murdoch *et al.*, 2014). Therefore, true cause-and-effect relationships could be determined and disease interventions established.

3.2.1. Study Design

The application of research questionnaires and/or surveys in healthcare can be deemed as a long-standing tradition that dates back to the 1960s and 1970s (Logan, 1966; Oberst, 1978). This tool allows researchers to gather primary data from specific populations (O'Connor, 2022). In comparison to interviews or observations, questionnaires allow the collection of less complex data, which can be utilised for the identification of patterns and relationships across the target population (Braun and Clarke, 2013). Moreover, questionnaires offer several practical advantages over interviews/observations in terms of resources and time.

Therefore, an anonymous questionnaire, which could be administered online, was deemed most appropriate for research in risk factors associated with CVDs/DM. Risk factors evaluated included 'comorbidities', 'diet', 'exercise', 'medicine use' and 'smoking' (Table 3.1). As demonstrated, the questionnaire was separated into two main sections, patient-related (sociodemographic and lifestyle) and clinical-related.

Questions included a range of closed-ended questions e.g. 'Do you smoke?', which required a 'yes' or 'no' answer and open-ended questions such as 'How healthy do you consider your diet? What does a normal mealtime consist of?'.

Part I: Patient-related Factors

This section of the questionnaire was separated into two parts, those being: sociodemographic (part I.A) and lifestyle (part I.B).

Part I.A: Sociodemographic

In this section of the questionnaire, participants were asked three questions regarding their demographic background including their age, biological sex and ethnicity. They were also asked questions relating to their sociodemographic background including education level, household size, marital status, occupation and religious practice. This section consisted of eight questions.

Part I.B: Lifestyle Factors

This part of the questionnaire aimed to collect information regarding the participants' lifestyle in terms of behaviours and habits. A total of 10 questions were asked regarding the participants diet, exercise and smoking habits, as well as their height and weight.

Part II: Clinical Factors

This section consisted of seven questions and investigated the absence or presence of any medical conditions and/or diseases. Participants were also asked to comment on their family history of illness, management of their conditions, medicine/vitamin use and visits to A&E or emergency doctors. Not only did this section look to identify the type of medicines and vitamins applied for medical condition/disease management, but also to indicate common themes in medical intervention and routine such as frequency of administration and skipping medicine intake.

Table 3.1. Questions deployed in questionnaire.

Factor	Questions
Patient-related	Sociodemographic
	• What is your age? • What is your biological sex? • What is your ethnic background? • Do you practice religion, if so, which religion do you practice? • What is your marital status? • What is your education level? • What is your occupation? • How many people live at your home?
	Lifestyle
	• Are you a smoker? • If you do smoke, what do you smoke? • What is your weight? • Do you suffer from being overweight? If yes, did the doctor advice you to lose weight? • What is your height? • How active do you consider your lifestyle? • What kind of exercise do you participate in? • How many meals do you have a day? • How healthy do you consider your diet? What does a normal mealtime consist of? • Do you eat fast food? If yes, how frequently do you eat fast food?
Clinical-related	• Do you have any medical conditions? If yes, please list all the conditions. • Do you have diabetes mellitus? Including Type 1 and Type 2 diabetes. If yes, please specify the diagnosis duration. • Do you take any medicines for diabetes mellitus? • Have you been to A&E or seen an emergency doctor because of diabetes mellitus? • Do have any cardiovascular disease(s)? If yes, please list all. • Do you take any medicines for any cardiovascular disease(s)? • If you have hypertension/hypotension, does your blood pressure fluctuate? • Do you have any family history of any disease(s)? If yes, please specify. • Do you take any medicines for other medical conditions? • How many medicines do you take? • How frequently do you take your medicines? • Do you skip your medicines intake for any reason? • Are you happy with the management of your conditions? Please specify.

3.2.2. Ethical Approval and Consent

In compliance with LJMU's Code of Research Practice (2020), ethical approval was granted from LJMU (23/PBS/009A), as well as the Lebanese University (LU) (2022-0104). Moreover, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Good Clinical Practice Standards (2023) (ICH, 2023), the WHO Good Clinical Practice (2005) (WHO, 2005) and the Data Protection Act (2018) (Legislation.Gov.uk, 2025) were considered. Additionally, all participants provided written/signed consent for providing a set of 6 – 10 fingernail clippings.

3.2.3. List of definitions

Comorbidities are defined as the coexistence of two chronic medical conditions (Li *et al.*, 2021). A comorbidity coexists with the index condition (specific condition under consideration) over an individual's lifestyle (Kuan *et al.*, 2023; Li *et al.*, 2021). Risk factors are described as conditions or measurements associated with an increased probability of disease (Nexøe, Halvorsen and Kristiansen, 2007; Reventlow, Hvas and Tulinius, 2009; Willadsen *et al.*, 2016).

3.2.4. Data Collection

Electronic copies of the questionnaire were sent to potential participants via the LJMU's Forensic Science community site. Potential participants were invited to complete questionnaires electronically or in written format.

During the design phase of questionnaires, a focus group discussion was carried out. The focus group consisted of healthy (n = 16, 80%) and unhealthy participants (n = 4, 20%). Rapport was created and maintained between the principal investigator (PI) and participants using a conversational approach. In the discussion, the PI highlighted key areas of the research such as disease detection, disease prognosis and treatment but allowed the participants to take control of the discussion. Common themes such as 'diet', 'exercise' and 'smoking' were identified on several occasions and were frequently associated with the presence of CVDs and/or DM. The focus group setting ensured that participants could freely give their own opinion and thoughts regarding

their disease susceptibility, experience and lifestyle. Moreover, participants were asked to discuss potential questions that could be utilised for the collection of data regarding risk factors of CVDs/DM. The themes highlighted during the discussion were then validated through a comparison with the literature and were used to construct the questionnaire.

3.2.5. Participant Recruitment and Selection

A convenience sampling method was utilised, which meant that samples were taken from participants that were readily available or convenient. For example, the staff and students at LJMU. The application of a convenience sample ensured that the sample's representation was strengthened, as well as generalisability of the research results (Golzar, Noor and Tajik, 2022).

3.2.5.1. Inclusion and Exclusion Criteria

Participants recruited were of adult age (≥ 18 years old). Individuals with no previous medical diagnosis of a long-term condition were included as healthy controls. Moreover, participants with prior diagnosis of medical conditions/disease unrelated to CVDs and/or DM e.g. anxiety, breast cancer or hyperthyroidism, were also included and were classified as 'unhealthy'. Those with a medical diagnosis of CVDs and/or DM were also included in the study sample. Potential participants who could not give their consent for any reason, were excluded from the study.

3.2.5.2. Identification of Participants

A total of 126 participants completed the questionnaire. These participants received a participant information sheet, which explained the overall aim of the study, its research and wider societal implication, their rights throughout the study and dissemination of findings.

3.2.6. Pilot Study

For piloting the questionnaire, a total of 30 participants took part in the pilot study. The purpose of this study was to first identify whether the produced questionnaire elicited valuable responses, which provided insight into the relationship between risk factors and disease, as well as common trends between participants, who had been

diagnosed with CVDs and/or DM. The pilot study was also carried out to validate the use of LR models for assessing the predictive capabilities of risk factors in relation to CVDs/DM.

3.2.7. Data Analysis

Data analysis was carried out using Microsoft Excel 2019 and SPSS V29.0.2.0. Categorical data was expressed as the number of (n) and frequency (%). To identify the relationship between risk factors and the diseases of interest, LR was applied to the participants' data. The produced odds ratio (OR) values, alongside confidence intervals (CI) of 95%, demonstrated the likelihood of disease presence in the face of certain characteristics and factors. The presence of CVDs or DM were classified as the dependent variable and risk factors e.g. smoker were categorised as the independent (predictor) value (Vetter and Schober, 2018). Theoretically, LR is an extension of linear regression (Schober and Vetter, 2021; Vetter and Schober, 2018). Instead of modelling a linear relationship between the independent variable and the outcome probability, the LR model assumes a linear, straight-line relationship with the logit of the outcome (Schober and Vetter, 2021). The regression coefficients represent the intercept and slope of the line (Equation 3.1).

Equation 3.1. LR model (Schober and Vetter, 2021).

$$\text{Log} \left(\frac{p}{1-p} \right) = b_0 + b_1 X$$

Where, log is the logit or log odds,

P is the probability,

b_0 is the intercept of the line,

and b_1 is the slope of the line (Schober and Vetter, 2021).

An OR value of > 1 is associated with higher odds of a particular outcome, in this case the presence of disease. While an OR value < 1 suggests lower odds of an outcome. An OR of 1 indicates that the factor does not affect the odds of the outcome (Szumilas, 2010). To estimate the precision of the OR values, 95% CI were utilised. Large CI values were indicative of low levels of OR precision, while smaller CI values signified higher OR precision. However, it should be noted, that while the CI values do provide an indication of OR precision, they should not be confused with p-values and do not provide a measure of statistical significance (Szumilas, 2010).

3.2.8. Validity and Reliability

The supervisory team was initially asked to critically assess the content of the questionnaire and therefore determined its (face) validity. The PI's supervisors expressed their views on the 28 questions and whether they would consider a correct and comprehensive reflection of the research aim.

The reliability of the questionnaire refers to the consistency of a measure. Therefore, a participant completing the questionnaire, meant to identify the presence of CVD and/or DM risk factors, should have approximately the same responses each time the questionnaire is completed (Zhang and Aryadoust, 2022). In the case of this research, equivalence was decided as a reliable measure of consistency among several responses of the questionnaire (Heale and Twycross, 2015). As several groups were investigated within this research, the PI assessed the reliability by examining responses made from individuals of the same group e.g. healthy controls.

3.3. Results

3.3.1. Pilot Study

The pilot study aimed to investigate the relationships between risk factors and CVDs/DM and included 30 participants, who were classified as healthy (no medical diagnosis) ($n = 6$, 20%), unhealthy (medical diagnosis unrelated to CVDs and/or DM) ($n = 7$, 23%), CVD ($n = 8$, 27%), diabetic ($n = 7$, 23%) and CVD-diabetic ($n = 1$, 3%) participants.

3.3.1.1. Demographic Factors

Respondents included 20 females (67%) and 10 males (33%). The reported age of respondents ranged from 18 – 84 years of age. The majority of respondents (n = 12, 40%) were aged 40 – 64 years old, the remaining participants were aged 18 – 24 (n = 2, 6.67%), 25 – 34 (n = 3, 10%), 35 – 39 (n = 2, 6.67%) and 65 – 84 years old (n = 11, 36.67%). Four ethnic groups were accounted for, those being: Arab (n = 12, 40%), Asian (n = 1, 3.33%), Lebanese Arab (n = 10, 33.3%) and White (n = 7, 23.3%).

3.3.1.2. Lifestyle Factors

Lifestyle characteristics investigated in relation to disease included diet, exercise and smoking habits. Four specific diet types were reported by participants and included gluten free (n = 1, 3.33%), halal meat (n = 3, 10%), high protein diet (n = 1, 3.33%) and low salt diet (n = 1, 3.33%). The remaining 24 participants reported not adhering to a specific diet or dietary requirement. Exercise was rarely reported by participants, with only eight responders reporting frequent exercise (26.67%). Across the 30 responders, eight reported smoking cigarettes (26.67%) and one shisha (3.33%). The remaining 21 (70%) responders did not smoke.

3.3.1.3. Clinical Factors

A total of 41 diseases were recorded and included: high blood pressure (n = 8, 15.1%), hypercholesterolaemia (n = 6, 11.3%) and hypertension (n = 6, 11.3%).

3.3.1.4. Medicinal Factors

In total, 80 medicines were accounted for, with the most common medicines being Aspirin (n = 4, 3.88%), followed by fluoxetine, furosemide and paracetamol, which were all recorded three (2.91%) times across the 30 participants. A wide range of vitamins (n = 19) were also reported. The most frequently recorded was vitamin D (n = 13, 25%), followed by vitamin C (n = 4, 7.69%) and omega-3 fish oil (n = 3, 5.77%).

3.3.1.5. LR

When applied to the LR models, the pilot study data revealed several key relationships between predictive values and the presence of CVDs or DM. For the prediction of CVDs, biological sex demonstrated the highest predictive capabilities and suggested that females were 2.15 (95% CI = 0.356 – 13.1) times more likely than males to have a CVD. CVDs were also linked to diet (OR = 0.531, 95% CI = 0.051 – 5.55), ethnicity (OR = 1.094, 95% CI = 0.169 – 7.061), exercise (OR = 1.4, 95% CI = 0.224 – 8.77),

presence of additional medical conditions (OR = 1.5, 95% CI = 0.293 – 7.68), smoking habits (OR = 1.25, 95% CI = 0.233 – 6.69) and religion (OR = 0.138, 95% CI = 0.015 – 1.302). The presence of disease was most strongly associated with religion, with those practicing a religion being 10.20 (95% CI = 1.55 – 67.2) times more likely than non-religious participants to have DM. Factors such as age (OR = 2.63, 95% CI = 0.264 – 26.1), biological sex (OR = 0.375, 95% CI = 0.070 – 1.99), ethnicity (OR = 2.63, 95% CI = 0.264 – 26.1), exercise (OR = 3.27, 95% CI = 0.334 – 31.9), number of meals (OR = 3.57, 95% CI = 0.658 – 19.3), medical conditions (OR = 2.5, 95% CI = 0.41 – 15.2) and smoking habits (OR = 1.6, 95% CI = 0.289 – 8.86) were also linked to DM.

3.3.2. Sample for the Main Study

The collection of participants' data for the main study started in November 2021 to June 2024. Participants were of different nationalities, ethnic groups and sociodemographic backgrounds. Prior to the study, participants were invited to the study via email and received the participant information sheet, consent form and questionnaire before they decided on their participation. Those who consented to participate, were provided with the questionnaires online or as a printed document. A total of 126 participants completed the questionnaire.

3.3.3. Patient-related Characteristics of Participants

A summary of the participants' sociodemographic characteristics can be found in Table 3.2. These characteristics showed that the main age range reported was 40-64 years old, the main sex was female and the main ethnicity was White. The other characteristics related to education, number of people in household, marital status and occupation had many unreported cases. The majority of the participants were aged 40 – 64 (n = 69, 54.8%) years old, followed by participants aged 18 – 24 (n = 21, 16.7%), 65 – 84 (n = 20, 15.9%), 24 – 34 (n = 11, 8.73%) and 35 – 39 (n = 5, 3.97%) years old. The results demonstrated that most of the participants recruited were female (n = 76, 60.32%). A total of seven ethnic groups were accounted for, with the majority being classified as White (n = 46, 36.5%). While 38.9% (n = 48) of the recruited participants

did not report the size of their household, 61.1% (n = 78) of participants made evident their household occupancy. The most frequently reported household size was two (n = 33, 26.2%), followed by one (n = 22, 17.5%) and four (n = 14, 11.1%). Less reported household sizes included three (n = 4, 3.17%) and five+ (n = 5, 3.97%). To further understand the participant's household and living arrangements, participants were asked about their marital status. The most frequently reported marital status was single (n = 38, 30.16%), followed by married (n = 21, 16.7%). Participants also reported cohabitation (n = 6, 4.76%), divorce (n = 4, 3.17%) and engagement (n = 8, 6.35%). The remaining participants (n = 49, 38.9%) did not report a marital status. Considering the UK Government's Standard Occupation Classification, participants were placed into six categories, those being: academic, engineers, laboratory-based, retired, students and other (bartender, cleaner, forensic accountant and salaried occupation) (Elias and Birch, 2010). While 41 (32.5%) participants reported no religious belief, 50 (39.7%) participants reported a religious belief of Hinduism (n = 4, 3.17%), Islam (n = 43, 34.1%), Catholicism (n = 1, 0.793%) and Methodism (n = 1, 0.793%).

Table 3.2. Sociodemographic characteristics of participants (n = 126).

Parameter		n (frequency, %)
Age group (years old)		
	18-24	21 (16.7)
	25-34	11 (8.73)
	35-39	5 (3.97)
	40-64	69 (54.8)
	65-84	20 (15.9)
Median		20
IQR		11-21
Biological sex		
	Female	76 (60.32)
	Male	50 (39.7)
Ethnicity		
	Arab	38 (30.16)
	Asian	13 (10.32)
	Indian	2 (1.59)
	Lebanese Arab	22 (17.5)
	Middle Eastern Arab	3 (2.38)
	Mixed	2 (1.59)
	White	46 (36.5)
Religion		
	Hinduism	4 (3.17)
	Islam	43 (34.1)
	Others (Catholicism, Methodism, unspecified)	3 (2.38)
	No	41 (32.5)
	Not reported	35 (27.8)
Marital status		
	Cohabiting	6 (4.76)
	Divorced	4 (3.17)
	Engaged	8 (6.35)
	Married	21 (16.7)
	Single	38 (30.16)
	Not reported	49 (38.9)
Educational level		
	Bachelor's	9 (7.14)
	College qualifications	6 (4.76)
	High school	6 (4.76)
	Master's	6 (4.76)
	No formal qualifications	13 (10.32)
	PhD +	37 (29.4)
	Others (HNC)	1 (0.794)
	Not reported	48 (38.1)
Occupation		
	Academic	36 (28.6)
	Engineer (including mechanic and auto electrician)	12 (9.52)
	Healthcare	5 (3.97)
	Laboratory-based	6 (4.76)
	Retired	7 (5.56)
	Student	7 (5.56)
	Other (bartender, cleaner, forensic accountant, salaried occupation)	4 (3.17)
	Not reported	49 (38.9)
Household		
	Five +	5 (3.97)
	Four	14 (11.1)
	Three	4 (3.17)

	Two	33 (26.2)
	One	22 (17.5)
	Not reported	48 (38.1)
Median		18
IQR		7.25-30.25

IQR: Interquartile range; HNC: Higher national certificate; n: Number of.

Regarding lifestyle factors, most participants showed unhealthy lifestyles in terms of smoking, diet and weight (Table 3.3). However, the vast majority of participants (n = 71, 53.4%) were non-smokers. Several factors were considered in relation to the participants' diets, including the diet type, fast-food consumption, number of meals and weight. While 11 specific diet types/requirements were reported across the 126 participants, several participants reported two or more diet types. For example, one participant reported practicing a mostly vegetarian diet, but was also lactose intolerant and when consuming meat, only halal meat was eaten. Across the 11 diet types reported, the most frequently reported diets were lactose intolerance (n = 22, 26.2%) and a balanced diet (n = 19, 15.1%). All participants reported the absence or presence of exercise, with most participants (n = 69, 54.8%) reporting frequent or occasional exercise.

Table 3.3. Lifestyle characteristics of participants (n = 126).

Parameters	n (frequency, %)
Smoking habits*	
Ex-smoker	2 (1.504)
Sheesha	8 (6.015)
Smoker (cigarettes or e-cigarettes)	51 (38.3)
Non-smoker	71 (53.4)
Passive smoker	1 (0.752)
Weight	
Healthy	9 (7.14)
Overweight	9 (7.14)
Not reported	108 (85.7)
Median	9
IQR	9-58.5
Exercise	
Exercise	69 (54.8)
Not active	57 (45.2)
Number of meals	
Three	61 (48.4)
Two	10 (7.94)
Two or three	17 (13.5)
Not reported	38 (30.16)
Median	27.5
IQR	15.25-43.75
Diet	
Balanced diet	19 (15.1)
Halal food	8 (6.35)
High protein	4 (3.17)
Misses meals	3 (2.38)
Lactose intolerance	22 (26.2)
Ultra-processed foods	4 (3.17)
Others (gluten free, high carbohydrate, low protein, low salt and vegetarian)	5 (3.97)
No specific diet	61 (48.8)
Fast-food	
Yes	18 (14.3)
No	108 (85.7)

IQR: Interquartile range; n: Number of.

*Participants reported the use of multiple smoking habits such as both cigarettes and e-cigarettes, therefore the total is higher than 126.

3.3.4. Clinical-related Characteristics of Participants

Clinical-related characteristics included comorbidities, CVDs, DM, medicine use and vitamin use (Table 3.4).

Table 3.4. Clinical-related characteristics of participants (n = 126).

Parameters	n (frequency, %)
CVD conditions	
Heart disease	2 (6.67)
Hypertension	24 (80)
Other (AF, CAD, issues with left ventricle and weakness of heart muscle)	4 (13.3)
DM conditions	
T1DM	3 (15.0)
T2DM	17 (85.0)
Comorbidity	
Anxiety	9 (6.98)
Asthma	5 (3.88)
Chronic sinusitis	2 (1.55)
Depression	3 (2.33)
High blood pressure	2 (1.55)
High cholesterol	13 (10.1)
Hyperthyroidism	5 (3.88)
Thyroid removed	3 (2.33)
IBS	2 (1.55)
Other (Alzheimer's disease, androgenic alopecia, bipolar, BPH, breast cancer, Celiac disease, chronic sinusitis, COPD, Crohn disease, FMF, kidney disease, Meniere's disease, neurological conditions, osteoporosis, Parkinson's disease, phenylketonuria, photosensitive epilepsy, rhinitis, tensional headaches)	17 (3.2)
No medical conditions	68 (48.9)
Family History of Disease	
Cancer	2 (1.55)
CVDs (angina, hypertension and stroke)	9 (6.97)
Dementia	1 (0.775)
DM	5 (3.88)
No	77 (59.7)
Not reported	35 (27.1)
Medical Use	
Angiotensin II receptor antagonist**	
Irbesartan	2(18.2)
Telmisartan	2(18.2)
Valsartan	4(36.4)
Others (candesartan cilexetil, irbesartan and hydrochlorothiazide*, losartan potassium)	3(27.3)
Angiotensin-converting enzyme inhibitors**	
Ramipril	7(77.8)
Others (perindopril, trandolapril and verapamil HCl*,	2(22.2)
Antiepileptics	
brivaracetam	1(33.3)
ethosuximide	1(33.3)

	valproic acid	1(33.3)
Antihistamine		
	cetirizine HCl	3(9.38)
	fexofenadine HCl	4(12.5)
	loratadine	21(65.6)
	unspecified antihistamine	3(9.38)
	Others (cinnarizine)	1(3.13)
Antimuscarinics		
	ipratropium bromide	20(80)
	solifenacin succinate	3(12)
	Others (hyoscine butylbromide, tiotropium bromide)	2(8)
Beta blocking agent**		
	bisoprolol fumarate	9(81.8)
	sotalol HCl	2(18.2)
Biguanides***		
	metformin HCl	14(93.3)
	Others (sitagliptin and metformin*)	1 (6.67)
Calcium-channel blockers**		
	amlodipine besylate	5(62.5)
	perindopril erbumine with amlodipine*	2(25)
	Others (diltiazem HCl)	1(12.5)
Corticosteroids		
	beclometasone with formoterol*	2(25)
	fluticasone with azelastine*	4(50)
	Others (fluticasone, umecclidinium and vilanterol*, montelukast)	2(25)
Insulins, long acting***		
	insulin degludec	1(33.3)
	insulin glargine	1(33.3)
	insulin glulisine	1(33.3)
Non-steroidal anti-inflammatory drugs		
	aspirin**	7 (17.5)
	diclofenac	11 (27.5)
	ibuprofen	22 (55)
SSRIs		
	citalopram	2 (28.6)
	fluoxetine	4 (57.1)
	Others (paroxetine)	1 (14.3)
Others		
	atorvastatin**	13 (8.5)
	bendroflumethiazide**	2 (1.31)
	betahistine dihydrochloride	2 (1.31)
	bromazepam	3 (1.96)
	clopidogrel**	2 (1.31)
	fenofibrate	4 (2.61)
	furosemide**	4 (2.61)
	mebeverine HCl	23 (15.03)
	omeprazole	25 (16.3)
	paracetamol	32 (20.91)
	risperidone	4 (2.61)
	spironolactone**	2 (1.31)
	sulodexide	3 (1.96)
	tramadol HCl	2 (1.31)
	Others (allopurinol, bumetanide**, bismuth subsalicylate, colchicine, desogestrel, docetaxel, docusate sodium, doxazosin**, duloxetine,	32 (20.91)

empagliflozin***, ethinylestradiol with levonorgestrel*, finasteride, flupentixol/melitracen*, glimepiride***, gliquidone***, isosorbide mononitrate**, isotretinoin, ivabradine**, letrozole, levothyroxine sodium, mesalazine, naftidrofuryl oxalate, nebivolol**, pantoprazole, propranolol HCl**, pseudoephedrine HCl and triprolidine*, risedronate sodium, salbutamol, simvastatin, sumatriptan, thiamazole, trimetazidine**, vildagliptin and metformin* ***)

Vitamin and Lifestyle Products Use		
	Biotin	20 (8.55)
	Calcium	6 (2.56)
	CBD oil	3 (1.28)
	Collagen	19 (8.12)
	Creatine monohydrate	2 (0.855)
	Hyaluronic acid	13 (5.56)
	Multivitamins	5 (2.14)
	Vitamin B	2 (0.855)
	Vitamin C	30 (12.8)
	Vitamin D	52 (22.2)
	Fish oil/Omega-3	6 (2.56)
	Zinc	4 (1.71)
	Other (Berocca, iron, Magne B6, magnesium, ten herbs, Perfectil, Phlexy-10, Phlexy vitamins, unspecified vitamins, vitamin A, vitamin B, vitamin B12 and vitamin B complex)	11 (4.701)
	No vitamin use	61 (26.1)

AF: Atrial Fibrillation; BPH: Benign prostatic hyperplasia; CAD: Coronary artery disease; CBD: Cannabidiol; COPD: Chronic obstructive pulmonary disease; CVDs: Cardiovascular diseases; DM: Diabetes mellitus; FMF: Familial Mediterranean fever; HCl: Hydrochloride; IBS: Irritable bowel syndrome; IQR: Interquartile range; SSRIs: Selective serotonin reuptake inhibitors; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

*Used in combination.

** Drug used to treat CVDs.

*** Drug used to treat DM.

3.3.4.1. Medical Conditions

Across the study population, 68 (54%) participants had no medical conditions. The remaining participants (n = 58, 46%) had a medical condition of which 30 (55.2%) were related to CVDs and 20 (34.5%) were related to DM. An additional 30 medical conditions (unrelated to CVDs/DM) were also recorded. Many suffered from two or more medical conditions (n = 37, 63.8%). The most frequently reported comorbidity was hypercholesterolemia (n = 13, 9.35%), closely followed by anxiety (n = 9, 6.47%) and asthma (n = 5, 3.62%).

Across the CVDs reported, hypertension demonstrated the highest prevalence and was recorded by 24 (80%) participants. This was followed by unspecified heart disease, which was recorded on two occasions (6.67%). Both T1DM and T2DM were represented within the main study sample and were reported by three (15%) and 17 (85%) participants, respectively.

3.3.4.2. *Family History of Disease*

The vast majority (n = 77, 59.7%) of participants reported no family history of disease. The lack of response was seen for 25 (27.1%) participants.

3.3.4.3. *Medicine Use*

A variety of medicines were reported and included antihistamines, antidepressants/antipsychotics, antimuscarinic, CVD medication, DM medication and others.

3.3.4.3. *Vitamin and Lifestyle Products Use*

While 61 (26.4%) participants reported no vitamin or lifestyle product use, the remaining 65 respondents reported a variety. The most frequently used was vitamin D (n = 52, 22.5%), followed by vitamin C (n = 30, 12.9%). Other common lifestyle products included biotin (n = 20, 8.66%), collagen (n = 19, 8.23%) and hyaluronic acid (n = 13, 5.63%).

3.3.5. LR

The results of the LR are presented in Table 3.5. Across the 16 variables examined, the strongest association between a risk factor and CVDs was age. Individuals aged ≥ 40 years old were 11.3 (95% CI = 1.46 – 87.4) times more likely to develop a CVD than individuals ≤ 39 years old. Similarly, those with DM were also 7.75 (95% CI = 2.68 – 22.4) times more likely to also have a CVD than those without DM. In like manner, participants with comorbidities such as high blood pressure and/or high cholesterol were 1.83 (95% CI = 0.665 – 5.029) times more likely to have a CVD than those with no additional medical conditions. However, due to the high CI values, seen noticeably for age, OR values were approached with caution and findings could not be generalised (Hazra, 2017; Irala, Navajas and Castillo, 1997). A family history of CVDs also increased the likelihood of developing a CVD by an OR of 10.5 (95% CI = 0.731 – 150.9). Interestingly, responders that reported a religious practice were more likely to have a CVD than non-religious responders (OR = 4.08, 95% CI = 0.400 – 41.6). The high CI of religion does however suggest that precision is low and therefore indicated that not all individuals who practiced religion also had a CVD and/or DM. As highlighted by the literature, common risk factors such as a lack of exercise, smoking

and weight were linked to CVDs (Adhikary *et al.*, 2022). The number of meals consumed by a participant daily was not associated with the prevalence of CVDs.

Akin to the prevalence of CVDs, the likelihood of developing DM was associated age. Similarly, those aged ≥ 40 years old were 4.44 (95% CI = 0.974 – 20.203) times more likely to develop DM than individuals ≤ 39 years old. The presence of a CVD demonstrated the strongest predictive capabilities for the presence of DM. Hence, participants with a CVD were 7.75 (95% CI = 2.68 – 22.4) times more likely to also have DM than non-CV participants. Similarly, participants with an additional medical condition or a family history of DM were 3.19 (95% CI = 1.071 – 9.504) and 3.64 (95% CI = 0.553 – 23.9) times more likely to have DM than those with no additional medical conditions or family history of DM, respectively. While diet was not associated with DM (OR = 0.759, 0.291 – 1.98), the number of meals strongly predicted the presence of DM. For example, participants who had two meals or fluctuated between two or three meals daily were 5.96 times (95% CI = 0.728 – 48.8) more likely to have DM than those who ate three consistent meals a day. However, the precision of this OR value is low and therefore was approached with caution. Marital status showed an extremely low predictive capability in relation to DM (OR = 0.90, 95% CI = 0.222 – 3.64). However, it is worth noting that an imbalance of responses was received with several participants ($n = 49$, 38.9%) not declaring their marital status. Therefore, strong conclusions regarding the relationship between marital status and presence of disease could not be drawn.

Table 3.5. LR of sociodemographic, lifestyle and clinical characteristics of participants (n = 126).

Parameter	CVDs		DM	
	OR	95% CI	OR	95% CI
Age	11.3	1.46-87.4	4.44	0.974-20.203
Biological sex	0.955	0.374-2.42	0.606	0.232-1.58
CVD	-	-	7.75	2.68-22.4
Educational level	0.044	0.005-0.378	0.044	0.005-0.38
Ethnicity	0.645	0.254-1.64	0.655	0.249-1.72
Exercise	1.64	0.650-4.14	1.59	0.607-4.14
Diet	0.786	0.312-1.98	0.759	0.291-1.98
DM	7.75	2.68-22.4	-	-
Family history	10.5	0.731-150.9	3.64	0.553-23.9
Household	0.267	0.031-2.27	0.00	0.00-0.00
Marital status	0.90	0.222-3.64	0.90	0.222-3.64
Medical condition	1.83	0.665-5.03	3.19	1.071-9.50
Number of meals	0.00	0.00-0.00	5.96	0.728-48.8
Occupation	0.223	0.051-0.98	0.119	0.023-0.62
Religion	4.08	0.400-41.6	1.44	0.432-4.80
Smoke	1.51	0.600-3.81	1.15	0.441-3.02
Weight	2.29	0.169-30.9	0.438	0.032-5.93

CI: Confidence intervals; OR: Odds ratio.

3.4. Discussion

The present study provided a real-world insight into the prevalence of CVDs/DM among the public. The findings of this research confirmed the relationship between risk factors related and CVDs/DM. Risk factors such as age, comorbidities and high BMI were strongly related to CVDs and DM. Similarly, patterns within the demographic characteristics of participants uncovered groups susceptible to CVDs or DM. For an illustration, females were 0.955 (95% CI = 0.374 – 87.4) and 0.606 (95% CI = 0.232 – 1.58) times more likely to develop a CVD and DM than males, respectively.

3.4.1. Patient-related Characteristics

3.4.1.1 Sociodemographic Characteristics

This study confirmed previous work in the literature that suggested a strong relationship between age and CVDs. Kim (2023) highlighted the ‘very high-risk’ of individuals aged ≥ 70 years old to CVDs, particularly atherosclerosis. Furthermore,

using age as a predictive risk factor, Heidenreich *et al.*, (2011) was able to predict that by the year 2030, 27 million people will have hypertension, eight million CAD, four million stroke and three million HF (Costantino, Paneni and Cosentino, 2015). Akin to the highlighted research, this study demonstrated that older participants (40 – 84 years old) were more likely to have a CVD than younger participants (18 – 39 years old). Functional changes in ageing adults' hearts are characterised by diastolic and systolic dysfunction and electrical dysfunction, both of which have been associated with a high prevalence of AF and HF (Rodgers *et al.*, 2019). Ageing was also linked to high levels of proinflammatory markers such as CRP and tumour necrosis factor- α (TNF α) (Meschiari *et al.*, 2017). Such markers contribute to cardiac remodelling such as significant extracellular matrix (ECM) remodelling. Dysregulation of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) expression levels are frequently associated to increased collagen deposition and in turn development of cardiac hypertrophy and fibrosis (Meschiari *et al.*, 2017). The aforementioned structural changes often lead to cardiac dysfunction in ageing participants (Martos *et al.*, 2007).

With regards to the sample population, CVDs and DM were linked to the female biological sex. While CVDs have historically been perceived as a 'male's disease', they are the leading cause of mortality and morbidity worldwide for both male and female patients (Gauci *et al.*, 2022; WHO, 2025). Work by Glasser *et al.*, (2024) suggested that the prevalence of CVDs within the male population can be attributed to male gender expressivity (MGE). Prevalent sociocultural pressures such as expectations, norms and values, of stereotypical male behaviours have often been said to shape the behaviour of males (Courtenay, 2000; Gal and Wilkie, 2010; Li and Gal, 2022; Stanaland, Gaither and Gassman-Pines, 2023). As a result, males often adopt risk factor behaviours such as smoking habits and poor weight control. Nonetheless, the presence of CVDs is severely understudied, underdiagnosed and undertreated in females (Burgess, 2022). Therefore, while previous work and figures suggest that males are more likely to manifest a CVD than females, care should be taken when carrying out diagnostic assignments. In fact, the literature revealed that the varying biological mechanisms related to sex play a significant role in the manifestation of CVDs (Clayton and Gaugh, 2022). A common misconception identified within the literature is the protective qualities of biological mechanisms

related to sex. Thus, while oestrogen does appear to exert a protective effect against CVDs such as atherosclerosis and IHD in premenopausal females, it is ineffective in postmenopausal females (Clayton and Gaugh, 2022; Connelly, Jandeleit-Dahm and Delles, 2020; Regitz-Zagrosek and Kararigas, 2017). Nonetheless, the relationship between oestrogen and CVD protection can be confirmed through examining the participants' characteristic data. For example, several ($n = 13$) of the female participants with CVDs, were of menopausal age. Thus, they did not have a sufficient level of oestrogen for CVD protection. Similarly, studies suggested that premenopausal females have greater skeletal muscle, hepatic insulin sensitivity and higher stimulated insulin secretion (Goossens, Jocken and Blaak, 2021; Kautzky-Willer, Harreiter and Pacini, 2016). Hence, have lower fasting glucose and HbA_{1c} values than males and are typically protected from DM. However, the protection of females from DM is reduced during their postmenopausal years, as the case for the participants within the study.

3.4.1.2. Lifestyle Characteristics

The LR model indicated a weak relationship between diet and the diseases of interest. The low OR value for diet in relation to CVDs/DM could be attributed to a variety of diet styles. Therefore, there was no specific category of diet more frequently reported than another category. Diet styles including a balanced diet and halal meat consumption have been found to positively impact the presence of disease. Current scientific research makes evident that Western dietary patterns including pre-packaged foods, red meat, refined grains, processed meat and ultra-processed food/snacks have been linked to excessive production of proinflammatory cytokines and with the reduction of anti-inflammatory cytokines (Denova-Gutiérrez *et al.*, 2016; Hu, 2002; Okada *et al.*, 2018; Viscogliosi *et al.*, 2013). Casas *et al.*, (2018) suggested that dietary intervention through a better combination of multiple foods and nutrients can act as an effective prevention for CVDs. Similarly, Sami *et al.*, (2017) also demonstrated the positive impact of diet intervention on the presence of T2DM. Studies have found a close link between a high intake of carbohydrates and/or fats in relation to T2DM (Hosseini *et al.*, 2022; Khatib, 2004; Ludwig, Peterson and Gortmaker, 2001). Halal meat has also been linked with healthier lipid profiles and improved medical outcomes (Carrasco *et al.*, 2022). Thus, the presence of such diets may not act as a causation for CVDs and/or DM but rather as a form of management.

In individuals that provided an indication of weight or specific height and weight values, the relationship between being overweight and presence of disease was examined. While weight predicted both CVDs and DM, a stronger predictive capability for CVDs was observed. Obesity, which is classified as a multifactorial disease, possesses a complex pathogenesis related to biological, psychosocial, socioeconomic and environmental factors (Franks and McCarthy, 2016; Gebreab *et al.*, 2018; Loos, 2012; Sallis and Glanz, 2009; Sommer *et al.*, 2015). Moreover, obesity disrupts the heterogeneity in pathways and mechanism that are associated to adverse health outcomes (Jastreboff *et al.*, 2019; Jensen *et al.*, 2013; Gordon-Larsen and Heymsfield, 2018). Through prolonged excess calorie intake, excessive fat storage surpasses the limited storage capacity of adipose tissue for fatty acids. As a result, increased levels of circulating free fatty acids occur, as well as abnormal storage of fatty acids in organs that play imperative roles in metabolic regulation including the liver, pancreas and skeletal muscles (Welsh *et al.*, 2024). The lipotoxicity of fatty acids causes chronic inflammation, oxidative stress and systemic metabolic dysregulation (Khanna, Welch and Rehman, 2022; Welsh *et al.*, 2024). Therefore, lipotoxicity is a major risk factor for several chronic diseases including CVDs, particularly hypertension, DM, kidney disease and multiple types of cancers (Welsh *et al.*, 2024). In the case of CVDs, obesity is both directly and indirectly impactful. For example, excessive adiposity induces endothelial dysfunction, alongside small vessel remodelling and cardiomyocyte. As a result, atherosclerotic and vasospastic CHD, arrhythmias, cardiomyopathy and congestive HF are promoted (Powell-Wiley *et al.*, 2021). Furthermore, obesity contributes to other common CVD risk factors including DM, dyslipidaemia and chronic kidney disease (Khanna, Welch and Rehman, 2022).

The relationship between obesity and disease urges not only for a balanced diet, but also for frequent exercise. The LR models indicated that individuals who did not frequently take part in exercise or physical activity were more likely to develop CVDs/DM than those who frequently took part in exercise/physical activity. This was confirmed by the literature which provided strong scientific evidence supporting the relationship between physical activity and a reduced risk of cardiometabolic risk factors, alongside clinical end points (Perry *et al.*, 2023). Moreover, Nystoriak and Bhatnagar (2018) made evident the relationship between a sedentary lifestyle, characterised by low levels of physical activity and poor CV health. In contrast, regular

exercise and physical activity positively impacts the risk and prevalence of developing CVDs (Paffenbarger *et al.*, 1986; Nystoriak and Bhatnagar, 2018). In like manner, death rates among females and males have been found to be inversely linked to cardiorespiratory fitness levels, even in the presence of other CVD risk factors including smoking and hyperlipemia (Blair *et al.*, 1996; Nystoriak and Bhatnagar, 2018). Additionally, increased levels of fitness have been shown to partially reverse the increased rates of CVD mortality that are associated with high BMI or obesity (Hu *et al.*, 2002; Nystoriak and Bhatnagar, 2018; Stevens *et al.*, 2002). Similarly, excess lipid accumulation surrounding key organs can cause impairment of insulin signalling and insulin resistance (Vodovotz *et al.*, 2020). Thus, the use of physical activity favourably impacts glycaemic control (Colberg *et al.*, 2016; Piercy *et al.*, 2018; Syeda *et al.*, 2023).

The predictive capability of smoking as a risk factor for CVDs/DM was confirmed within the literature. Through meta-analysis, Lv *et al.*, (2015) was able to determine that among non-smokers, those exposed to second-hand smoke were 1.23 times more likely to develop CHD than those unexposed (Fu *et al.*, 2024). The increased CV risk has been attributed to adrenergic effects created by the nicotine, which results in increased coronary microvascular resistance, heart rate, inotropic status and reduced insulin sensitivity (Gallucci *et al.*, 2020). In similar fashion, the habit of smoking was also associated with DM. Cigarette smoke has been associated with blood-clotting, endothelial dysfunction and vascular damage (Cacciola, Guarino and Polosa, 2007). A systematic review carried out by Willi *et al.*, (2007) demonstrated that smokers were 1.44 (CI = 1.13 – 1.58) times more likely to develop T2DM than non-smokers. Similarly, Pan *et al.*, (2015) found that smokers were 1.37 (95 CI = 1.33 – 1.42) times more likely to have T2DM than non-smokers. Roderick *et al.*, (2019) went as far to describe the relationship between smoking and DM-related mortality, with diabetic smokers being 1.48 (95% CI = 1.34 – 1.64) times more at risk for all-cause mortality than non-smokers. Thus, it is not uncommon for smokers to develop both CVDs and DM, alongside other respiratory diseases such as chronic obstructive pulmonary disease (COPD), interstitial lung disease and lung cancer (Ishii, 2013; Varghese and Gharde, 2023). Research by Mangoni *et al.*, (2002) also suggested that smokers are more likely to adopt poor lifestyles such as a poor diet, which is commonly characterised by a low intake of fibre and a high intake of fats and sugars (Dallongeville *et al.*, 1998;

Morabia, Bernstein and Antonini., 2000; Norouzzadeh *et al.*, 2024). Empirical evidence also highlighted that smokers that lacked an adequate nutritional status were at a higher risk of CVD development (Dallongeville *et al.*, 1998; Norouzzadeh *et al.*, 2024; Stamler *et al.*, 1997).

3.4.2. Clinical Characteristic

Comorbidities are associated with complex clinical management, increased health costs and poor health outcomes (Brown and Thorsteinsson, 2020; Valderas *et al.*, 2009). As highlighted by the LR analysis, the manifestation of CV comorbidities is common. Buddeke *et al.*, (2019) made apparent that the most common comorbidity of HF was PAD and of stroke was CHD (Buddeke *et al.*, 2019). This work also highlighted non-CV comorbidities including back/neck problems, cancer, COPD, DM, low vision and osteoarthritis. (Buddeke *et al.*, 2019). Research by Rayman *et al.*, (2022) made evident key comorbidities related to DM including obesity, heart disease and renal disease. Moreover, this research indicated that poor management of DM often led to additional comorbidities including DKA and hyperosmolar hyperglycaemic syndrome (Rayman *et al.*, 2022). Within the sample population, several participants with CVDs and/or DM also suffered frequently from high cholesterol and high blood pressure. The presence of the aforementioned comorbidities is not unexpected and has been raised on several occasions within the literature (Fuchs and Whelton, 2020; Jung *et al.*, 2022; Ohishi, 2018). Among CVD risk factors, high blood pressure was the strongest evidence for causation and prevalence of exposure (Fuchs and Whelton, 2019). Ohishi (2018) demonstrated the close link between elevated blood pressure and DM. For illustration, this work showed that patients with DM were also likely to suffer peripheral artery resistance caused by vascular remodelling (a consequence of high blood pressure) and increase body fluid volume, which is associated with insulin resistance-induced hyperinsulinemia and hyperglycaemia (Ohishi, 2018).

A close relationship was also observed between CVDs and DM, with sufferers of CVDs being more likely to also suffer from DM and vice versa. Work by Canto *et al.*, (2019) indicated that a significant number of health burdens associated with DM were also related to macrovascular and microvascular complications including CHD, HF, PAD and stroke. CVDs are the main cause of morbidity and mortality in sufferers of T2DM

(Wang *et al.*, 2016a). For example, individuals with T2DM are likely to experience a cardiac event 15 years earlier than those without DM (Booth *et al.*, 2006). Factors such as autonomic neuropathy, endothelial dysfunction, increased coagulability and oxidative stress, commonly found in diabetic patients, directly contribute to the development of CVDs (Matheus *et al.*, 2013). Similarly, hypertension is extremely prevalent in both T1DM and T2DM, with a prevalence of 30% and 60%, respectively (Matheus *et al.*, 2013).

The relationship between family history of CVDs or DM and their prevalence was confirmed through the literature. For example, Taylor *et al.*, (2023) suggested that a parental history of CVDs conferred a greater risk of future CVDs amongst offspring. Moreover, Lenarda *et al.*, (2024) further confirmed a relationship between CAD and a family history of CVDs. Similarly, research also suggested that family history of DM acted as a non-modifiable risk factor for DM. To illustrate, Papazafiropoulou *et al.*, (2017) made evident that individuals that have either one or two parents with T2DM were 2 – 4 times more likely to develop T2DM than those with no diabetic parents. Alrashed *et al.*, (2023) also indicated family history as a risk factor for DM and showed amongst a sample of 713 participants, that the risk of DM development was 76.3% higher amongst participants with a family history of DM than those with no history.

3.5. Strengths and Weaknesses

A key strength of this study was that data had been collected from participants diagnosed with CVDs and/or DM by trained healthcare experts. Therefore, their experiences in terms of comorbidities, medication/vitamin use and disease management were highly reflective of CVD/DM cases. Moreover, results from this study increased the awareness of CVD/DM risk factors and demonstrated key areas for medical intervention. Thus, this work can be used to advice healthcare practitioners, researchers and sufferers of CVDs/DM on improved disease management and outcomes. Moreover, data gathered from this study could help create cost-effective healthcare interventions such as dietary plans, exercise routines and smoking cessation schemes.

The first limitation of this research was the small sample size of 126 participants. It is also worth noting that certain questions regarding height and weight yielded low

response rates. This may be attributed to two reasons, the first being the participant did not know their specific height/weight and the second being that they felt uncomfortable declaring this information. However, it should be noted that questionnaires were made anonymous in an attempt to protect participants from any form of discomfort. As data was only taken from the UK and Lebanon, caution should be taken when interpreting the generalisability of findings globally. In addition, as a self-report, questionnaires can pose risks for response bias, which is characterised by a participant providing inaccurate answers (Elston, 2021). Two common types of response bias include social desirability bias, which refers to the desire to conform to perceived social norms, and the Hawthorne effect, which is the desire to appear favourably to the researcher (McCambridge, Witton and Elbourne, 2014; Teh *et al.*, 2023). Thus, in the case of this work, participants may have felt embarrassed about their current diet style and chosen to describe it in a positive light such as a balanced diet or no specific diet type. However, the PI hoped that through the application of anonymity, the likelihood of biased results was limited (Kang and Hwang, 2023).

Furthermore, it is important to consider the type of responders. As previously demonstrated, a high number of responders were academics ($n = 36$, 28.6%), while the sample represented only a small number of students ($n = 7$, 5.56%). This supports the idea of research informed teaching, whereby, research underpins the curriculum content (Joseph-Richard *et al.*, 2021). Therefore, academics, who are also carrying out their own research, are more likely to appreciate and participate in research. On several occasions, responders also chose not to report social characteristics including education, household size, marital status and occupation, as well as weight. The reason for missing responses can be attributed to participants feeling embarrassed about sensitive data or fearful of how data will be used (Lounsbury *et al.*, 2021). This is not only seen for research, which has publishable findings, but social media as well. In the age of digital interconnectivity, the caution regarding digital footprints has become increasingly significant. A digital footprint refers to the trail of data through the internet, online purchases, shared multimedia, social media and website visits (Kaku, 2024). Concerns for protecting digital footprints have been associated with academic opportunities, job prospects and social interactions (Kaku, 2024). Social media offers a unique opportunity for the promotion of scientific literature and allows authors/publishers to reach a wider audience (Abou-Ismael *et al.*, 2024). Therefore, it

is likely that participants were more cautious and protective over their data, creating low response rates of certain questions. Missing data can not only create gaps and plausibility of conclusions but can also weaken the accuracy of results. Moreover, if non-response rates are high in certain groups, generalisation of the group's population is not plausible. Thus, bias can be introduced into the sample and models, reducing representation and statistical significance (Choi and Pak, 2004). However, the response rates observed within this study did not come from a sole group e.g. age group or ethnicity. Hence, statistical analysis and findings were not drastically skewed.

3.6. Conclusions

This chapter identified several modifiable, semi-modifiable and non-modifiable risk factors leading to CVDs/DM. The aforementioned risk factors were categorised into sections, namely, sociodemographic, lifestyle and clinical-related factors. Sociodemographic factors leading to CVDs and DM included age, biological sex, educational level, ethnicity, household size, religion, marital status and occupation. Lifestyle factors comprised diet and fast-food consumption, exercise, number of meals, smoking habits and weight. Finally, clinical characteristics referred to the presence of additional medical conditions (unrelated to CVDs/DM), CVD diagnosis, DM diagnosis, family history of disease, medicine use and vitamin use.

LR models were produced to confirm the predictive capabilities of risk factors. Factors including age, family history of CVDs, religion and the presence of DM demonstrated the highest predictability for CVDs. In like manner, age, family history of DM and presence of additional medical conditions showed the strongest predictive relationship with DM. In several cases, findings were confirmed by the literature and ensured that the results found in this study were not unique to the sample population. Therefore, ensuring generalisability to sufferers of CVDs and/or DM in the UK and Lebanon. Moreover, the confirmation of such risk factors, allows the dissemination of medical intervention. This research made evident that unhealthy lifestyle factors such as smoking, infrequent exercise and poor diets, can be modified to prevent and improve disease manifestation and outcomes.

As described by Al-Hamid *et al.*, (2024), CVDs are commonly perceived as a 'man's disease'. This misconception contributes to the under-diagnosis and treatment of

females on a global scale (Schenck-Gustafsson, 2009). Diagnosis of CVDs in females is not often clear, due to the different and delayed presentation of CVD symptoms in comparison to males (Al-Hamid *et al.*, 2024). For example, it is not uncommon for females suffering with acute myocardial infarction (AMI) to present atypical symptoms such as abdominal pain, back/neck pain, dyspnoea, indigestion, palpitations and unexplained fatigue (Eastwood and Doering, 2005; Gold and Krumholz, 2006). In contrast, males often display typical symptoms including chest pains, which are better recognised by doctors. As a result, females often do not report their symptoms or are under-diagnosed (Brewer, Svatikov and Mulvagh, 2015; Xhyheri and Bugiardini, 2010). A delayed or lack of diagnosis can be detrimental to the clinical outcomes. However, the findings of this chapter demonstrate the prevalence of CVDs in females and urges for a greater level of care and consideration to be carried out during the diagnosis process.

The disparity between previous research and the study's results, as well as the complexity of a patient, in terms of sociodemographic, lifestyle and clinical characteristics, urges for the development of unique patient profiles. Through applying a patient centred approach, the management of chronic conditions have greatly improved (Inzucchi *et al.*, 2012). Thus, clinical practices should take into consideration several of the patient's characteristics and risk factors, during the prediction of disease. A combination of risk factors such as ethnicity, inadequate exercise and smoking habits can not only provide aid during the diagnosis process but also provide insight into disease management (Elissen *et al.*, 2016). The complexity of the term ethnicity even encapsulates several factors such as 'culture' and 'religion', which in turn impact lifestyle characteristics such as diet and smoking habits (Ford and Harawa, 2011; Hamer *et al.*, 2018).

3.7. Key Messages

- The questionnaire revealed modifiable, semi-modifiable and non-modifiable risk factors that were categorised as sociodemographic, lifestyle and clinical factors and were present for several CVD and/or diabetic participants.

- LR models demonstrated the predictive capabilities of several risk factors including age, biological sex, presence of additional medical conditions, smoking and lack of physical activity for CVDs/DM.
- Age, family history, religion and the presence of DM possessed the highest predictive capabilities for the development of CVDs. While factors including age, family history, the presence of comorbidity and the presence of CVDs showed the highest predictive capability for DM.
- Medical intervention including cessation of smoking, frequent exercise and nutritional diets can aid in disease management and outcomes.

Chapter four: Evaluation of Raman spectroscopy as a diagnostic tool for cardiovascular diseases and diabetes mellitus

The objective of this chapter was to explore the feasibility of Raman spectroscopy for detecting CVDs and/or DM in fingernails. The work utilised a handheld Raman spectrometer equipped with 830 nm laser wavelength. Fingernails were measured via glass vials without any pre-treatment. The findings confirmed the ability of Raman spectroscopy to detect endogenous constituents including amino acids (cysteine, cystine, tyrosine, etc.), lipids and proteins. In this respect, the presence of disease influenced the Raman activity of endogenous constituents in terms of Raman intensity. CVD, DM or CVD-diabetic fingernails showed lower Raman intensities compared to healthy fingernails. The fingernails' Raman activity was further influenced by confounding factors such as age, biological sex and ethnicity. Raman spectroscopy was also successful in detecting disease-related biomarkers. Specifically, diagnostic biomarkers were identified at 1128 (C-C trans conformation), 1129 (skeletal C-C trans conformation), 1312 (in-plane CH deformation) and 1591 cm^{-1} (C=C stretching) and were attributed to the presence of glucose, cytochrome B and cytochrome C, respectively (Atkins *et al.*, 2017). MLAs including CWS and PCA were successful in performing synchronous classification of fingernails of healthy, CVD, DM and CVD-DM, respectively.

4.1. Introduction

Raman spectroscopy has shown great success in the diagnosis of breast cancer (Cervo *et al.*, 2015; Hanna *et al.*, 2022; Nargis *et al.*, 2021; Pichardo-Molina *et al.*, 2007), cervical cancer (Shaikh, Daniel and Lyng, 2023; Wang *et al.*, 2021), gastric cancer (Liu *et al.*, 2022), prostate cancer (Artemyev *et al.*, 2021; Gaba *et al.*, 2022; Medipally *et al.*, 2017), lung cancer (Li *et al.*, 2018; Zhang *et al.*, 2019) and skin cancer (Auner *et al.*, 2018; Bratchenko *et al.*, 2022; Bratchenko *et al.*, 2021; Choo-Smith *et al.*, 2002; Kong *et al.*, 2015).

In these studies, Raman has been used both in labelled and unlabelled modes. In labelled mode, Raman was able to give specific signatures for biomarkers increased or absent in specific diseases. On the other hand, label-free Raman characterised biological matrices (fluids or tissues) and changes that occurs in them as a result of a disease. Subsequently, this chapter builds on previous research in evaluating SORS as a detection tool for CVDs and DM. The chapter addressed the following objectives:

- To determine whether spectral pre-processing was imperative for the interpretation of Raman spectra.
- To explore the Raman activity of healthy, CVD, DM and CVD-diabetic fingernails.
- To identify spectral bands corresponding to disease specific biomarkers.
- To investigate the relationship between demographic characteristics (age, biological sex and ethnicity) and Raman activity of fingernails.
- To classify healthy versus CVD and/or DM fingernails' Raman spectra using MLAs.

4.2. Methods and Materials

4.2.1. Materials

Fingernails samples were collected by the participants themselves and consisted of 6-10 fingernail clippings. Details regarding the number of fingernails per set and their dimensions can be found in Appendix B2. Fingernails were stored in 2 mL VWR glass vials prior to analysis and were then transferred into clear zip-locked bags when measured (5" / 100 x 137 mm).

Fingernail sets were collected from 72 participants (676 clippings) aged between the ages 18 – 85 years old. Participants were of female (n = 47, 65.8%) and male (n = 25, 34.7%) sex and four ethnic groups. Ethnic groups included Arab (n = 31, 43.1%), Asian (n = 4, 5.56%), Lebanese Arab (n = 14, 19.4%) and White (n = 23, 31.9%). Of these participants, 18 (25%) were diagnosed with a CVD including hypertension (n = 15, 83.3%), AF (N = 1, 5.56%), CAD (n = 1, 5.56%) and unspecified heart disease (n = 1, 5.56%) In addition, six (8.33%) participants had DM including T1DM (n = 2, 33.3%) and T2DM (n = 4, 66.7%).

4.2.2. Instrumentation

The Agilent Resolve Raman spectrometer (Agilent Technologies, Inc. Santa Clara, CA) was equipped with an 830 nm laser excitation wavelength and a maximum laser power of 475 mW (Figure 4.1). The chosen wavelength was 830 nm and was selected to avoid fluorescence, often encountered in biological samples (Goldrick *et al.*, 2018). Moreover, the choice of handheld spectrometer was justified in terms of its light weight (2.2 kg), long battery life (4-6 hours), and portability (dimensions of 73 mm x 290 mm x 155 mm). Subsequently, such technique would be sustainable and beneficial in different settings such as hospitals and clinics. The instrument utilised three modes of operation, those being: through barrier scan, surface scan (conventional point and shoot Raman) and vial holder mode. The Resolve utilised SORS to non-invasively measure materials through packaging. Hence samples were measured 'as received' in clear zip-locked bags. Three adaptor tools were available for use, including the pointer, universal tablet holder and the vial holder. However, to ensure that direct contact was made and that the fingernail was flat against the Raman source, the pointer tool was utilised. Raman spectra were collected over the range 350 – 2,000 cm^{-1} . SORS mode was utilised and allowed information from the fingernail's three layers (ventral, intermediate and dorsal) to be collected for maximised diagnostic potential (Matousek *et al.*, 2005). The fingernails measured in this study ranged between 0.13 – 1.41 mm in depth.



Figure 4.1. The Agilent Resolve Raman spectrometer, equipped with an 830 nm laser wavelength.

4.2.3. Method

Fingernails were measured through polyethylene bags, which made contact with the spectrometer's pointer tool. If fingernails were dirty, then they were soaked briefly in acetone prior to measurements. A black cloth (supplied by Agilent) was gently placed over the sample to prevent the interference of environmental light sources during measurements.

For optimisation of spectral number, the equal frequency ellipse was considered and showed no significant difference between 20 spectra and infinity for statistical analysis purposes (Equation 4.1, Figure 4.2) (Jee, 2007).

Equation 4.1. Equal frequency ellipse.

$$\text{Equal frequency ellipse} = \sqrt{\frac{(n-1)}{(n-2)}} F_{\alpha(2, n-2)}$$

Where, f is frequency,

N is the number of samples.

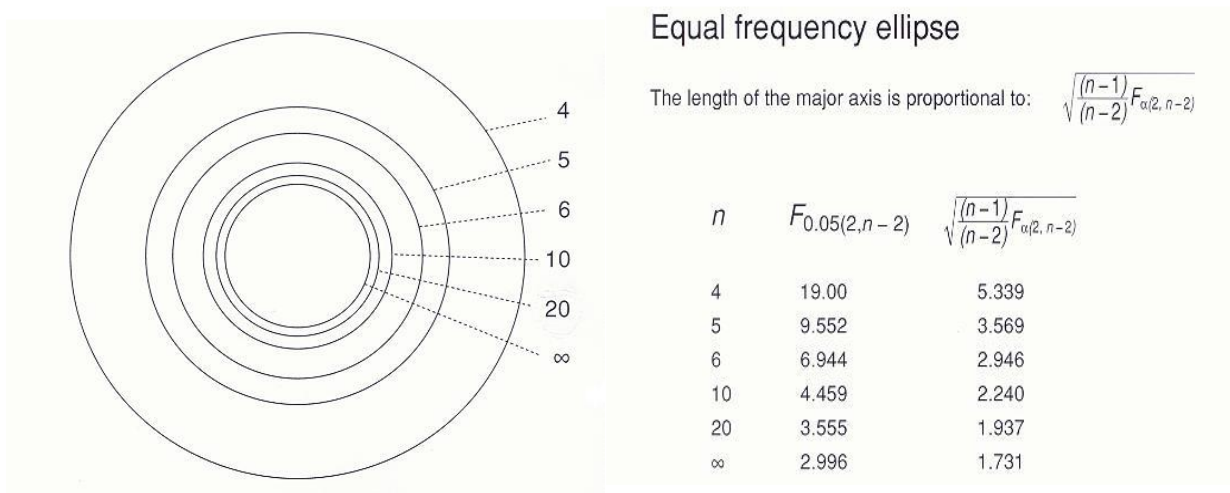


Figure 4.2. Equal frequency ellipses (Jee, 2007).

Post measurement, spectra of samples were matched against the in-built instrumental library and the values of the top three matches were recorded. These values were based on a hit quality index (HQI) that in turn indicated the % similarity between the test spectrum and library spectra. Moreover, spectra were then exported as .csv files to Microsoft Excel 2019 and MATLAB R2019a for additional spectral analysis and application of MLAs.

4.2.4. Spectral Pre-Processing

For comparison of untreated vs pre-processed data, several pre-processing methods were utilised, including MSC, SNV, D1 and D2 (Figure 4.3).

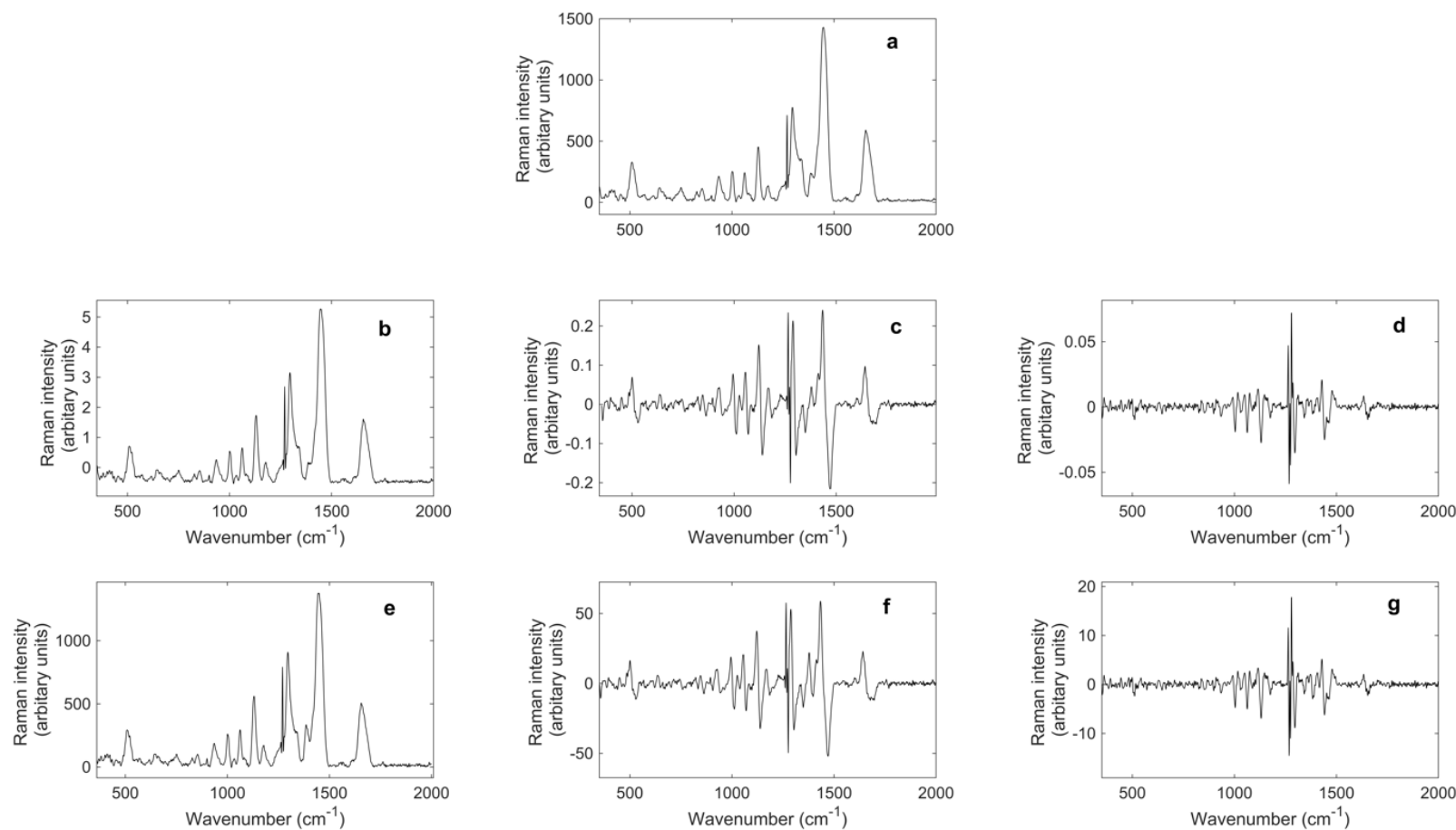


Figure 4.3. Untreated versus treated Raman spectra. (a) Raw Raman spectra compared to (b) SNV, (c) SNV-D1, (d) SNV-D2, (e) MSC, (f) MSC-D1 and (g) MSC-D2 measured using the Agilent Resolve Raman equipped with an 830 nm laser wavelength.

4.2.5. Qualitative Analysis

For qualitative analysis, the following MLAs were used: CWS, PCA and SOMs. As three variations within the SOMs were expected, those being: the fingernail's physical properties, fingernail's water content and the presence/absence of disease, a 3 x 3 SOM was applied and 100 epochs trained.

4.3. Results and Discussion

Raman spectroscopy successfully identified fingernails' key endogenous constituents and biomarkers including cysteine, cystine, tyrosine, tryptophan, cytochrome B, cytochrome C and glucose. Diseases such as IBS and high cholesterol, alongside CVDs and/or DM, disrupted the healthy activity of endogenous constituents such as cysteine and cystine.

4.3.1. Raman Signature of Fingernails

Raman signature of fingernails showed bands corresponding to endogenous constituents such as amino acids, lipids and proteins (Tables 4.1 and 4.2) (Kourkouvelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012; Towler *et al.*, 2007; Widjaja, Lim and An, 2008).

Key bands relating to amino acids were identified at 512 (S-S stretching), 647 (C-S stretching), 750 (symmetric breathing), 828 (aliphatic CCH bending), 855 (CCH bending), 1002 (aromatic C-C stretching), 1034 (in-plane C-H bending) and 1178 cm^{-1} (C-C stretching) and were associated with cystine, cysteine, tryptophan, tyrosine and phenylalanine, respectively (Kourkouvelis *et al.*, 2018; Towler *et al.*, 2007; Widjaja, Lim and An, 2008). Protein was identified at 855, 939, and 1448 cm^{-1} and was attributed to C-C stretching, skeletal C-C stretching and methylene CH_2 deformation, respectively (Kourkouvelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012; Towler *et al.*, 2007; Widjaja, Lim and An, 2008). The protein bands identified at 855, 939, and 1448 cm^{-1} could be attributed to the fingernail plate's keratin. Within the fingernail, this scleroprotein exists in the form of α -keratin that is present as both hard and soft in a

ratio of 8:2, respectively (Baswan *et al.*, 2017; Lynch *et al.*, 1986) Unspecified fatty acids or lipids (cholesterol) were located at 1448 cm^{-1} and were attributed to methylene CH_2 deformation (Baswan *et al.*, 2017; Olsztyńska-Janus *et al.*, 2012). Additional constituents were identified at 1556 cm^{-1} (NH bending) and 1656 cm^{-1} ($\text{C}=\text{O}$ bending) and corresponded to amide II, amide I and collagen type I, respectively (Kourkoumelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012).

Table 4.1. Spectral interpretation of fingernails measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

Band (cm^{-1})	Functional group	Association	Reference
512	S-S stretching	Sulphide, disulphide bridges and cystine	Kourkoumelis <i>et al.</i> , 2018 Towler <i>et al.</i> , 2007
522	S-S stretching	Cystine	Kourkoumelis <i>et al.</i> , 2018
647	C-S stretching	Cystine and cysteine residues	Kourkoumelis <i>et al.</i> , 2018
750	Symmetric breathing	Tryptophan	Widjaja, Lim and An, 2008
828	Aliphatic CCH bending	Tyrosine	Kourkoumelis <i>et al.</i> , 2018
847	C-C stretching CCH bending	Proteins Tryptophan	Kourkoumelis <i>et al.</i> , 2018
855	C-C stretching CCH bending	Hydroxyproline Tryptophan	Kourkoumelis <i>et al.</i> , 2018
937	Skeletal C-C stretching	Tyrosine	Widjaja, Lim and An, 2008
1002	Aromatic C-C-stretching	Phenylalanine	Kourkoumelis <i>et al.</i> , 2018
1032	In-plane C-H bending	Phenylalanine	Widjaja, Lim and An, 2008.
1126	C-methyl stretching	Valine	Birech <i>et al.</i> , 2017.
1127	C-OH and C-O-H stretching	Glucose	Birech <i>et al.</i> , 2017.
1128	Skeletal C-C, trans conformation	Cytochrome B	Horiue <i>et al.</i> , 2020.
1172	C-C stretching	Tyrosine	Kourkoumelis <i>et al.</i> , 2018.
1207	C-C ₆ H ₅ stretching	Tyrosine and phenylalanine	Kourkoumelis <i>et al.</i> , 2018.
1243	NH bending, CN stretching	Amide II, proteins and leucine	Widjaja, Lim and An, 2008.
1300	CH deformation	Leucine and glucose	Birech <i>et al.</i> , 2017.
1337	CH ₃ CH ₂ twisting	Cytochrome C	Managò, Zito and De Luca, 2018.
1448	Methylene CH ₂ deformation	Lipids and proteins	Olsztyńska-Janus <i>et al.</i> , 2012. Towler <i>et al.</i> , 2007.
1547	NH bending C-N stretching	Amide II Tryptophan	Kourkoumelis <i>et al.</i> , 2018.
1580	C-N stretching N-H bending	Amide II Protein	Surmacki <i>et al.</i> , 2018.
1591	Olefinic C-C stretching	Isoleucine, cytochrome C	Widjaja, Lim and An, 2008.
1608	In-plane C=C bending	Phenylalanine and tyrosine	Widjaja, Lim and An, 2008.
1657	C=O bending	Amide I, collagen type 1 and protein	Olsztyńska-Janus <i>et al.</i> , 2012.

Table 4.2. Frequency of Raman bands present in healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernails measured using the Agilent Resolve Raman Spectrometer equipped with an 830 nm laser wavelength.

Band position (cm ⁻¹)																								
Participa nt	512	522	647	750	828	847	855	937	1002	1032	1126	1127	1128	1172	1207	1243	1300	1337	1448	1547	1580	1591	1608	1657
MWS1																								
MWS2																								
MWS3																								
MWS4																								
MWS5																								
MWS6																								
MWS7																								
MWS8																								
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MWS61																							

4.3.2. Spectral Quality

Spectral quality was then performed to determine Raman activity of fingernails in the absence/presence of disease (Table 4.3). The first parameter to be assessed was the number of bands, which ranged from 12 – 21 (median = 17, IQR = 16 – 18). Fingernail set MWS57, showed the highest number of bands ($n = 21$), while set MWS58 displayed the least bands ($n = 12$). The additional bands identified within set MWS57 included 827, 1033 and 1206 cm^{-1} and were attributed to tyrosine (aliphatic CCH bending, in-plane C-H bending and C-C stretching) and phenylalanine (C-C₆H₅ stretching) (Kourkoumelis *et al.*, 2018; Widjaja, Lim and An, 2008). These bands were not seen in MWS58 that indicated the absence of phenylalanine and tyrosine due to CVD (Hu *et al.*, 2023).

The second parameter was maximum band intensity, which varied amongst the fingernails and was in the range of 734.96 – 22993.46 arbitrary units (median = 1680.63 arbitrary units, IQR = 1275.35 – 2105.79 arbitrary units). Set MWS25 demonstrated the lowest band intensity of 734.96 arbitrary units, whereas set MWS22 showed the highest band intensity of 22993.46 arbitrary units. Fingernail sets demonstrated band positions between 1445 – 1451 cm^{-1} (median = 1449 cm^{-1} , IQR = 1448 – 1449 cm^{-1}), which were associated with methylene CH₂ deformation (Chiriac *et al.*, 2021; Towler *et al.*, 2007; Sihota *et al.*, 2019). An outlier was observed within spectra of set MWS5, which presented a maximum band position of 1297 cm^{-1} . This region has previously been associated with C-H deformation and amide III (Chiriac *et al.*, 2021). It was also suggested that bands present between 1206 – 1305 cm^{-1} were evidence of tissue degradation caused by cigarette smoke altering the fingernail's chemical and physical structure, a risk factor reported by participant MWS5, who reported smoking 2 – 8 cigarettes a day.

The third parameter was signal-to-noise (S/N) and ranged from 12.64 – 129 (median = 46.05, IQR = 32.58 – 60.13). The lowest S/N ratio was observed in set MWS58, whereas fingernail set MWS47 demonstrated the highest S/N (median = 46.05, IQR = 32.5626 – 60.125). The variability of S/N ratio across the fingernail sets could be explained through the hydration of the participant and fingernails prior to sampling. For example, conditions such as hypertension and hyperthyroidism have been associated with dry brittle fingernails (Rosenberg and Lipner, 2022; Stern *et al.*, 2007). This was

the case for participant MWS47, who reported the presence of the highlighted conditions (hypertension and hyperthyroidism) and therefore was more susceptible to dehydrated brittle nails. Lower Raman activity of water present within the fingernail produced less interference and a higher Raman signal (Kim, Byun and Lee, 2017). Overall, fingernails produced either a strong (n = 33, 45.8%), medium (n = 36, 50%) or weak (n = 3, 4.2%) Raman activity.

Table 4.3. Spectral quality of fingernails taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser.

Fingernail Set	N	Band Intensity (Arbitrary units)	Band positions (cm ⁻¹)	S/N	Raman activity
MWS1	14	1472.07	1448	56.5	M
MWS2	16	857.17	1449	26.0	M
MWS3	19	1277.62	1449	64.5	S
MWS4	17	1758.35	1448	59.5	S
MWS5	19	855.23	1297	23.0	W
MWS6	15	1105.59	1447	36.3	M
MWS7	16	1027.50	1446	33.3	M
MWS8	13	1162.52	1447	38.0	M
MWS9	14	808.97	1447	22.0	W
MWS10	14	1273.78	1450	27.3	M
MWS11	15	1766.44	1448	40.0	M
MWS12	14	2120.83	1449	97.0	M
MWS13	14	2198.45	1449	100.0	M
MWS14	19	1650.05	1451	37.7	M
MWS15	17	1227.94	1448	35.0	M
MWS16	16	1199.98	1449	51.5	S
MWS17	15	1718.23	1447	59.0	M
MWS18	16	1630.58	1449	56.5	S
MWS19	16	1753.09	1448	60.5	S
MWS20	18	2233.85	1449	102.0	S
MWS21	16	1904.40	1449	43.7	S
MWS22	16	22993.46	1449	105.0	S
MWS23	17	1877.34	1449	43.0	S
MWS24	18	2121.93	1451	98.0	S
MWS25	16	734.96	1448	19.2	M
MWS26	18	827.68	1449	19.0	M
MWS27	18	1099.31	1447	25.5	M
MWS28	16	1785.24	1449	60.5	S

MWS29	16	2156.41	1448	24.8	M
MWS30	17	1994.24	1446	55.0	S
MWS31	17	1637.34	1449	25.3	M
MWS32	17	2253.08	1449	51.5	S
MWS33	16	1675.62	1448	56.5	S
MWS34	18	2865.29	1450	114.0	S
MWS35	19	2165.62	1449	97.0	S
MWS36	15	2154.76	1449	49.0	M
MWS37	17	1969.27	1448	64.0	S
MWS38	18	1911.37	1449	44.0	M
MWS39	20	935.32	1448	26.5	M
MWS40	16	1580.33	1448	60.0	S
MWS41	15	2927.14	1450	111.0	S
MWS42	18	2392.21	1450	55.0	S
MWS43	18	1567.71	1449	30.0	M
MWS44	18	1972.60	1449	36.0	M
MWS45	18	1685.64	1449	58.0	S
MWS46	19	1466.16	1450	28.0	M
MWS47	16	1894.50	1449	129.0	S
MWS48	16	2094.82	1449	47.5	S
MWS49	17	2316.45	1450	50.5	S
MWS50	18	1703.26	1448	39.0	M
MWS51	16	1570.73	1447	40.0	M
MWS52	17	2321.02	1449	53.0	S
MWS53	19	2212.09	1449	50.5	S
MWS54	18	1665.90	1448	28.5	M
MWS55	18	1499.99	1449	38.3	M
MWS56	20	1798.73	1449	62.0	S
MWS57	21	1352.79	1448	28.8	M
MWS58	12	886.45	1445	12.6	W
MWS59	19	1177.70	1447	26.8	M
MWS60	19	1465.33	1449	44.6	S
MWS61	16	2998.03	1449	68.5	M
MWS62	21	1212.64	1449	39.0	M
MWS63	15	811.59	1448	24.4	M
MWS64	19	1335.56	1445	65.5	S
MWS65	18	1388.61	1449	34.0	M
MWS66	18	2268.28	1448	62.0	S
MWS67	17	2234.97	1449	61.5	S
MWS68	18	2082.96	1449	57.0	S
MWS69	18	1275.87	1448	31.3	M
MWS70	16	2100.77	1449	57.5	S
MWS71	17	1490.50	1449	44.0	M

MWS72	21	1171.64	1447	33.0	M

M: Medium; N: number of bands; S: Strong; S/N: signal-to-noise ratio. All spectra Showed scattering in the range of 350 – 2,000 cm⁻¹.

Raman spectral pre-processing strategies were systematically evaluated using MSC, SNV and D1 or D2. Spectra acquired from the fingernail sample set MWS1 were used as a representative test dataset to enable a direct and unbiased comparison between untreated and pre-processed spectra. Spectral quality was assessed through the aforementioned parameters and determined the Raman activity of spectra as either strong, medium or weak. The evaluation demonstrated that spectral quality was consistently degraded following the application of pre-processing treatments. Raw Raman spectra exhibited medium (n = 10, 50%) and weak (n = 10, 50%) activity. In comparison SNV-treated spectra demonstrated medium (n = 5, 25%) and weak (n = 15, 75%). Furthermore, derivative-based treatments (SNV1-D1 and SNV-D2) resulted in exclusively weak Raman activity. Similarly, MSC, MSC-D1 and MSC-D2 combinations produced weak Raman activity across all 20 spectra and confirmed substantial signal degradation. Although the highlighted pre-processing techniques have previously been applied to Raman spectra, they are more commonly optimised for the treatment of NIR spectra (Huang, Romero-Torres and Moshgbar, 2010). However, in the present study, pre-processing hindered meaningful feature extraction and resulted in inaccurate clustering (González-Vidal, Pérez-Pueyo and Soneira, 2017; Smulko, Wróbel and Barman, 2015; Wu *et al.*, 2021). Therefore, based on the spectral quality assessments performed, untreated Raman spectra were selected for subsequent spectral interpretation and modelling.

4.3.3. Impact of demographic characteristics on Raman activity of fingernail

Demographic characteristics including age, biological sex and ethnicity impacted the Raman activity with clear differences between subcategories of each demographic characteristic.

4.3.3.1. Age

Raman spectra of fingernails showed differences in bands' intensities across the five age groups evaluated. Age groups included: 18 – 24 (Group one, n = 13, 18.1%), 25

– 34 (Group two, n = 3, 4.17%), 35 – 39 (Group three, n = 2, 2.78%), 40 – 65 (Group four, n = 43, 59.7%) and 66 – 85 years old (Group five, n = 11, 15.3%) (Figure 4.4, Table 4.4). Bands related to proteins showed higher Raman scattering in older individuals in contrary to bands related to lipids. For instance, the band corresponding to S-S and C-S stretching of cystine and cysteine (512 cm^{-1} and 647 cm^{-1}) showed the highest scattering for elderly participants (66-85 years old) (Kourkouvelis *et al.*, 2018). This confirmed other studies that showed that cystine contributed to ageing in elderly individuals (Akbaraly *et al.*, 2013).

All age groups showed close median intensities for tryptophan that was seen at 750 cm^{-1} and corresponded to the symmetric breathing of tryptophan (Widjaja, Lim and An, 2008). Yet, Raman scattering of tryptophan at 750 cm^{-1} was low for all groups in the range of 20-30 arbitrary units. Tryptophan showed further scattering at 855 cm^{-1} related to CCH bending (Kourkouvelis *et al.*, 2018). This band could also be linked to C-C stretching of proteins (Kourkouvelis *et al.*, 2018). Proteins were also featured at 936 cm^{-1} (skeletal C-C stretching) across all age groups, however scattering at this band mostly occurred for those aged 25 – 34 years old (Widjaja, Lim and An, 2007). High Raman scattering of phenylalanine (aromatic C-C stretching), proteins (C-N stretching) and collagen type 1 (C=O bending) were also seen in participants aged 25 – 34 years old, at 1005 , 1172 , 1550 and 1656 cm^{-1} , respectively (Kourkouvelis *et al.*, 2018, Olsztyńska-Janus *et al.*, 2012; Towler *et al.*, 2007; Surmacki *et al.*, 2018). However, bands corresponding to tyrosine showed higher scattering intensity in individuals of age < 35 years old at 828 cm^{-1} . Lipids were seen at lower Raman intensity in elderly and that was featured in bands at 1449 and 1580 cm^{-1} , which corresponded to the methylene CH_2 deformation of lipids and the C-N stretching of amide II, respectively (Olsztyńska-Janus *et al.*, 2012; Surmacki *et al.*, 2018). It is also worth noting that the latter two bands could also correspond to proteins.

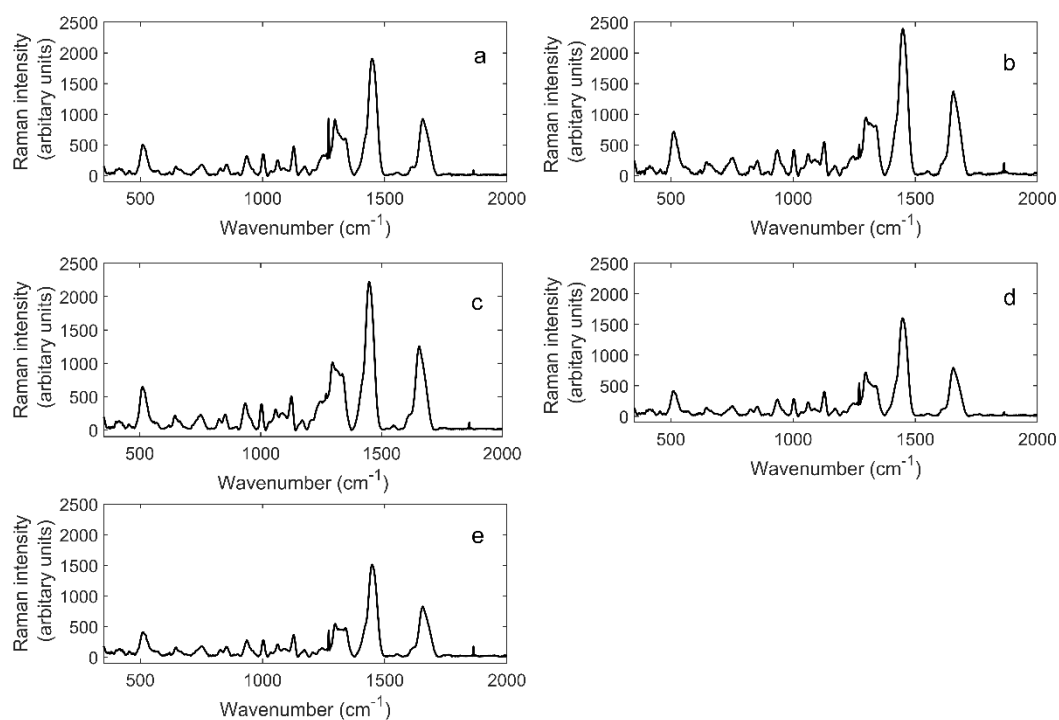


Figure 4.4. Average raw Raman spectra of fingernails taken from participants aged (a) 18 – 24, (b) 25 – 34, (c) 35 – 39, (d) 40 – 65 and (e) 66 – 85 years old, measured with the Agilent Resolve Raman spectrometer and equipped with an 830 nm laser wavelength.

Table 4.4. Measures of variability of key Raman bands and their Raman scattering found in fingernails of participants aged (Group one) 18 – 24, (Group two) 25 – 34, (Group three) 35 – 39, (Group four) 40 – 65 and (Group five) 66 – 85 years old.

Band (cm ⁻¹)	Median (IQR1-IQR3)				
	1	2	3	4	5
512	10.44 (5.05-12.94)	35.23 (6.40-73.57)	17.55 (7.57-84.57)	32.86 (12.29-79.24)	40.52 (11.16-68.97)
647	118.88 (69.24-190.42)	134.12 (106.07-178.300)	89.12 (62.19-138.67)	122.57 (63.13-188.48)	204.32 (98.39-312.45)
750	23.54 (10.66-39.35)	25.22 (6.61-62.48)	22.52 (12.71-36.76)	27.15 (11.58-46.59)	25.05 (13.61-42.19)
828	29.46 (3.83-74.16)	66.54 (29.22-109.32)	42.14 (11.54-55.32)	37.16 (7.99-75.7)	32.97 (5.41-70.39)
855	17.05 (6.73-35.10)	23.09 (8.61-56.47)	17.19 (4.55-42.81)	22.50 (9.12-41.83)	26.68 (7.84-44.28)
936	19.64 (8.69-34.81)	33.51 (19.99-48.94)	14.96 (14.95-47.80)	30.73 (14.95-47.80)	27.57 (11.75-47.32)
1005	176.73 (119.56-230.63)	169.14 (133.78-216.19)	135.34 (98.19-212.44)	166.17 (97.95-232.23)	159.94 (95.22-250.48)
1172	10.69 (4.46-25.09)	12.18 (2.89-22.61)	10.09 (5.17-17.39)	11.41 (4.43-26.46)	8.26 (2.48-21.34)
1449	812.37 (528.52-1145)	566.23 (395.10-863.9)	521.09 (383.34-725.19)	660.06 (474.17-895.11)	558.84 (339.67-797.68)
1550	185.46 (92.71-265.06)	197.31 (135.37-275.69)	184.14 (114.62-255.24)	176.50 (94.77-258.97)	178.24 (95.60-276.05)
1580	937.59 (776.1-1155)	898.83 (647.60-1230)	905.87 (647.99-1040)	826.54 (603.23-1110)	859.67 (519.78-1120)
1656	15.73 (6.79-27.10)	20.27 (12.96-21.86)	6.87 (3.28-15.57)	16.97 (7.67-29.46)	15.79 (7.51-26.66)

IQ1: Inter quartile range 1; IQ3: Inter quartile range 3.

4.3.3.2. Biological Sex

Raman spectra of the two biological sexes evaluated showed differences in the intensity of scattering of amino acids, proteins and lipids. Both sexes showed scattering at 12 bands, but females showed higher Raman intensity. The 12 bands were 512, 647, 750, 828, 855, 936, 1005, 1172, 1449, 1550, 1580 and 1656 cm^{-1} (Figures 4.5-4.6, Table 4.5).

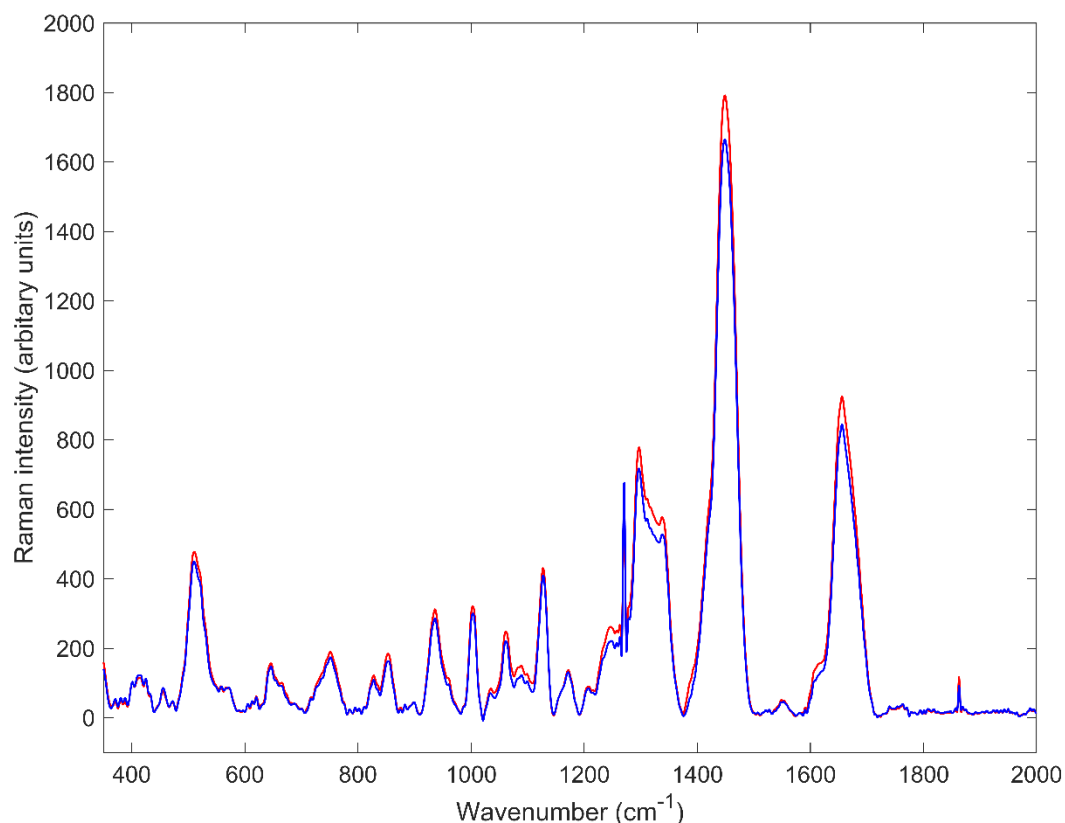


Figure 4.5. Raw Raman spectra of fingernails taken from female (red) and male (blue) participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

For cysteine, the intensity of bands 512 and 646 cm^{-1} showed higher scattering in females' fingernails' spectra than males ones (Kourkoumelis *et al.*, 2018). For example, spectra of females' fingernails showed a median band intensity of 443.40 arbitrary units, while males' fingernails' spectra showed a median band intensity of

394.18 arbitrary units at band 512 cm^{-1} . This suggested that when applying cysteine as an indicator for detecting CVDs and DM in fingernails, clinical practices must consider the biological sex of patients prior to disease detection.

Bands relating to lipids (1448 and 1580 cm^{-1}), proteins (855, 936 and 1656 cm^{-1}), phenylalanine (1005 cm^{-1}) tryptophan (855 and 1550 cm^{-1}) and tyrosine (750, 828 and 1172 cm^{-1}) also showed higher Raman scattering intensities in fingernail spectra taken from females than males. This indicated an accumulation of amino acids and proteins in females' fingernails. The metabolism of energy in humans is highly dependent on biological sex, which display distinct sex-specific functions. Energy metabolism in females is required for gestation and lactation and in males, energy is necessary for a default state e.g. metabolism of food, respiration and the regulation of water and body temperature (Varlamov, Bethea and Roberts, 2015).

Table 4.5. Measures of variability of key Raman bands and their Raman activity found in fingernails of female and male participants.

Band (cm^{-1})	Median (IQR1-IQR3)	
	Female	Male
512	443.40 (265.82-644.55)	394.18 (250.19-631.65)
647	142.36 (85.57-206.69)	138.92 (74.36-210.96)
750	181.99 (99.97-263.69)	158.42 (84.87-242.41)
828	113.13 (56.35-181.03)	91.48 (49.05-154.64)
855	177.46 (110.15-239.65)	150.58 (91.13-214.32)
936	302.85 (199.04-412.22)	260.36 (153.67-401.24)
1005	303.18 (215.10-407.67)	275.90 (188.63-394.18)
1172	132.35 (90.79-175.08)	123.87 (81.55-174.6)
1449	1777.33 (1271.93-2275.21)	1568.62 (1075.57-2254.05)
1550	48.21 (21.65-74.26)	39.68 (15.61-68.21)
1580	17.98 (8.47-32.72)	17.30 (7.44-32.67)
1656	869.85 (549.05-1265.56)	773.52 (415.87-1176.30)

IQ1: Inter quartile range 1; IQ3: Inter quartile range 3.

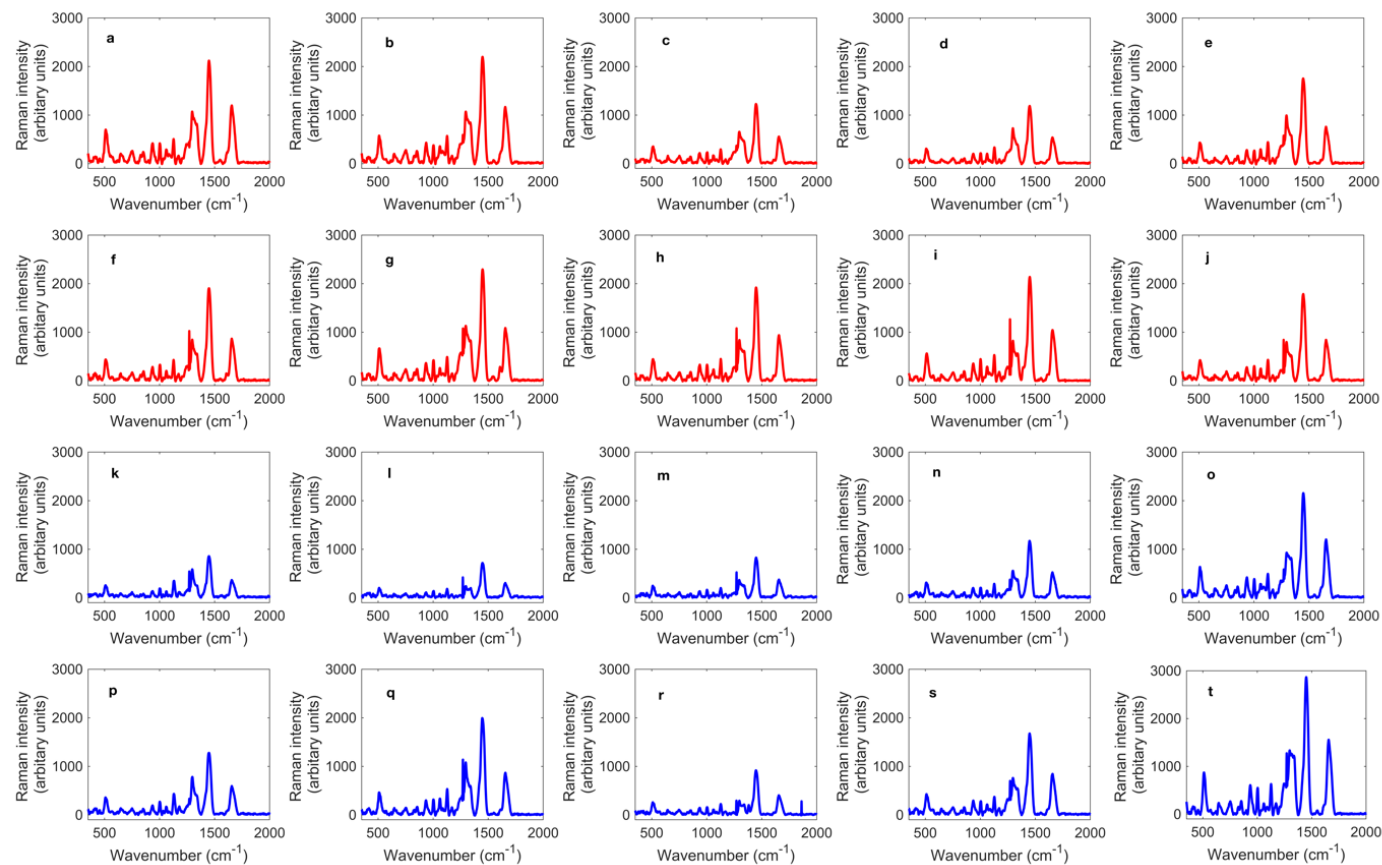


Figure 4.6. Subplot of raw Raman spectra of fingernails taken from female (a – j, red) and male (k – t, blue) participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

4.3.3.3. Ethnicity

Raman spectra of fingernails showed differences based on the evaluated ethnic groups that were: Arab, Asian, Lebanese Arab and White (Figure 4.7, Table 4.6). Variation between the highlighted ethnic groups was first seen at 512 cm^{-1} (corresponding to cystine and cysteine) wherein White participants showed the highest median band intensity of 548.81 arbitrary units and Asian and Lebanese Arab participants displayed the lowest band intensity of 413.28 and 417.28 arbitrary units, respectively. This could be attributed to diet, as cystine levels are related to the consumption of protein (Amin, 2013; Larsson, Håkansson and Wolk, 2015; Xiao *et al.*, 2013). For example, several Arab and Asian participants' diets were limited to halal meat and included no dairy products (due to lactose intolerance). As a result, their protein intake was reduced and this was evident at bands 936 and 1656 cm^{-1} , which demonstrated decreased Raman activity. At the same bands, White participants showed higher Raman activities, indicating high protein levels (Olszynska-Janus *et al.*, 2012; Widjaja, Lim and An, 2008). A similar trend was seen with bands 647 , 828 , 1005 and 1172 cm^{-1} , which corresponded to cysteine and cystine residues, tyrosine, phenylalanine and tyrosine respectively.

Likewise, Raman bands linked to tryptophan (750 and 1550 cm^{-1}) were of higher scattering intensity in White than Arab/Asian participants. Furthermore, White participants showed an increased Raman scattering of lipids at 1449 cm^{-1} (Olsztyńska-Janus *et al.*, 2012). Research suggested race-ethnic differences in lipid profiles further echoing the relationship between endogenous constituent deposition and ethnicity (Wilcox *et al.*, 2018; Willey *et al.*, 2011). Therefore, independent thresholds should be established to account for the variation of endogenous constituents between age, biological sex and ethnicity in order to demonstrate spectroscopic diagnostic accuracy in fingernails.

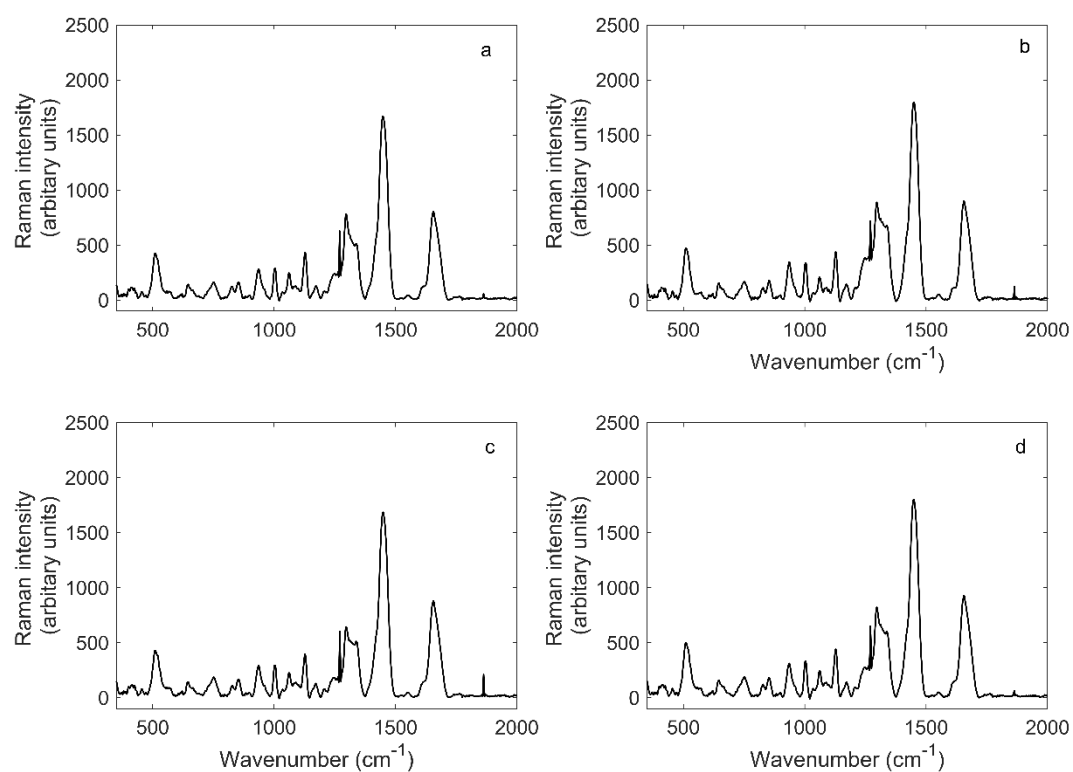


Figure 4.7. Average Raman spectra of fingernails taken from (a) Arab, (b) Asian, (c) Lebanese Arab and (d) White participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

Table 4.6. Measures of variability of key Raman bands and their Raman activity found in fingernails of Arab, Asian, Lebanese Arab and White participants.

Band (cm ⁻¹)	Median (IQR1-IQR3)			
	Arab	Asian	Lebanese Arab	White
512	417.79 (253.27-617.18)	413.28 (247.37-533.26)	417.28 (266.41-637.98)	548.81 (337.98-759.84)
647	136.36 (78.75-204.38)	138.59 (93.52-195.87)	133.87 (75.65-196.25)	173.73 (97.28-246.71)
750	169.24 (83.77-258.35)	163.59 (91.62-231.29)	183.08 (120.38-250.37)	203.44 (114.28-286.52)
828	105.17 (49.83-173.1)	99.02 (45.51-149.38)	116.12 (52.64-169.91)	143.17 (76.56-205.22)
855	178.49 (97.49-239.73)	172.86 (119.41-208.98)	165.5 (105.35-228.75)	201.18 (121.04-269.77)
936	274.1 (166.78-389.51)	303.47 (209.24-400.21)	299.13 (203.94-406.95)	365.71 (233.27-446.89)
1005	291.01 (193.87-389.95)	317.82 (235.30-363.75)	290.25 (187.64-400.43)	356.83 (270.10-449.80)
1172	128.03 (85.85-168.51)	131.33 (92.78-193.95)	124.47 (97.31-171.56)	148.96 (111.57-187.42)
1449	1754.99 (1060.43-2259.07)	1575.30 (1350.94-2108.55)	1728.96 (1337.15-2167.71)	1961.38 (1393.37-2396.54)
1550	49.04 (21.70-72.54)	38.06 (13.73-81.84)	48.69 (22.81-72.10)	53.22 (29.32-79.70)
1580	879.85 (593.78-1120)	957.91 (731.91-1255)	824.73 (592.29-1027.5)	885.74 (631.46-1130)
1656	773.1 (426.54-1191.1)	798.97 (558.8-1258.3)	868.75 (558.34-1258.3)	1055.6 (654.04-1368.18)

IQ1: Inter quartile range 1; IQ3: Inter quartile range 3.

4.3.4. Diagnostic Signature of Fingernails Using Handheld Raman Spectroscopy

Spectral interpretation revealed the presence of biomarkers relating to CVDs in fingernails. The Raman spectral band linked to cytochrome B was located at 1128 cm^{-1} and was attributed to skeletal C-C trans conformation (Horiue *et al.*, 2020). Moreover, cytochrome C was encountered at 1337 and 1590 cm^{-1} , which were related to CH_3CH_2 twisting and C=C stretching, respectively (Managò, Zito and De Luca, 2018; Brazhe *et al.*, 2013).

Biomarkers related to DM were also identified from fingernails taken from diabetic participants. The first indication of DM was found at 1128 cm^{-1} (C-C trans conformation) and was attributed to increased levels of glucose. Isoleucine and leucine were also identified from spectra of diabetic fingernails at 1250 (amide III), 1585 (olefinic C-C stretching) and 1242 cm^{-1} (C-C stretching), respectively. As highlighted in Chapter two of the thesis, section 2.3.4.2, the aforementioned amino acids were frequently reported in literature as biomarkers for DM (Lin *et al.*, 2020; Long *et al.*, 2020; Shi *et al.*, 2018; Wang *et al.*, 2013).

The combination of MLAs (CWS, PCA and SOMs) and Raman spectroscopy demonstrated success in detecting CVDs and/or DM from fingernails' spectra. CWS did not show success in classifying CVDs and/or DM from fingernails' spectra that showed r values > 0.98 against healthy fingernails' spectra (Figure 4.8). This indicated type I error and was the consequence of fingernail sets sharing the same basic biochemical profile regardless of disease. Yet, these mismatches were not encountered with all fingernails' sets. For instance, fingernail set MWS5 (healthy) showed an r value of 0.75 against MWS58 (CVD-diabetic) and that indicated successful comparison. Fingernail set MWS5 also produced an r value of 0.75 against set MWS59, who had previously been diagnosed with a CVD and DM.

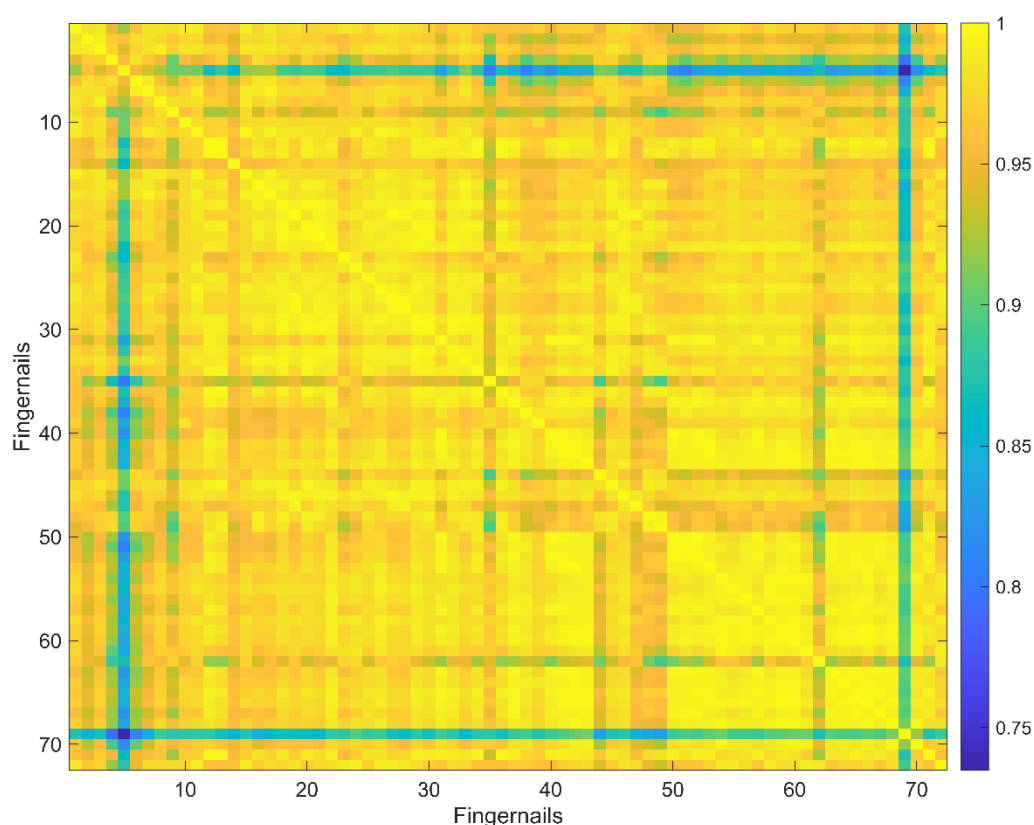


Figure 4.8. CWS of Raman spectra taken from healthy (1-43), unhealthy (44-53) CVD (54-65), diabetic (66-67) and CVD-diabetic participants (68-72). A dark blue colour represents a minimum r value of 0.75 and a dark yellow colour represents a maximum r value of 0.9999. In between r values of 0.77 – 0.82 correspond to a light blue colour, 0.83 – 0.87 correspond to a cyan colour, 0.88 – 0.92 correspond to a green, 0.93 – 0.97 correspond to a greenish yellow colour and 0.98 – 0.99 correspond to an orange colour.

PCA was then utilised to explore clustering patterns amongst healthy, unhealthy and diseased fingernails. The PC scores plot of 72 fingernails contributed to 85.61% of the variance amongst the data (Figure 4.9). However, the model was unsuccessful at differentiating between healthy, unhealthy CVD, diabetic, CVD-diabetic fingernails. As seen in Figure 4.9, an overlap was observed between the aforementioned groups and was attributed to all fingernails sharing the same set of amino acids, lipids and proteins. Type I and type II errors were also encountered. Type I errors were frequently

visualised in scores associated with healthy, CVD and diabetic fingernails. Moreover, one set of CVD-diabetic fingernails were clustered away from the remaining fingernail sets. This is likely to be attributed to the differences between T1DM and T2DM.

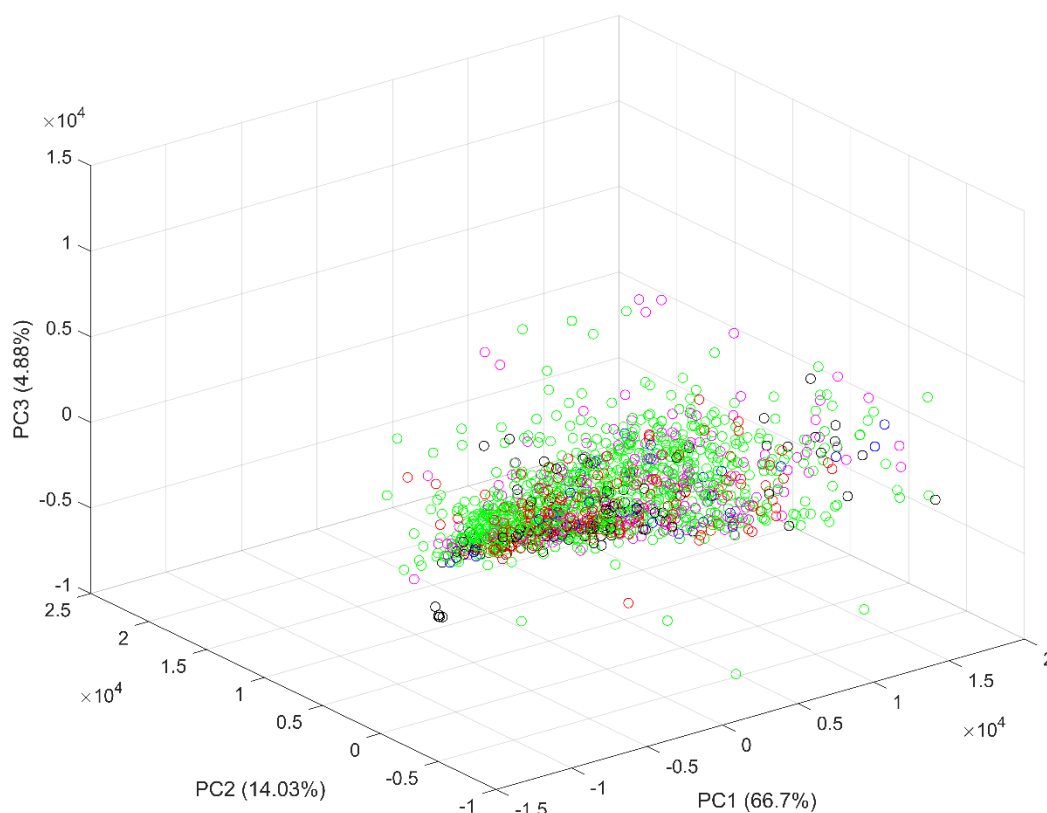


Figure 4.9. PCA plot of fingernails taken from healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

PC loading plots were explored to identify the significance of Raman bands and their related endogenous constituents/biomarkers. Spectral interpretation revealed key bands in PC1 (Figure 4.10a) were related to cystine, cystine/cysteine residues, tryptophan, tyrosine, proteins, phenylalanine and lipids and were encountered at 512 (S-S stretching), 645 (C-S stretching), 752 (symmetric breathing), 828 (aliphatic CCH bending), 854 (C-C stretching, CCH bending), 936 (skeletal C-C stretching), 1004 (in-plane C-H bending) and 1451 cm^{-1} (methylene CH_2 deformation), respectively

(Kourkoumelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012; Towler *et al.*, 2007; Widjaja, Lim and An, 2008). PC2 (Figure 4.10b) captured the variance of bands related to cystine (514 and 1656 cm^{-1}), lipids and proteins (1449 cm^{-1}) and were attributed to S-S stretching, C=O bending and methylene CH deformation, respectively (Kourkoumelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012). PC3 loading (Figure 4.10c) was comprised of bands related to phenylalanine at 1004 cm^{-1} (aromatic C-C stretch) and lipids and proteins at 1450 cm^{-1} (methylene CH₂ deformation) (Kourkoumelis *et al.*, 2018; Olsztyńska-Janus *et al.*, 2012).

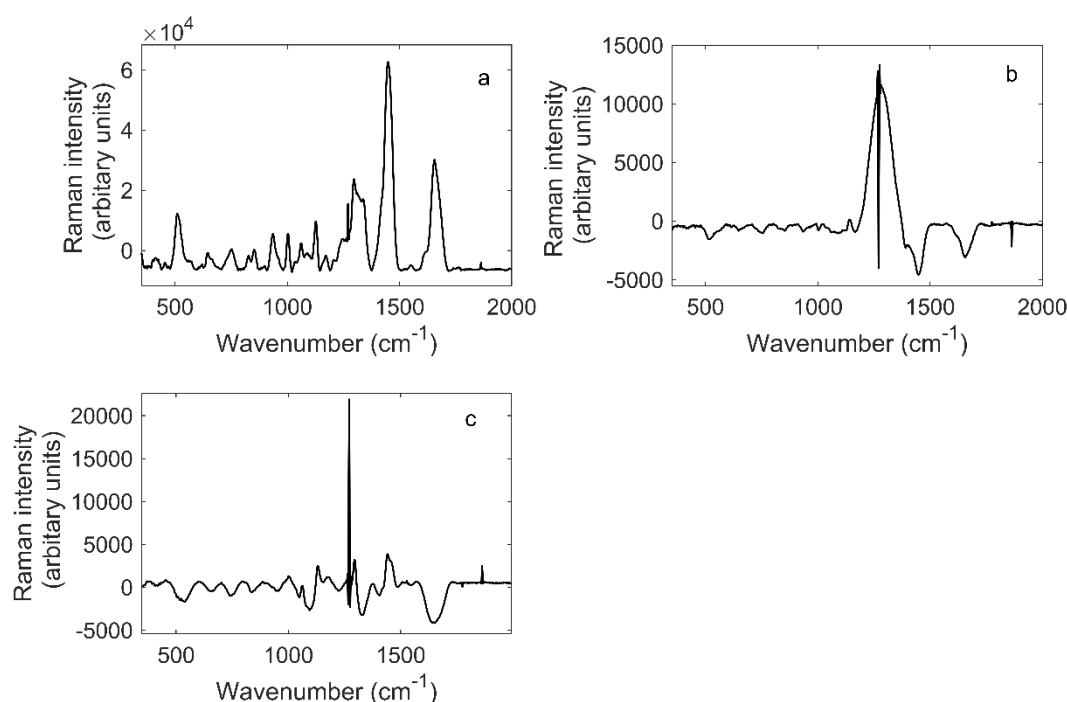


Figure 4.10. (a) PC1 (66.7%), (b) PC2 (14.03%) and (c) PC3 (4.88%) loading plots of fingernails taken from healthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength.

The presence of key endogenous constituents across the PC loadings suggested their importance in relation to the detection of disease. Therefore, the highlighted constituents and biomarkers should be utilised by clinical practices in future CVD and DM diagnosis.

SOMs confirmed the overlap seen in the PCA model. The U-Matrix demonstrated the weight distances and expressed the distances between the neighbouring neurons. Figure 4.11 demonstrates that neurons are connected to one another via red lines, which are representative of distances between neurons. Larger distances possessed lower densities and smaller distances had larger densities (Yang, Ouyang and Shi, 2012).

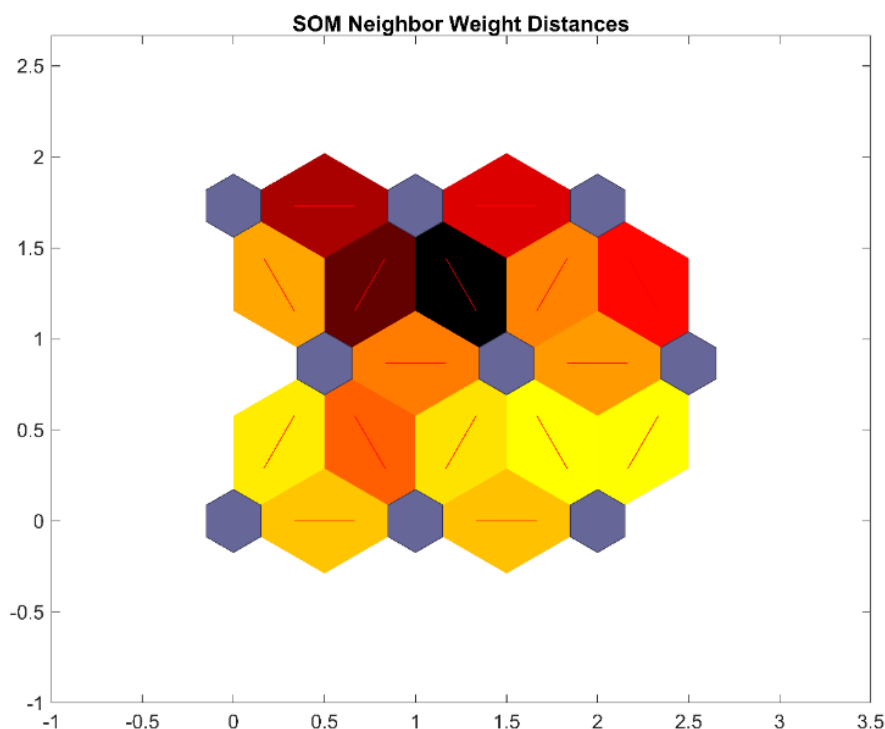


Figure 4.11. U-Matrix of SOM applied to Raman spectra of fingernails taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser wavelength. Black and dark red represented closely related variables and smaller distances. Dark orange and orange indicated moderately related variables and medium distances. Light orange and yellow highlighted loosely related variables and larger distances.

PCA findings were further complemented with the addition of sample hits (Figure 4.12). As highlighted in the PCA model, overlap was observed between the five groups of interest. The overlap between fingernail sets lends itself to the sharing of the same set of endogenous constituents across the fingernail sets regardless of disease.

Additionally, through considering the participants' characteristics, further explanation can be provided. Many of the unhealthy participants (medical condition unrelated to CVDs and/or DM) reported other medical conditions such as cancer, cholesterol and IBS. The presence of the aforementioned conditions influences the Raman activity of endogenous constituents found in fingernails.

Nevertheless, nine interpretable groups were identified. The first group ($n = 63$, 4.47%) being fingernails taken from diabetic participants. Groups two ($n = 12$, 0.852%) and three ($n = 59$, 4.19%) were also attributed to diabetic fingernails. Overlap was seen with groups four ($n = 164$, 11.6%) between CVD, diabetic and CVD-diabetic fingernails. Groups five ($n = 229$, 16.3%) and six ($n = 79$, 5.61%) were comprised of fingernails taken from healthy, unhealthy and CVD participants. Groups seven ($n = 271$, 19.2%), eight ($n = 237$, 16.8%) and nine ($n = 295$, 20.9%) were composed of healthy and unhealthy fingernail sets. While there was some overlap between the groups, the sample hit model suggested that Raman spectroscopy showed some capabilities with regards to the differentiation of healthy, CVD, diabetic and CVD-diabetic fingernails.

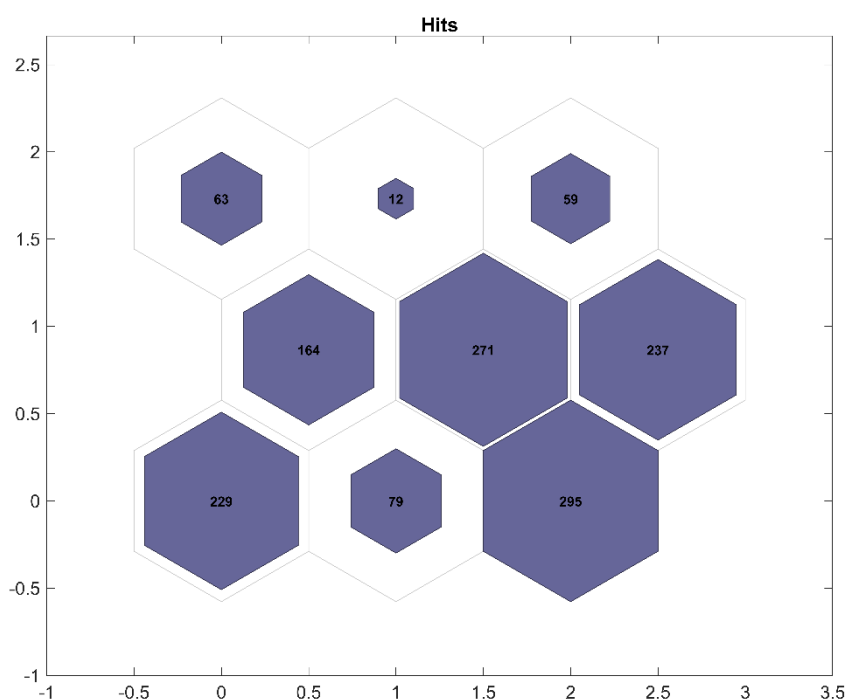


Figure 4.12. Sample hit map of the SOM applied to Raman spectra of healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails measured using the Agilent Resolve Raman spectrometer equipped with an 830 nm laser.

4.4. Conclusion

Raman spectroscopy was successful in the detection of endogenous constituents deposited in fingernails such as cysteine, cystine, tryptophan, tyrosine, lipids, phenylalanine and proteins. Furthermore, CVD- and DM-related biomarkers were detected in Raman spectra of fingernails. These biomarkers were cytochrome B and C for the detection of CVDs and glucose, isoleucine and leucine for the detection of DM.

Fingernails' Raman activity was influenced by demographic characteristics that were age, biological sex and ethnicity. Demographic characteristics played a significant role in the Raman scattering of endogenous constituents found in fingernails. As a result, clinical practices should consider these characteristics during the detection process and establish biological thresholds for disease detection based on patients' individual characteristics.

The combination of Raman spectroscopy and MLAs showed Raman's capability to detect differences between healthy and diseased fingernails. CWS showed more mismatches than PCA and SOMs. This was due to the common bands among healthy individuals and those with CVDs and/or DM. Future research (chapters five and six) will look at exploring FTIR and NIR spectroscopy's potential in detecting CVDs and/or DM in fingernails.

4.5. Key Messages

- Raman spectral interpretation revealed key endogenous constituents found in fingernails including cysteine, cystine, lipids, phenylalanine, proteins, tryptophan and tyrosine.
- Demographic characteristics such as age, biological sex and ethnicity played a role in the Raman scattering of endogenous constituents within fingernails and therefore, should be considered during future disease detection cases.

- Raman spectra of fingernails detected disease related biomarkers including cytochrome B and C for the detection of CVDs and isoleucine, glucose and leucine for the detection of DM.

Chapter five: Classification of healthy, cardiovascular and diabetic fingernails using near-infrared spectroscopy and machine learning algorithms

The aim of this chapter was to explore the classification of CVD and/or DM from fingernails using NIR spectroscopy and MLAs. A total of 85 fingernails sets were measured using the Perkin Elmer Spectrum Two N Fourier transform (FT)-NIR Spectrometer equipped with a NIR reflectance module (NIRM). NIR spectroscopy successfully characterised disease related spectral features including the absorbance of diabetic fingernails, which was the lowest of the five groups. Further spectral features were identified within the regions 4,400 – 4,240, 5,100 – 4,600 and 7,100 – 6,000 cm^{-1} . Through the application of PCA, the presence of glycated proteins and variation between the spectral features of healthy versus diabetic fingernails was confirmed. Further MLAs including CWS and SOMs were applied to NIR spectra for the identification of patterns among the data. However, due to the presence of the same endogenous constituents across all fingernails regardless of the presence/absence of disease, CWS and SOMs were unsuccessful in separating the defined groups. Considering the limitations in these models, ML classification models were trained and hyperparameters applied through MATLAB R2019a's Classification toolbox. Cubic and quadratic SVM models were used with multiclass and binary classification of CVDs and DM. The training accuracy for CVD and DM was 89.4% and 87.9% respectively with multiclass classification. However, this accuracy improved to 100% when binary classification was used.

5.1. Introduction

NIR spectroscopy offers the advantage of rapid non-destructive analysis, which requires little-to-no sample preparation. Its ability to detect physicochemical properties has shown great success in a range of disciplines including clinical, forensic and

pharmaceuticals, wherein NIR spectroscopy has been utilised for disease management, vaccine authentication and quality assurance/control, respectively (Assi *et al.*, 2022; Monteyne *et al.*, 2018; Sodata and Peerapattana, 2020; Vitorino *et al.*, 2023). Moreover, the marked versatility of NIR spectroscopy allows the further advantage of working with a wide range of sample forms including crystals, gases, fibres, powders, liquids and solids (Ozaki, 2012).

The weak energy of NIR spectroscopy, allows it to be applied to alternative biological matrices including fingernails (Beć, Grabska and Huck, 2020). Previous research successfully showed the ability of NIR spectroscopy to detect endogenous constituents such as amino acids, lipids and proteins (Wilson *et al.*, 2023a). By monitoring the regulation of endogenous constituents, insight into the subject's health status can be determined (Ho *et al.*, 2018; Mengstie *et al.*, 2022). For example, work by Vitorino *et al.*, (2023) demonstrated that NIR spectroscopy could sensitively detect differences in lipid concentration in diabetic and non-diabetic blood samples. Thus, NIR spectroscopy can offer rapid, non-invasive analysis of fingernails in a cost-effective manner. Therefore, the aim of this chapter was to explore the classification of CVD and diabetic fingernails using NIR spectroscopy and MLAs. To fulfil this aim, the objectives have been set as followed:

- To explore the NIR activity of fingernails taken from healthy, CVD and diabetic participants, their endogenous constituents and the presence of diagnostic bands.
- To determine the ability of NIR spectroscopy for the detection of CVD and/or DM from fingernails.
- To apply MLAs to explore patterns in NIR spectra of healthy, CVD and diabetic fingernails.

5.2. Methods and Materials

5.2.1. Materials

A total of 85 fingernail sets were collected (769 clippings). Measured fingernail sets were provided by female (n = 50, 58.8%) and male (n = 35, 41.2%) participants aged between 18 – 85 years old. Participants were one of five ethnicities including Arab (n

= 33, 38.8%), Asian (n = 10, 11.8%), Indian (n = 2, 2.35%), Lebanese Arab (n = 15, 17.6%) and White (n = 25, 29.4%). Of these participants, 11 were diagnosed with a CVD, three with DM and seven with both a CVD and DM (Appendix B1, Tables 1-5).

5.2.2. Instrumentation

For collection of NIR spectra the Perkin Elmer Spectrum Two N FT-NIR Spectrometer (Perkin Elmer, Waltham, MA) equipped with a temperature stabilised InGaAs detector and NIR reflectance module (NIRM) was employed (Figure 5.1). The NIR spectral range was between 12,500 – 4,000 cm^{-1} and had a spectral resolution of 8 cm^{-1} .

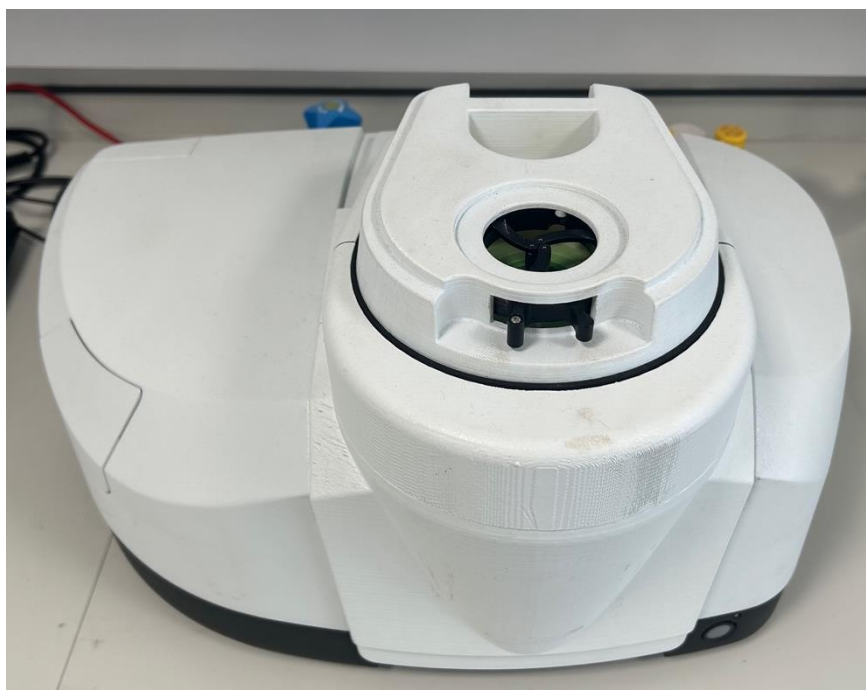


Figure 5.1. The Perkin Elmer Spectrum Two N FT-NIR Spectrometer equipped with NIRM.

5.2.3. Method

The number of NIR spectra collected per set was decided considering the equal frequency ellipses (chapter four, section 4.2.3). Therefore, 22 spectra were taken per fingernails set. The glass vial was placed directly over the NIR source and secured using the vial sampling accessory. Between the collection of spectra, the glass vial

was vortexed using the SciQuip Vortex VarlMix to ensure that different areas of the fingernail clippings were targeted and the distribution of endogenous constituents across the fingernails accounted for. Spectra were exported for offline spectral interpretation and spectral quality assessments.

5.2.4. Spectral Pre-Treatment

Exported NIR spectra were imported into MATLAB R2019a and Microsoft Excel 2019 for the application of pre-processing. Several combinations of pre-processing algorithms were made, those being MSC, MSC-D1 and MSC-D2. MSC was first applied to the NIR spectra for the correction of offset and baseline, while also minimising light-scattering effects (Thennadil, Martens and Kohler, 2006). Due to the frequent occurrence of broad overlapping NIR bands, which make spectral interpretation overly complex, derivatisation was imperative (Dehghani *et al.*, 2011).

5.2.5. Data Analysis

For data analysis, MLAs explored were CWS, PCA and SOMs (chapter one, section 1.7.5.). Additional ML was applied using the MATLAB R2019a Classification Learner Toolbox. A total of 32 models were trained to first identify the most suitable classification models for spectral data. As a preliminary study, a total of 1403 spectra were applied to all models after the removal of outlier spectra. Models were trained on the full range (3,000 features) and a 10 cross-validation method was applied to validate the models. Additionally, 20% of the imported data was utilised as a testing set, to blindly assess the models' predictive abilities. The full list of models available through the MATLAB R2019a Classification Learner Toolbox included: fine tree, medium tree, coarse tree, linear discriminant, quadratic discriminant, efficient LR, efficient linear SVM, gaussian naïve bayes, kernel naïve bayes, linear SVM, quadratic SVM, cubic SVM, fine gaussian SVM, medium gaussian SVM, fine K-nearest neighbour (KNN), medium KNN, coarse KNN, cosine KNN, cubic KNN, weighted KNN, boosted trees, bagged trees, subspace discriminant, subspace KNN, RUSBoosted trees, narrow neural network, medium neural network, wide neural network, bilayered neural network, trilayered neural network, SVM kernel and LR kernel. The top five models with the highest accuracy were selected for further training. The accepted

models included: bagged tree ensemble, boosted tree ensemble, cubic SVM, SVM kernel and quadratic SVM.

Models were then fine-tuned through the alteration of hyperparameters, as followed. For the bagged tree model, a maximum of 11,956 splits were allowed with the number of learner nodes being set to 30 (Harper, 2023). The ensemble method for this model was bag, with the learner type as decision tree. For the boosted tree model, the ensemble method AdaBoost was selected, and the learner type was a decision tree. The maximum number of splits was 20, the number of learners was 30 and the learning rate was 0.1 (Harper, 2023). For the cubic SVM model, hyperparameters included a cubic kernel function, a box level of one and a multiclass method of one-versus-one. The quadratic SVM employed similar hyperparameters, but varied in terms of kernel function, which was quadratic.

All models were tested using a training:testing split of 80:20, with 80% of the data being utilised for training and 20% for testing. A K-left out method was also applied to test the models for subjectivity. For this project, a 10-fold cross validation method was employed, whereby the data was first randomly divided into ten parts and tested using 10% of the overall data (Colakoglu, Alis and Yergin, 2019). Through withholding the random 10% of data, the researcher was able to determine and evaluate the models' performance when confronting unseen data and therefore, suggesting how the selected classification models may interact with future data.

Models were evaluated through the following parameters: accuracy, area under the curve (AUC), F1-score, false negative rate (FNR) false positive rate (FPR), misclassification rate, recall, specificity, precision and prevalence (Table 5.1).

Table 5.1. Evaluation metrics for classification models.

Parameter	Equation	Reference
Accuracy	$\frac{TN + TP}{TN + FP + FN + TP}$	Kulkarni, Chong and Batarseh, 2020.
FPR	$\frac{FP}{FP + TN}$	Niu et al., 2020; Solana and Santiago, 2022.
FNR	$\frac{FN}{FN + TP}$	Niu et al., 2020.
Specificity	$\frac{TN}{TN + FP}$	Niu et al., 2020.
Misclassification rate	$\frac{FP + FN}{TP + TN + FP + FN}$	Karimi, 2021.
Prevalence	$\frac{\text{Actual yes}}{\text{Total}}$	Sathyanarayanan and Tantri, 2024.
Precision	$\frac{TP}{TP + FP}$	Bruce, Bruce and Gedeck, 2020; Kulkarni, Chong and Batarseh, 2020.
Recall	$\frac{TP}{TP + FN}$	Kulkarni, Chong and Batarseh, 2020.
F1 score	$2 * \frac{\text{precision} * \text{recall}}{\text{precision} + \text{recall}}$	Kulkarni, Chong and Batarseh, 2020.

FP: False positive; FN: False negative; TP: True positive; TN: True negative.

To determine the AUC, receiver operating characteristic (ROC) curves were visualised. The ROC was plotted through calculating the FPR (x-axis) against the true positive rate (TPR) (y-axis). For a model to be identified as a good classifier, it must have reached the top left of the ROC's curve (Jiménez-Valverde, 2021). The diagonal line present within ROC curves was representative of random guessing (Kulkarni, Chong and Batarseh, 2020). In cases, whereby the ROC curve was below the diagonal line, the classifier was deemed as performing poorer than if random guessing was utilised (Hajian-Tilaki, 2013). Hence, good classifiers possessed an ROC curve that was upper the diagonal line. The AUC was then calculated to provide a numerical score for the evaluation of classification. ROC curves that were present in the upper diagonal showed an AUC value of > 0.5 , whereas ROC curves within the lower diagonal demonstrated an AUC of < 0.5 .

5.3. Results and Discussion

NIR spectroscopy demonstrated the ability to detect disease related biomarkers, as well as spectral regions that can be targeted not only for initial disease detection but for disease monitoring. Key NIR regions were associated with the presence of

advanced glycation end-products (AGEs) and glycated proteins, which indicated the presence of DM (Monteyne *et al.*, 2018). While PCA was unable to successfully differentiate between the five groups of interest, classification models including bagged tree ensemble, boosted tree ensemble, cubic SVM, SVM kernel and quadratic SVM showed a moderate ability to differentiate between healthy and diseased fingernails. However, classification of disease presence was greatly improved when applied to binary classification models for the classification of healthy versus CVD fingernails and healthy versus diabetic fingernails.

5.3.1. NIR Signature of Fingernails

Prior to establishing NIR spectroscopy as a diagnostic tool, it was first imperative to assess its ability to identify key bands within the spectra of healthy, CVD and diabetic fingernails. As previously highlighted in chapter four of the thesis, section 4.3.4, the fingernails shared the same set of endogenous constituents including cysteine, cystine, phenylalanine, tryptophan, tyrosine, lipids (cholesterol) and proteins (keratin). Therefore, the collected NIR spectra were reflective of one another and several NIR bands occurred across all fingernail sets ($n = 85$ sets) (Table 5.2). It is worth noting that spectral interpretation was carried out on raw NIR spectra, prior to the application of spectral pre-processing. This was to ensure that the bands identified were representative of the endogenous constituents found in fingernails, as spectral pre-processing can create computational noise and D1 can shift the positioning of bands.

Across the 85 sets of fingernails, the number of bands ranged from 7 – 17 bands. Spectra taken from healthy participants ($n = 50$, 58.8%) showed 8 – 16 bands (median = 11, IQR = 9 – 11), unhealthy participants ($n = 14$, 16.5%) had 8 – 17 bands (median = 10, IQR = 8.75 – 13), CVD ($n = 11$, 12.9%) participants displayed 9 – 16 (median = 11.5, IQR = 9.25 – 15.25), diabetic participants ($n = 3$, 3.53%) demonstrated 7 – 12 (median = 11, IQR = 10 – 12) and CVD-diabetic participant ($n = 7$, 8.24%) possessed 9 – 14 bands (median = 12, IQR = 11 – 13). Across the five groups, diabetic participants demonstrated the lowest number of bands. The reduced number of bands within this group lends itself to the gradual alteration of intrinsic material properties and structural tissue integrity damage caused by DM (Sihota *et al.*, 2019). Prolonged hyperglycaemia causes chronic damage to fingernails. Furthermore, DM has been

associated with poor circulation (Yachmaneni *et al.*, 2023). High levels of glucose can cause damage to the blood vessels' linings and often impedes blood flow (Poznyak *et al.*, 2022). As a consequence, the blood supply of endogenous constituents to the fingernail bed and germinal matrix is reduced below the healthy level.

Table 5.2. Spectral band assignments of bands present within healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer and equipped with NIRM (Jee, 2021).

Wavenumber (cm ⁻¹)	Band assignment	Region	Overtone
4186	CH ₂ , CHCl ₃	III	First
4248	CH ₂ , CHCl ₃	III	First
4330	CH ₂ , CH ₃ , CHCl ₃	III	First
4864	RNHR', RCONHR', ROH	III	First
5148	RNH ₂ , RNHR' RCONHR'	III	First
5784	SH	II	Second
5890	CH ₃ , SH	II	Second
6106	RCONHR'	II	Second
6362	RCONHR'	II	Second
6632	RNH ₂ , RCONH ₂ , ROH	II	Second
7068	CH ₂	II	Second
8410	CH, CH ₃	II	Second

Spectral visualisation was made to assess the spectral similarities and differences between the chosen five groups, those being: healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) (Figure 5.2.). While NIR spectra shared similarities, in terms of the overall shape and trend of the NIR spectra, the absorbance was dissimilar. As observed in Figure 5.2 the absorbance between 10,000 – 5,000 cm⁻¹ was CVD-diabetic > healthy > unhealthy > CVD > DM and later changed between 5,000 – 4,000 cm⁻¹ to CVD-diabetic > healthy > CVD > unhealthy > DM. Variation observed between the different absorbance levels can be attributed to the offset and background of NIR spectra.

Across the full range, spectra taken from diabetic participants displayed the lowest absorbance due to decreased blood supply to fingernails (Poznyak *et al.*, 2020; Yachmaneni *et al.*, 2023). The highest absorbance was observed in spectra taken from MWS57, who had been previously diagnosed with CAD and T1DM. NIR spectra of

MWS57 showed two water bands at 5,148 and 7,068 cm^{-1} that had absorbance values in the range of 1 – 1.3 absorbance units (Jee, 2019). Healthy individuals on the other hand had absorbance < 0.85 absorbance units at 7068 cm^{-1} .

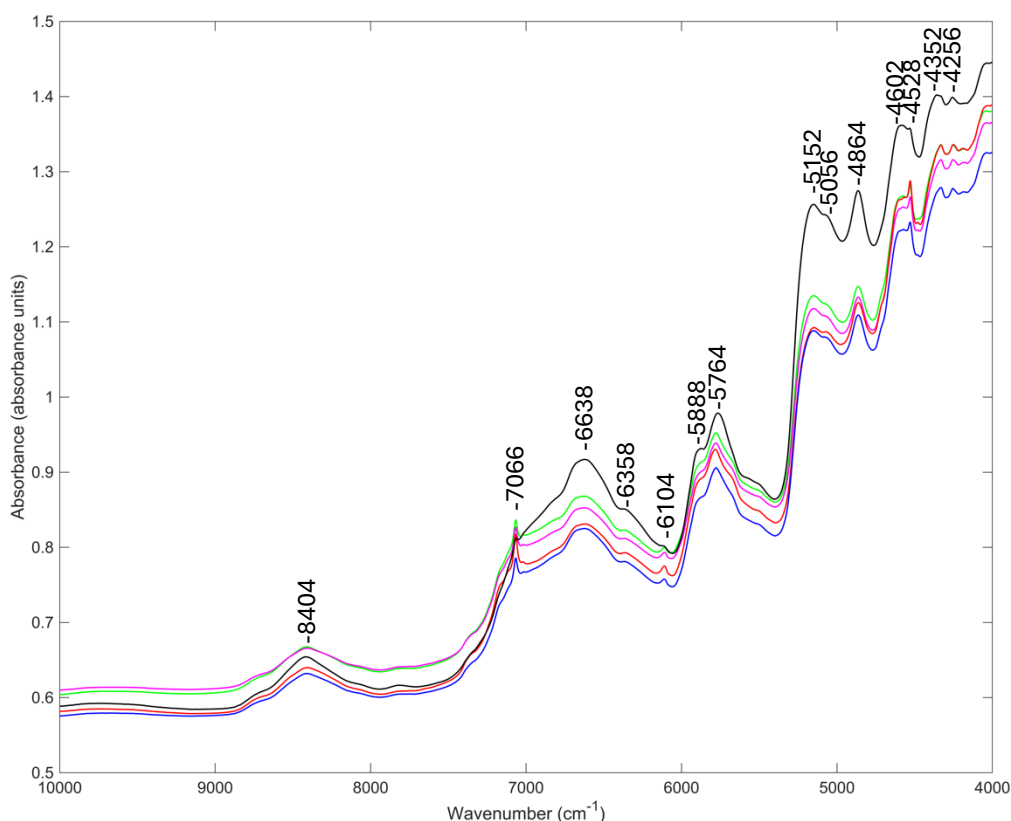


Figure 5.2. Average NIR spectra of fingernails taken from a healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) participant, measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer and equipped with NIRM.

5.3.1.1. Glycation of Proteins in Diabetic Fingernails

NIR spectra of fingernails showed key bands corresponding to glycated proteins (Figure 5.3a). These bands were in the range of 4,400 – 4,300 cm^{-1} and were linked to C-H stretching, C-H bending and O-H bending of glucose (Monteyne *et al.*, 2018). Additional research also suggested that this area was linked to the α -anomer of glucose (Awichi *et al.*, 2002; Cocciardi *et al.*, 2006). Closer examination of this area indicated clear differences between healthy versus diabetic fingernails. As highlighted within Figure 5.3b, the spectrum taken from a diabetic participant showed a broader

band between $4,400 - 4,300 \text{ cm}^{-1}$ in comparison to the spectrum taken from a healthy non-diabetic participant. The broadness of this band revealed an increased level of glycation in diabetic versus healthy participants and therefore suggested the presence of disease (Monteyne *et al.*, 2018). Furthermore, this region offered disease monitoring opportunities, whereby the fingernail sample could be sampled once a month for six to nine months to monitor the fluctuation of glycation during growth.

Further spectral differences were observed between diabetic and healthy participants between the region $7,100 - 6,000 \text{ cm}^{-1}$ (Figure 5.3c). This area was associated with combination OH antisymmetric and symmetric stretching and was the result of glycation (Monteyne *et al.*, 2018). As shown in Figure 5.3c, the prominent band at $6,636 \text{ cm}^{-1}$ increased in both intensity and broadness for the diabetic set of fingernails (blue) and decreased in the healthy set of fingernails (green). The lower intensity of this band detected in healthy fingernails, owes itself to the successful metabolism of glucose, whereas diabetic patients have little to no insulin for glucose metabolism. As a result, AGEs accumulate in the blood, fingernails and other tissues that are high in lysine-rich residues. Similarly, between $5,100 - 4,600 \text{ cm}^{-1}$, the spectrum taken from diabetic fingernails displayed a higher intensity than the healthy fingernails (Figure 5.3d). This region is attributed to CONH_2 stretching bands and a combination of NH stretching and bending (Monteyne *et al.*, 2018). Within this region, the band located at $4,864 \text{ cm}^{-1}$ provided a key indication of AGEs and glycation. The sharper band observed in spectrum collected from a diabetic participant highlights an increased level of glycation activity. In contrast, the less intense band present in the spectrum collected from a healthy individual was reflective of the lower level of glycation. Hence, this band allowed the differentiation of healthy and diabetic fingernails and could be used by clinical practices for the detection of DM, as well as monitoring its prognosis.

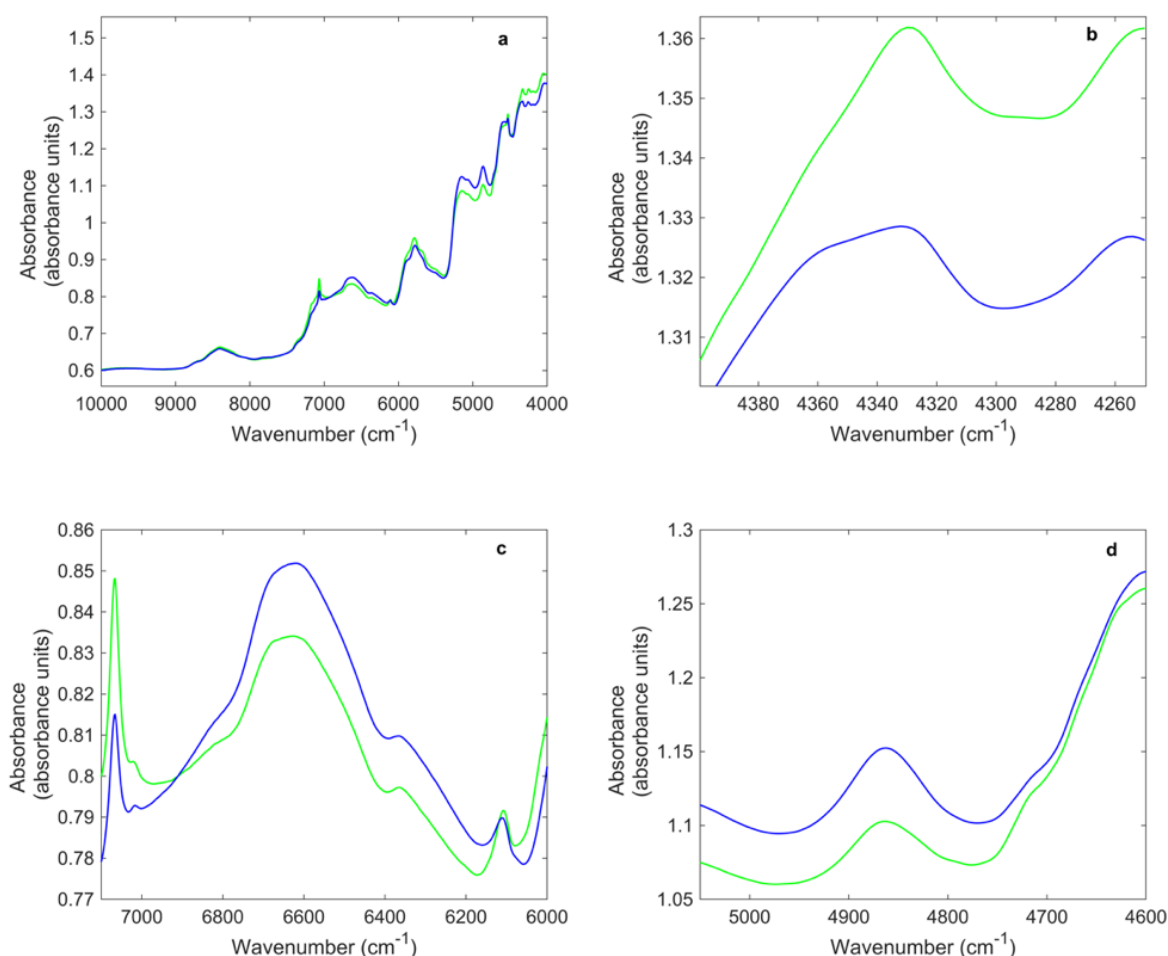


Figure 5.3. (a) NIR spectra of fingernails taken from healthy (green) and diabetic (blue) participants and measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM. Partial NIR spectra of regions (b) 4,400 – 4,250, (c) 7,100 – 6,000 and (d) 5,100 – 4,600 cm^{-1} measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

To validate spectral differences observed in Figure 5.3, between healthy and diabetic fingernails, raw NIR spectra were treated using MSC-D1 and PCA models were applied to the regions 7,100 – 6,000, 5,100 – 4,600 and 4,400 – 4,250 cm^{-1} . Figure 5.4a, focussing on the region 4,400 – 4,240 cm^{-1} , captured 96.04% of the variance within the first two PCs. While some separation was seen between scores of healthy and diabetic fingernails, several overlapping scores were identified. Separation between the aforementioned groups was improved across the region 5,100 – 4,600

cm^{-1} , where a variance of 94.7% was present within the first two PCs (Figure 5.4c). The final selected region, $7,100 - 6,000 \text{ cm}^{-1}$, demonstrated the clearest separation between scores of healthy and diabetic fingernails (Figure 5.4b). Within Figure 5.4b, two adjacent clusters were observed and 98.8% of the variance was captured within the first two PCs. The clear separation between the healthy and diabetic fingernails, indicated that NIR spectroscopy could effectively detect spectral differences between fingernails of different health and disease status. Figure 5.4 also validated that NIR spectroscopy paired with PCA analysis was successful in the clustering of healthy and diabetic fingernails. While clustering was observed to some degree across the selected regions, regions $7,100 - 6,000$ and $5,100 - 4,600 \text{ cm}^{-1}$ (Figures 5.4b and 5.4c) showed the highest level of clustering and therefore should be utilised in future research and disease detection.

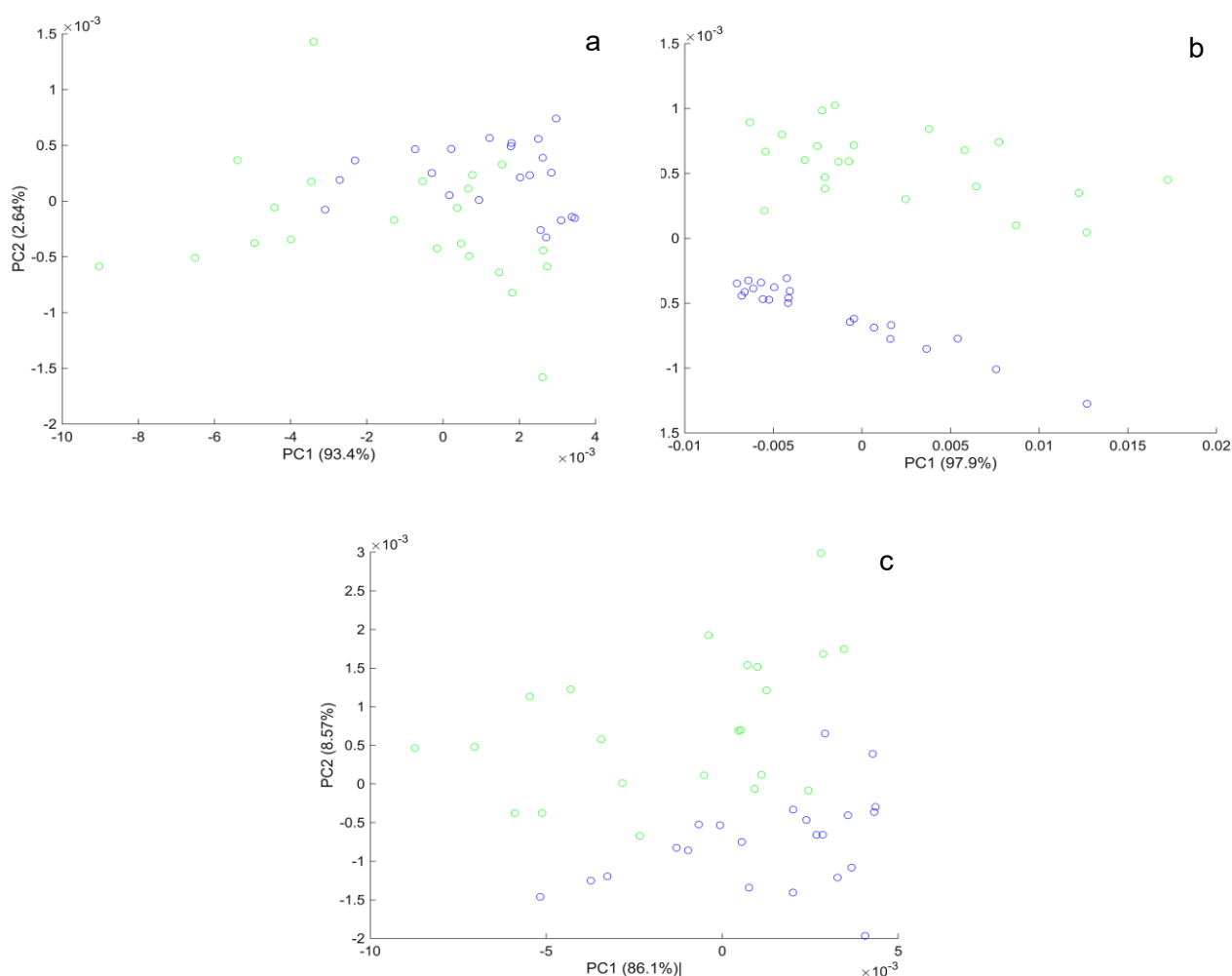


Figure 5.4. PCA scores plot of MSC-D1 NIR spectra of healthy (green) and diabetic (blue) fingernails within the regions (a) 4,400 – 4,240, (b) 7,100 – 6,000 and (c) 5,100 – 4,600 cm^{-1} (c) measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

5.3.2. Assessment of Spectral Pre-treatments

Spectral pre-processing algorithms were applied to NIR spectra to improve the interpretation and visualisation of spectra, while not impeding the overall quality through the introduction of noise. As broad overlapping bands were characteristic to NIR spectra, the application of spectral pre-processing also offered band separation.

Spectral quality assessments were applied to the average of each fingernail set before and after the application of pre-processing algorithms (Figure 5.5). These metrics were

used to evaluate the impact of different pre-processing strategies on spectral interpretability and data quality prior to model development.

Raw spectra displayed strong ($n = 31$, 36.5%) and medium ($n = 54$, 63.5%) NIR activity (Figure 5.5a). The number of bands ranged from 9 – 13 for spectra of fingernail sets with strong NIR intensity and 7 – 16 for spectra of fingernails sets with medium NIR activity (median = 11, IQR = 9 – 12). Raw NIR spectra shared a maximum band position of $4,860 - 7,068 \text{ cm}^{-1}$ (median = $6,618 \text{ cm}^{-1}$, IQR = $5,778 - 6,618 \text{ cm}^{-1}$) and maximum band intensity of 0.76 – 1.3 absorbance units (median = 0.92 absorbance units, IQR = 0.89 – 0.97 absorbance units). Within this region several combination bands and overtones were observed, including CH_2 , SH, RNH_2 , RNHR' , RCONHR' and ROH (Jee, 2019). The S/N ratio of raw NIR spectra ranged from 60 – 240 (median = 180, IQR = 140 – 200). The variance seen within the S/N ratios of sets MWS1 – MWS85, indicated that pre-processing was imperative for the analysis of NIR spectra, as well as the application of MLAs and classification models. This was supported by previous work, which suggested that the sample's physical properties, the instrument's response and the environment itself can act as confounding variables and increase the variation seen within the spectral data (Robert and Gosselin, 2022). Hence, it was necessary to conduct spectral pre-processing.

NIR spectra were first treated using MSC, which compensated for additive and/or multiplicative effects within spectral data (Maleki *et al.*, 2007). This pre-processing step also removed physical effects such as particle size and surface blaze from the spectra, confounding variables that were indicated during the analysis of raw NIR spectra (Silalahi *et al.*, 2018). MSC treated NIR spectra showed strong ($n = 79$, 92.9%) and medium ($n = 6$, 7.059%) NIR intensity (Figure 5.5b). After band separation, 9 – 20 bands (median = 13, IQR = 11 – 15) were identifiable. MSC pre-treated spectra produced maximum band positions between $4,860 - 7,068 \text{ cm}^{-1}$ (median = $6,634 \text{ cm}^{-1}$, IQR = $6,620 - 7,066 \text{ cm}^{-1}$) and maximum band intensities of 0.83 – 1.9 absorbance units (median = 0.89 absorbance units, IQR = 0.87 – 0.92 absorbance units). This region was associated with combination and overtone bands such as RNH_2 , RCONH_2 , RCONHR' and ROH (Jee, 2019). S/N ratio for MSC treated NIR spectra ranged from 60 – 220 (median = 120, IQR = 100 – 160).

A combination of MSC-D1 (Figure 5.5c) or MSC-D2 (Figure 5.5d) data pre-processing algorithms were then applied to the NIR spectra. The combination of MSC and derivatives have previously shown great success for many applications such as authentication of medicines, drug and disease detection and food quality-control (Assi *et al.*, 2021; Rambo *et al.*, 2019; Vitorino *et al.*, 2023). MSC-D1 spectra showed 45 – 97 bands (median = 73, IQR = 69 – 79) and MSC-D2 spectra demonstrated 16 – 66 bands (median = 37, IQR = 31 – 43). The increased number of bands seen within the derivatised spectra can be attributed to the derivatisation pre-processing algorithms narrowing broad, overlapping NIR bands and removing baseline effects. Therefore, MSC-D1 (n = 85, 100%) and MSC-D2 (n = 85, 100%) spectra received a classification of strong NIR activity. Bands observed in the MSC-D1 spectra possessed a maximum band position of 4,520 – 7,060 cm^{-1} (median = 5,282 cm^{-1} , IQR = 4,520 – 5,186 cm^{-1}) and captured combination bands and overtones related to CH_2 , CH_3 , RNH_2 , RNHR , RCONHR' , ROH and SH (Jee, 2019). The maximum band intensity of MSC-D1 treated spectra ranged from -0.0032 – 0.0076 absorbance units. The introduction of D1 also greatly improved the S/N ratio of NIR spectra from 60 – 240 in raw NIR spectra, to 73 – 470 (median = 210, IQR = 185 – 225) in the MSC-D1 treated spectra. A similar trend could be seen for MSC-D2 treated spectra, which displayed maximum band positions across the range 4,094 – 7,070 cm^{-1} (median = 4,968, IQR = 4,530 – 7,068 cm^{-1}) and were attributed to combination bands and overtones CH_2 , CH_3 , CHCL_3 , RNH_2 , RNHR' , RCONHR' , ROH and SH (Jee, 2019). The aforementioned bands manifested at intensities between -0.0012 – 0.000089 absorbance units (median = -0.00022 absorbance units, IQR, = -0.00046 – -0.0010 absorbance units).

Among the tested approaches, the combination of MSC followed by D1 produced the most favourable balance between spectral enhancement and noise suppression. The application MSC-D2, although capable of further resolving overlapping spectral bands, resulted in a noticeable increase of noise and a decrease in spectral interpretability. Consequently, MSC-D2 was not considered suitable for subsequent modelling. SNV pre-processing was not explored in detail for the NIR datasets as preliminary assessments indicated that multiplicative scattering effects were more effectively corrected through MSC treatment.

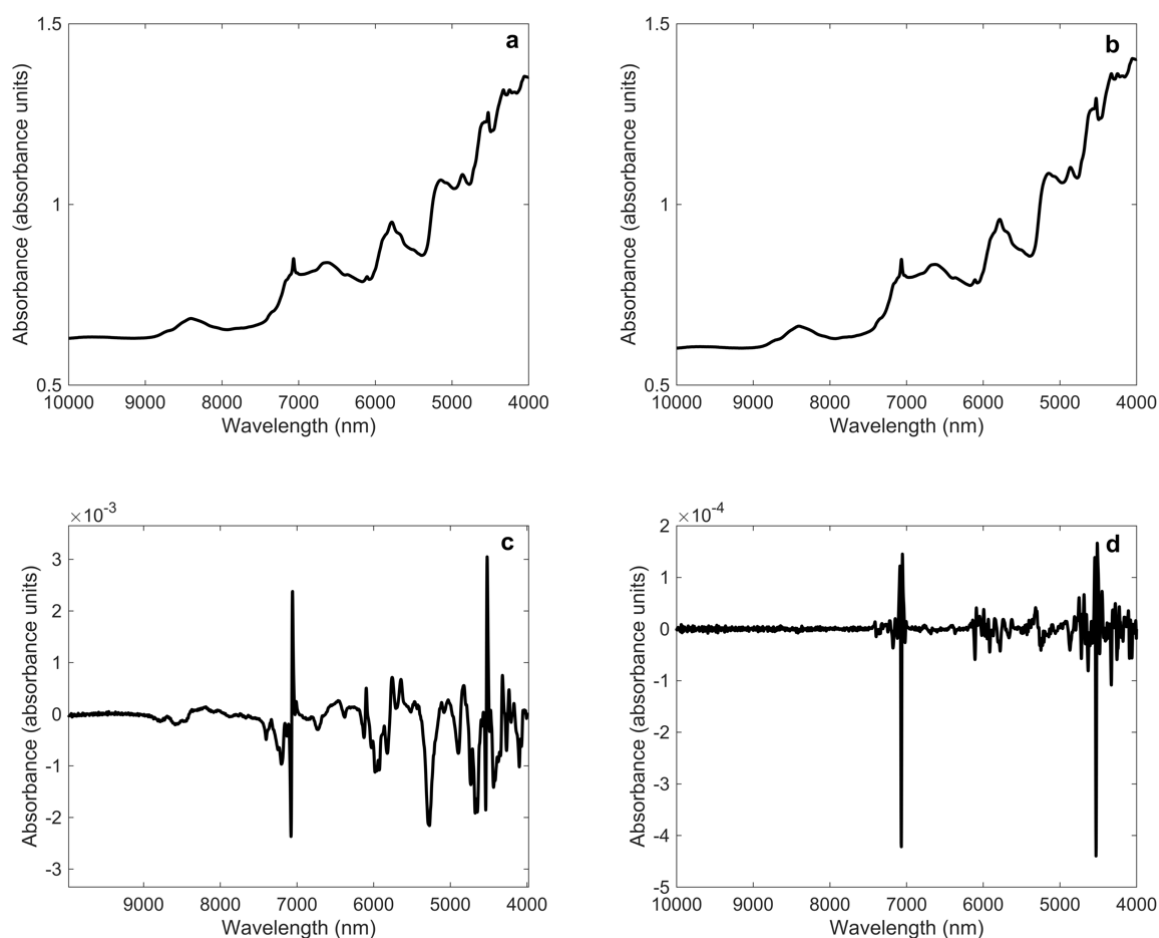


Figure 5.5. Average of (a) 22 raw NIR spectra, (b) 22 MSC treated NIR spectra, (c) 22 MSC-D1 treated NIR spectra and (d) 22 MSC-D2 treated NIR spectra of fingernails taken from participant MWS1 and measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

5.3.3. Application of MLAs to classifying fingernails' NIR spectra

MLAs were applied to aid in the differentiation of the fingernail groups, as well as the detection of disease.

5.3.3.1 Application to All Fingernails

CWS paired with NIR spectroscopy, successfully classified fingernail sets against themselves to produce a maximum r value of 1 (Figure 5.6). A match was considered if an r value of ≥ 0.95 was achieved. In some cases, fingernails received zero r values of ≥ 0.95 and therefore no matches. While others received 54 r values of ≥ 0.95 and

indicated a high number of matches. Several matches occurred between fingernails of participants of different characteristics (type II errors). For example, MWS26 (healthy) was incorrectly correlated against MWS35 (unhealthy) (r value = 0.98) (Assi *et al.*, 2020).

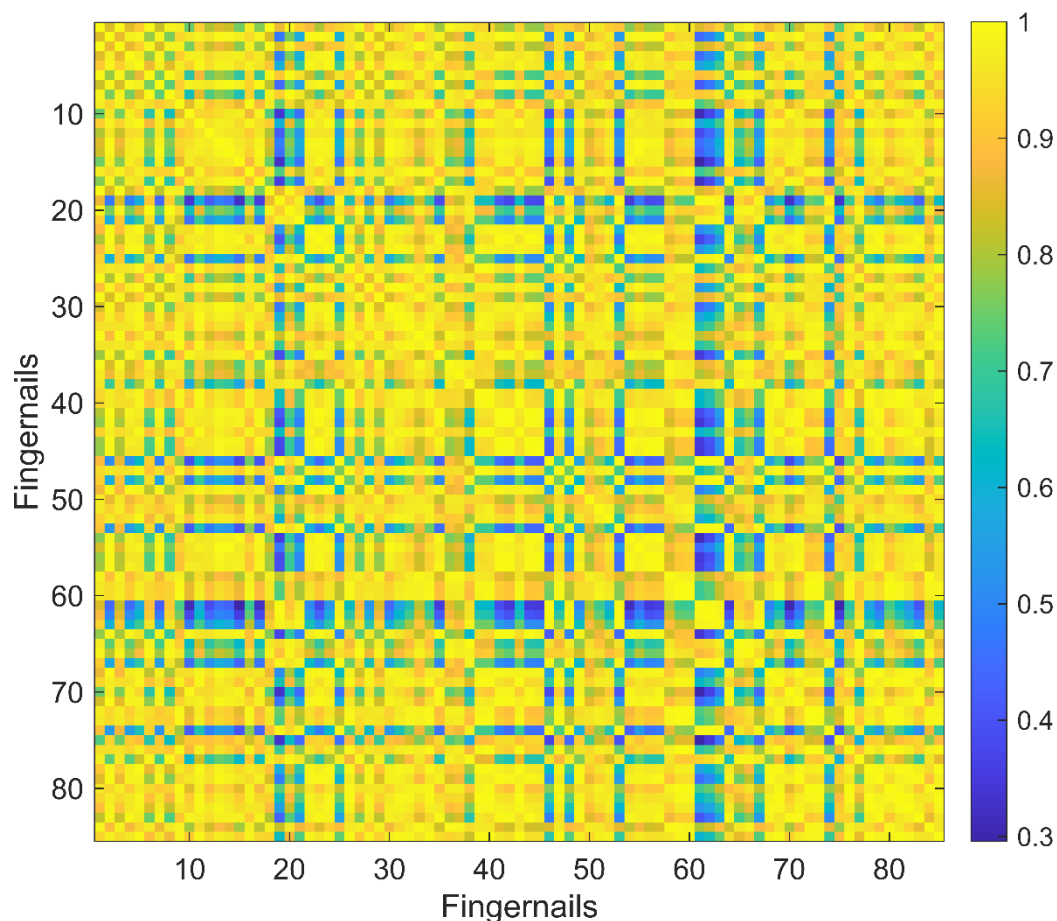


Figure 5.6. CWS of average MSC-D1 treated NIR spectra of fingernails taken from healthy (1-50), unhealthy (51-64), CVD (65-75), DM (76-78) and CVD-diabetic participants (79-85) and measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with a NIRM. A dark blue colour represents a minimum r value of 0.30 and a dark yellow colour represents a maximum r value of 0.9999. In between the r values 0.40 – 0.50 correspond to a light blue colour, r value of 0.51 – 0.69 correspond to a cyan colour, 0.70 – 0.75 correspond to a green colour, 0.76 – 0.80 correspond to a greenish yellow colour and 0.81 – 0.90 correspond to an orange colour.

To further understand mismatches in CWS, PCA was applied to MSC-D1 treated NIR spectra, with PC1 (87.6%), PC2 (3.52%) and PC3 (2.14%) scores accounting for 93.3% of the overall variance (Figure 5.7). However, an overlap between the five groups was observed and prevented accurate clustering. The overlapping of groups could be attributed to one of three reasons: (1) fingernails sharing the same set of endogenous constituents, (2) spectral range not targeting where changes happened or (3) imbalance among the categories fed into the PCA model. Moreover, the PCA confirmed the relationship between CVDs and DM and how they impacted the fingernails' physicochemical properties in a similar way. Similar results were found when a selected wavelength was utilised and this is likely attributed to the imbalance of healthy, CVD and diabetic spectra.

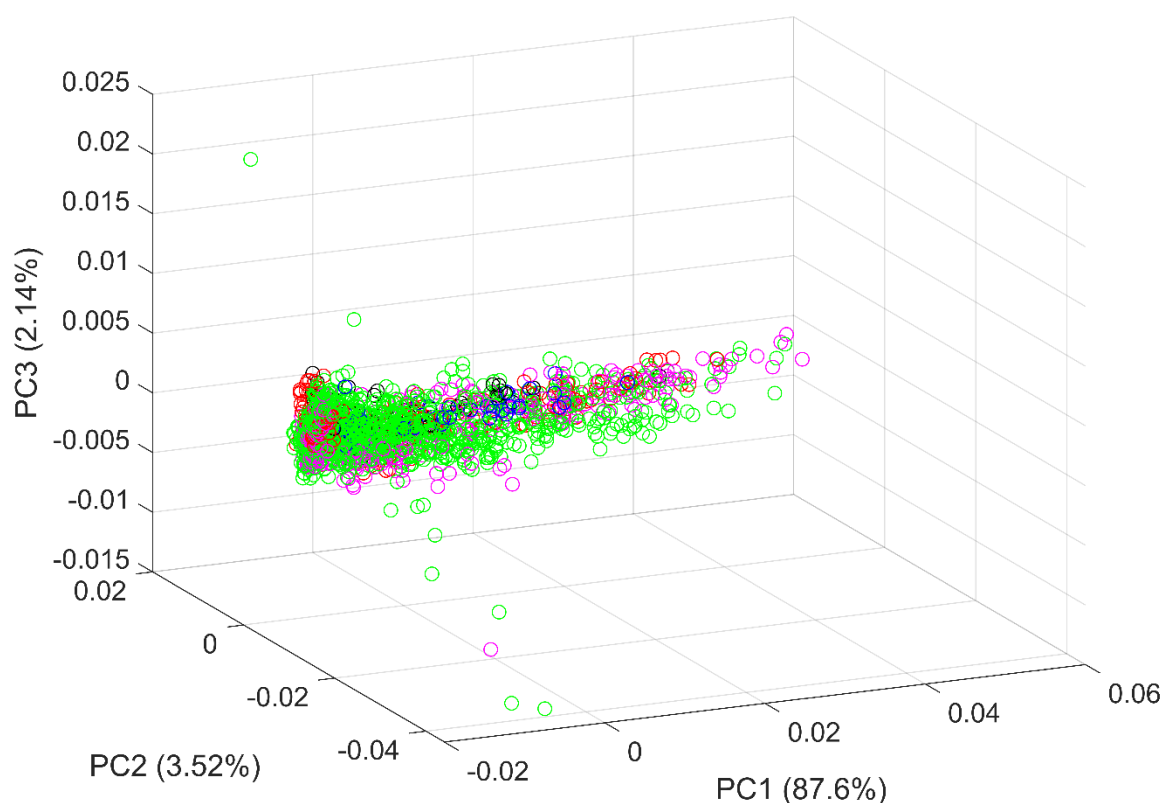


Figure 5.7. PCA of MSC-D1 treated NIR spectra of fingernails taken from healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Regarding the range, the PC loading plots showed bands over the wavenumber regions of 7,484 – 6,910, 6,294 – 5,516 and 5,378 – 4,068 cm^{-1} (Figure 5.8). These regions were associated with CH_3 , CH_2 , CH , RNH_2 , RCONH_2 and ROH (Jee, 2019). Within this region, bands at 4,864 and 4,320 cm^{-1} were linked to the fingernails' glycated proteins (Monteyne *et al.*, 2019).

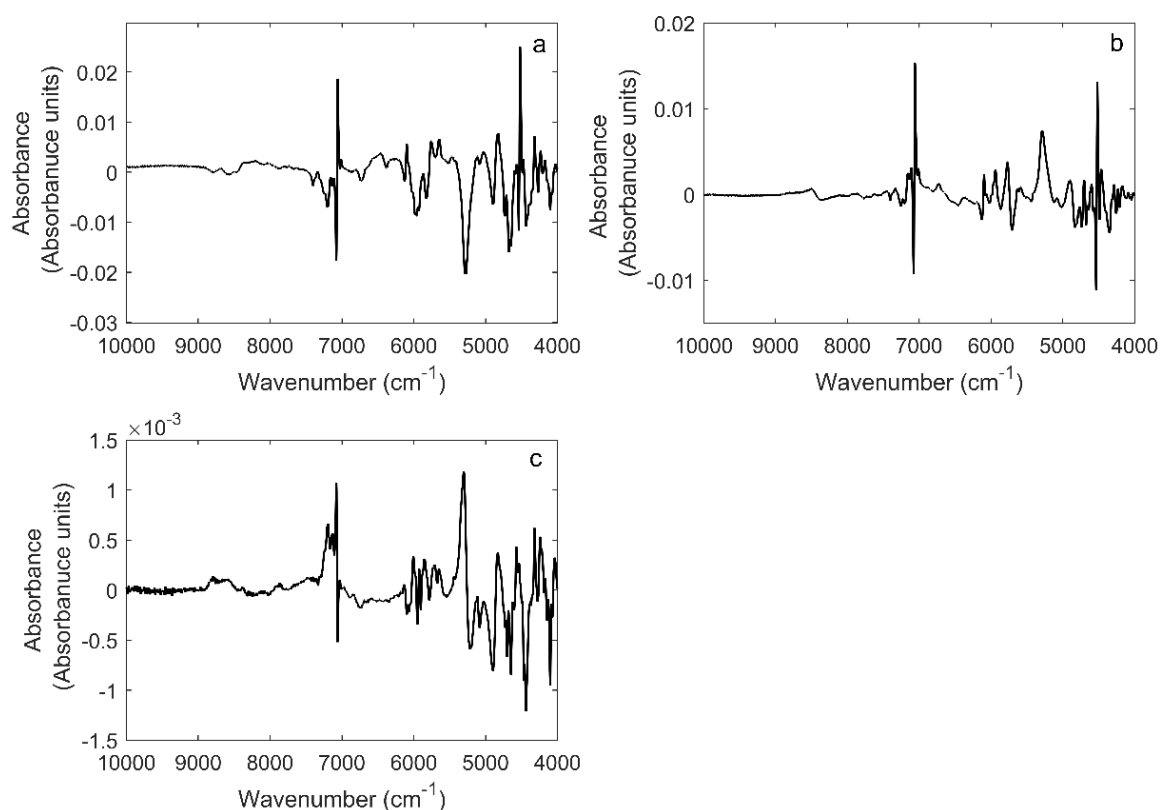


Figure 5.8. PCA loading (a) PC1, (b) PC2 and (c) PC3 of MSC-D1 treated NIR spectra of fingernails taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

This suggested the need for using further algorithms for detecting CVDs and/or DM in fingernails. SOMs were explored as an additional unsupervised clustering approach. Figure 5.9 demonstrated and confirmed PCA findings of the overlap between the five groups of participants. The U-Matrix highlighted the weighted distances between the neighbouring neurons. As previously explained in chapter four of the thesis (section

4.3.4), the red lines connecting the neurons (purple hexagons) indicated the distances between neighbouring neurons. Hence, variables that were closely related showed smaller distances between neurons and were portrayed with higher densities (black/red/dark orange). Whereas, variables that were dissimilar, displayed greater distances with lower densities (light orange/yellow). As shown in Figure 5.9, the vast majority of the U-Matrix contained higher densities, indicating the similarities between the groups of fingernails.

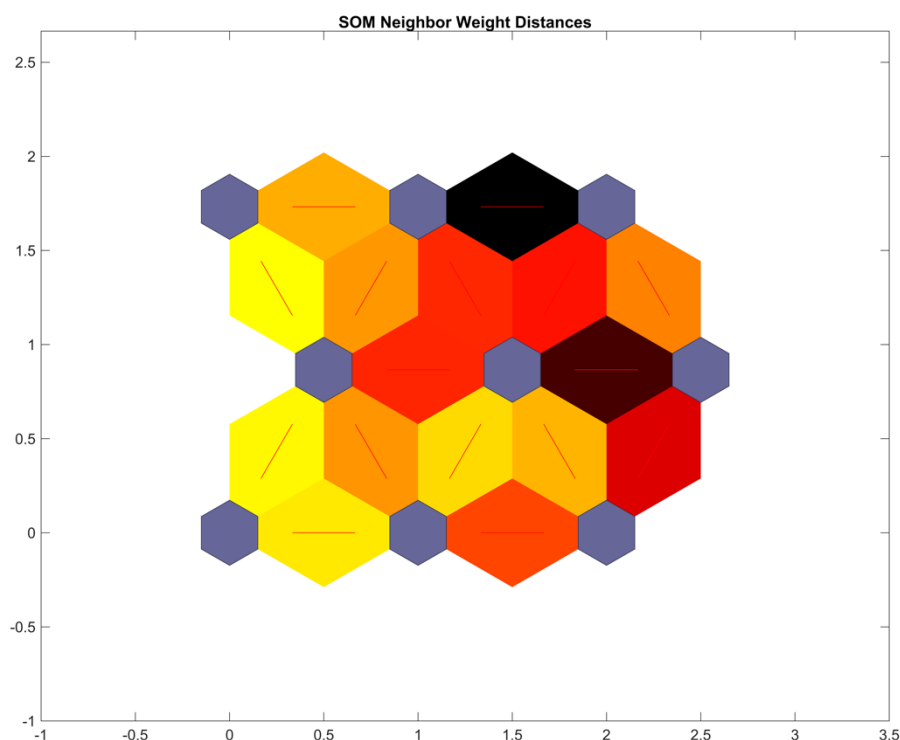


Figure 5.9. Neighbour weight distances SOM of MSC-D1 treated NIR spectra of fingernails taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Within Figure 5.10, nine groups were visualised. The first group ($n = 253$, 18%) consisted of healthy, unhealthy and CVD fingernails. The second and largest group ($n = 327$, 23%) corresponded to spectra taken from healthy, CVD and diabetic fingernails. Group three ($n = 270$, 19%) was comprised of healthy, unhealthy, diabetic and CVD-diabetic fingernails. Further overlap was seen for groups four ($n = 50$, 4%), group five

($n = 113$, 8%), group six ($n = 187$, 13%), group seven ($n = 79$, 6%) and group nine ($n = 91$, 7%), which were attributed to fingernails taken from healthy, unhealthy, CVD and diabetic fingernails. Group eight ($n = 33$, 2%) represented spectra of unhealthy fingernails. The overlap between groups confirmed the implications of a data imbalance, as well as the presence of endogenous constituents across healthy, unhealthy, CVD, DM and CVD-diabetic fingernails. Better balancing of groups could improve future MLA applications.

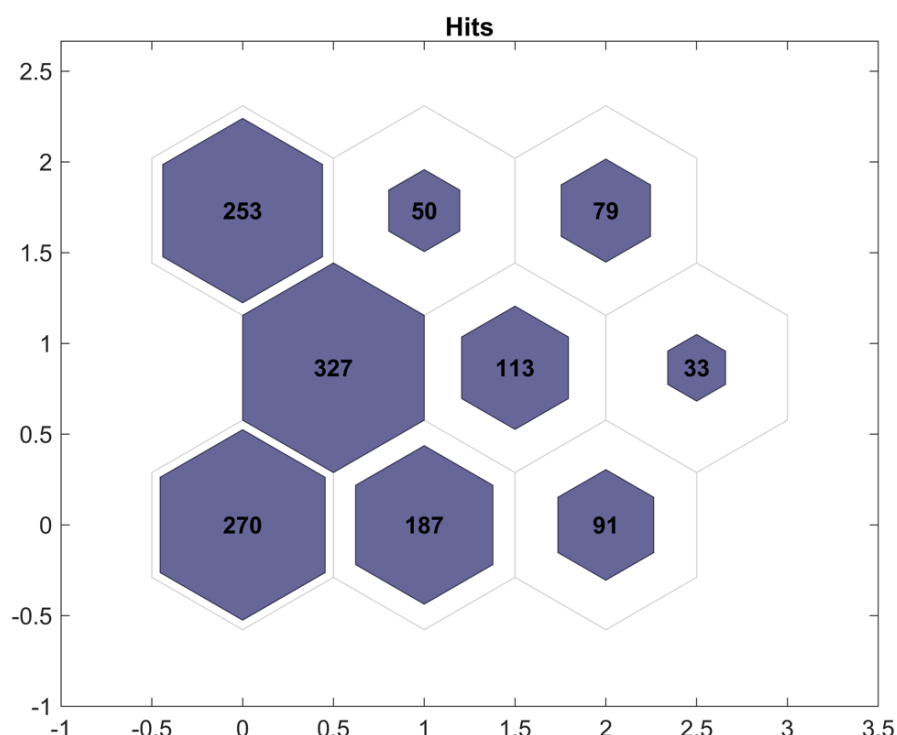


Figure 5.10. Sample hits SOM of MSC-D1 treated NIR spectra of fingernails taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

5.3.3.2. Applications to Fingernails Taken from CVD Participants

To explore NIR's ability to differentiate between different categories of CVDs, CWS method was applied (Figure 5.11). For this, 11 sets of fingernails were employed, and belonged to participants that had been previously diagnosed with AF (MWS44), hypertension (MWS46, MWS47, MWS52, MWS55, MWS56, MWS63, MWS64, MWS66 and MWS71) and unspecified heart disease (MWS48). Figure 5.11

demonstrated that the produced r values showed no clear variation between fingernails of different CVDs. For instance, MWS64 (hypertension) shared an r value ≥ 0.95 with MWS44 (AF). Thus, CWS was not successful in indicating specific CVD conditions.

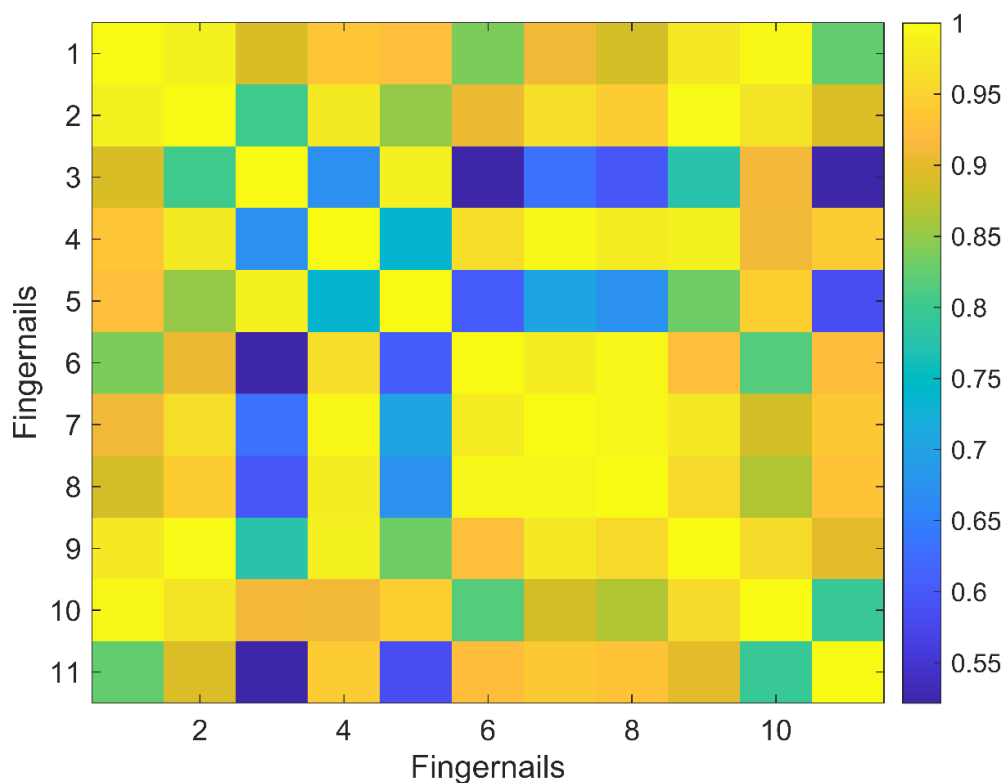


Figure 5.11. CWS of MSC-D1 treated NIR spectra of fingernails taken from participants MWS44 (1), MWS46 (2), MWS47 (3), MWS48 (4), MWS52 (5), MWS55 (6), MWS56 (7), MWS63 (8), MWS64 (9), MWS66 (10) and MWS71 (11), who have previously been diagnosed with a CVD, measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM. A dark blue colour represents a minimum r value of 0.55 – 0.65 and a dark yellow colour represents a maximum r value of 0.999 – 1.00. In between the r values 0.0.67 – 0.74 correspond to a light blue colour, r value of 0.75 – 0.80 correspond to a cyan colour, 0.80 – 0.84 correspond to a green, 0.85–0.90 correspond to a greenish yellow colour and 0.91 – 0.95 correspond to an orange colour.

Subsequently, PCA was applied to NIR spectra of individuals with CVDs (Figure 5.12). The first two PCs accounted for 94.6% of the variance across the data. The PC scores

plot showed one set (MWS71) differentiated from the remaining sets. This set corresponded to an individual with hypertension, which was seen in another six sets. On the other hand, the other sets were grouped together regardless of the specific CVD diagnosis. This demonstrated that NIR spectroscopy paired with PCA was not accurate in differentiating between fingernails of different types of CVDs.

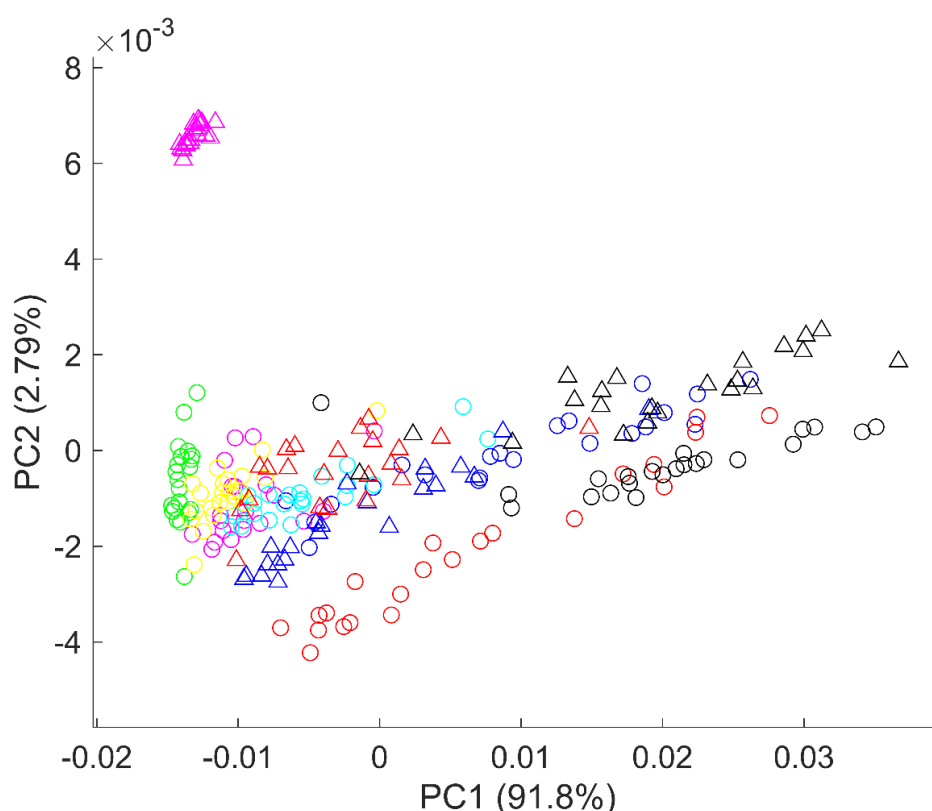


Figure 5.12. PCA of MSC-D1 treated NIR spectra of fingernails taken from participants MWS44 (blue circle), MWS46 (red circle), MWS47 (black circle), MWS48 (magenta circle), MWS52 (cyan circle), MWS55 (green circle), MWS56 (yellow circle), MWS63 (blue triangle), MWS64 (red triangle), MWS66 (black triangle) and MWS71 (magenta triangle) who have previously been diagnosed with a CVD measured using the using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

The same findings were obtained for SOMs applied to the NIR spectra of CVD individuals (Figure 5.13 and 5.14). For example, fingernail sets MWS48, MWS52,

MWS55, MWS56, MWS63 and MWS64 were closely related and therefore were linked via neurons of yellow and light orange. In contrast, fingernail set MWS71 was represented by the dark black neuron, highlighting the lowest density and largest distance. Therefore, suggesting that this fingernail set was unrelated to the other sets and confirmed the PCA findings. The dark red and dark orange neurons indicated medium related spectra and medium distances.

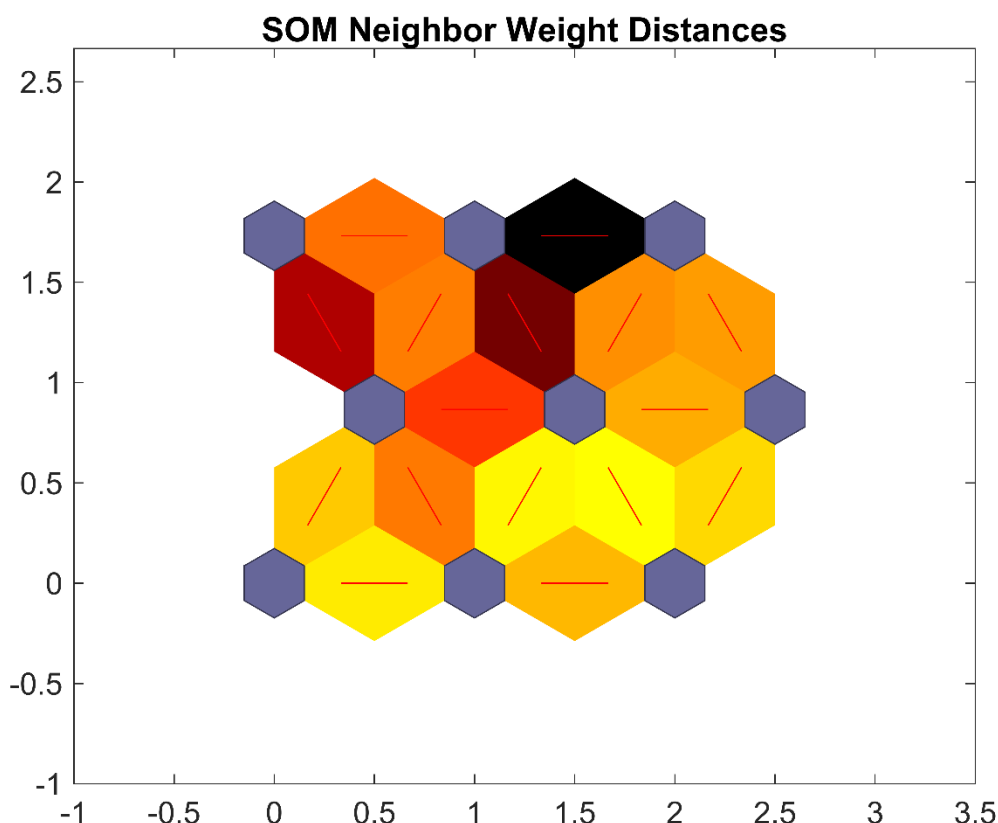


Figure 5.13. Neighbour weight distances SOM of MSC-D1 treated NIR spectra of fingernails taken from participants diagnosed with a CVD measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

During the visualisation of sample hits, nine groups were identified (Figure 5.14). Group one ($n = 16$, 6.61%) consisted of spectra taken from set MWS44, MWS46, MWS47 and MWS66. Group two ($n = 32$, 13.2%) corresponded to MWS46 and MWS47. Group three ($n = 22$, 9.091%) was made up of spectra from MWS71. Group four ($n = 15$, 6.2%) was attributed to MWS44, MWS46, MWS47 and MWS66. Group

five ($n = 36$, 14.9%) to fingernail sets MWS52, MWS63 and MWS64. Group six ($n = 25$, 10.33%) lends itself to MWS46 and MWS63. Group seven ($n = 25$, 10.33%) represented sets MWS44 and MWS63. Groups eight ($n = 23$, 9.5%) and nine ($n = 48$, 19.8%) signify sets MWS48, MWS52, MWS55 and MWS56. Several of the collected fingernails have been sampled from hypertensive participants or participants that likely displayed hypertensive symptoms. As a result, are likely to share similar physicochemical properties, thus have been clustered together. To improve the clustering of the PCA model, further CVD groups could be introduced in future work or further spectral ranges optimised.

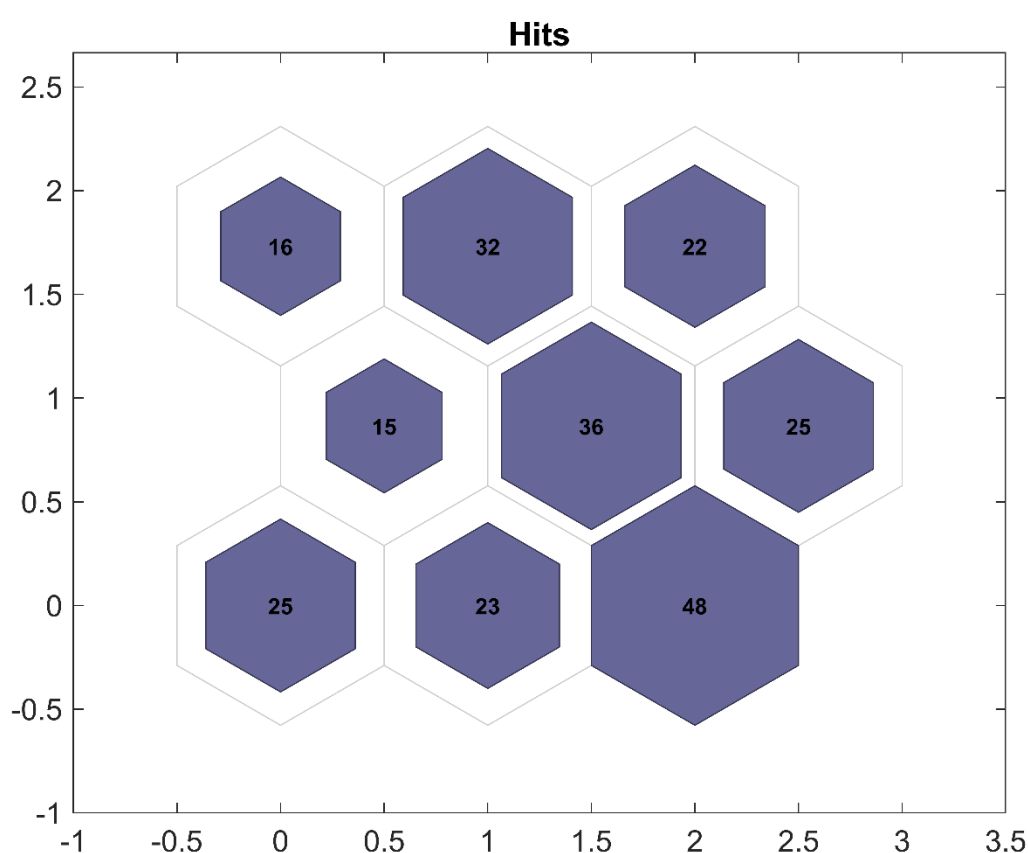


Figure 5.14. Weighted neighbours SOM of MSC-D1 treated NIR spectra of fingernails taken from CVDs participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

5.3.3.3. Application to Fingernails Taken from Diabetic Participants

For the differentiation of DM, both T1DM (MWS51) and T2DM (MWS43 and MWS79) were included in the comparison. The CWS method was applied to NIR spectra of diabetic fingernails for determining the differences between T1DM and T2DM (Figure 5.15). Yet as only three participants were available, it could not be concluded that CWS could accurately differentiate between T1DM and T2DM.

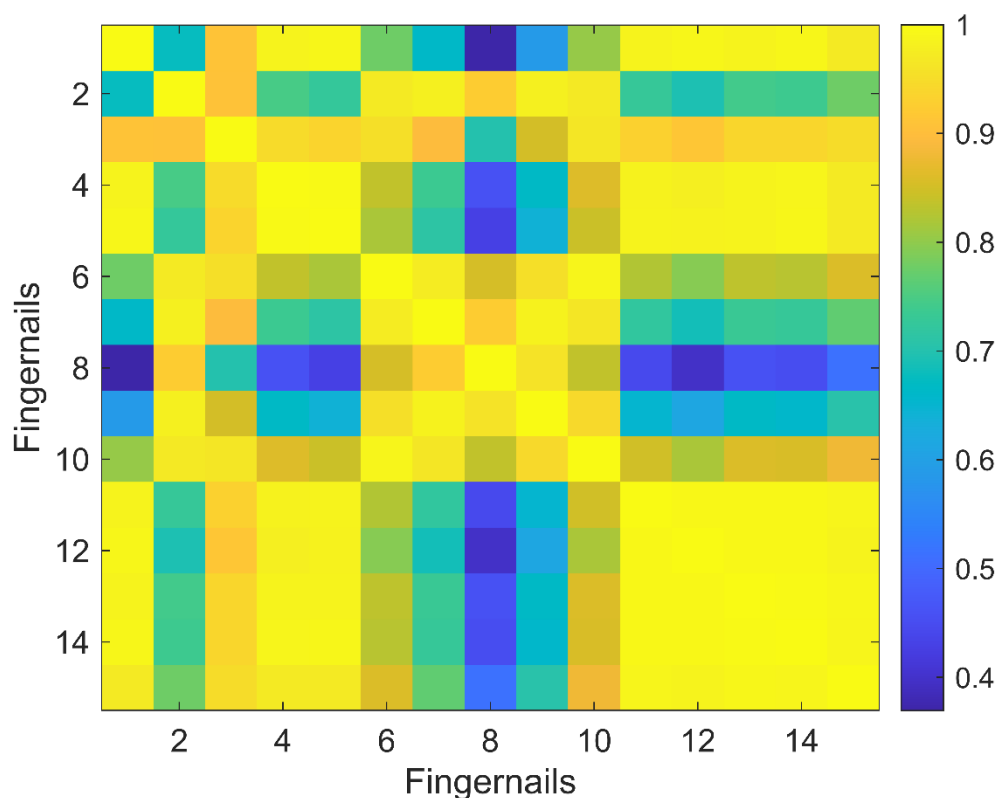


Figure 5.15. CWS of MSC-D1 treated NIR spectra of fingernails taken from participants MWS43 (1-5), MWS51 (6-10) and MWS79 (11-15), who have previously been diagnosed with DM, measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM. A dark blue colour represents a minimum r value of 0.40 and a light-yellow colour indicates a maximum r value of 0.9999. In between the r values of 0.41 – 0.6 correspond to a blue colour, 0.61 – 0.70 correspond to a cyan colour, 0.71 – 0.80 correspond to a green colour and 0.81 – 0.90 correspond to orange colour.

PCA applied to the NIR spectra of diabetic fingernails did not show clear clustering between T1DM and T2DM participants (Figure 5.16). The first three PCs captured 95.6% of the variance. PC1 captured the largest variance (91.4%), followed by PC2 (2.99%) and PC3 (1.22%). Overlap between scores of T1DM and T2DM were seen and this indicated a type II error. However, this result could not be conclusive as only three sets were evaluated.

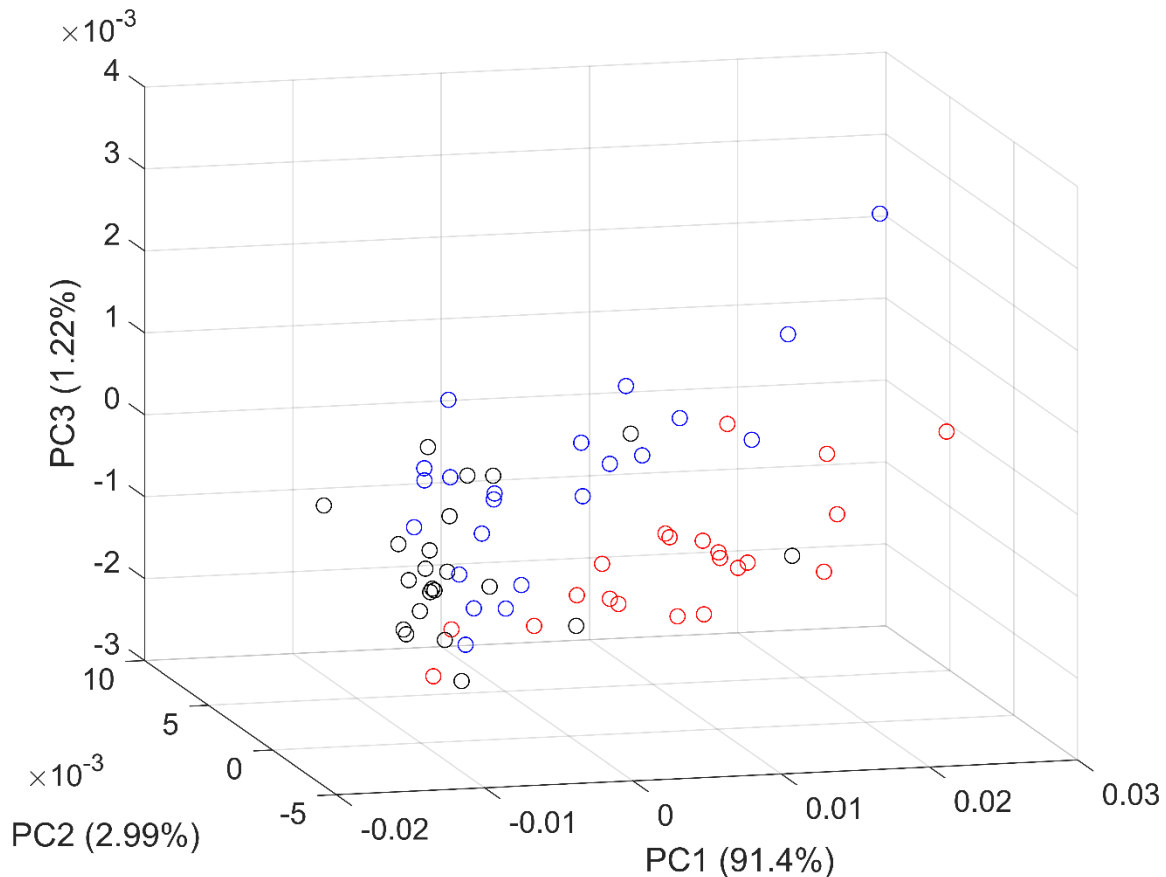


Figure 5.16. PCA of MSC-D1 treated NIR spectra of fingernails taken from participants MWS43 (blue circle), MWS51 (red circle) and MWS79 (black circle), who have been previously diagnosed with DM measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

SOMs showed better classification between T1DM and T2DM than PCA (Figure 5.17 and 5.18). The light-yellow coloured neurons indicated T2DM sets (MWS43 and MWS79) and the darker colour neurons (red, dark orange and black) corresponded to the T1DM set (MWS51) (Figure 5.17).

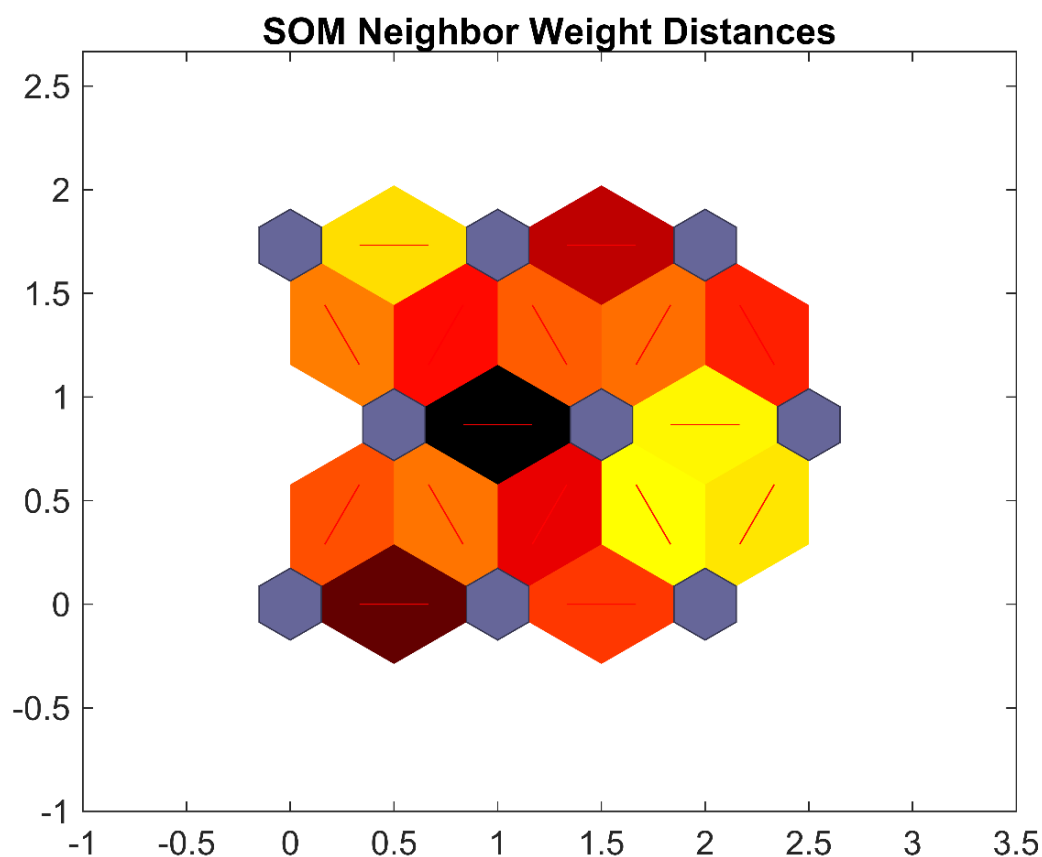


Figure 5.17. Neighbour weight distances SOM of MSC-D1 treated NIR spectra of fingernails taken from participants diagnosed with DM and measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Sample hits SOM (Figure 5.18) showed nine groups: groups one ($n = 4$, 6.06%), two ($n = 6$, 9.09%) and three ($n = 1$, 1.51%) solely corresponded to MWS43. The crossover between the T2DM spectra could be observed for group four ($n = 12$, 18.2%). Group five ($n = 18$, 27.3%) consisted of MWS79 spectra. Group six ($n = 7$, 10.61%) represented both MWS51 and MWS79. Finally, groups seven ($n = 6$, 9.09%), eight ($n = 4$, 6.06%) and nine ($n = 8$, 12.1%) indicated the presence of MWS51.

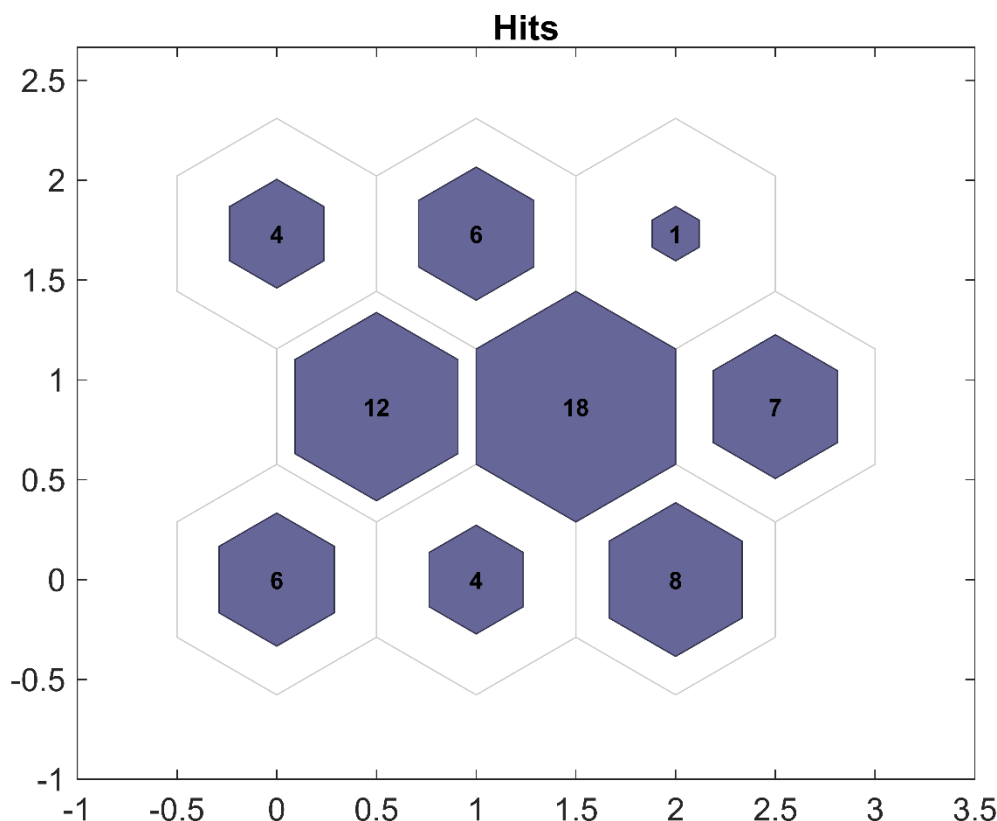


Figure 5.18. Sample hits SOM of MSC-D1 treated NIR spectra of fingernails taken from participants diagnosed with DM measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

5.3.4 Classification of CVDs and DM

Considering the limitations in the previous models in terms of lack of differentiation of CVDs subcategories and overall classification of fingernails, MLAs were applied to more inclusive fingernails' datasets for individuals with CVDs and DM.

5.3.4.1. Application of Classification Models to NIR Spectroscopy

Unsupervised classification ML models were applied to a full dataset of fingernails ($n = 1403$ NIR spectra) including fingernails from individuals with CVD, DM and other conditions. Following the removal of outlier spectra, a total of 32 models (section 5.2.5) were tested. From the tested models, five models produced the highest quality during the testing stage. These models included: bagged tree (Figure 5.19a), boosted tree

(Figure 5.19b), cubic SVM (Figure 5.19c), SVM kernel (Figure 5.19d) and quadratic SVM (Figure 5.19e). The aforementioned models produced training accuracies of 72.7%, 74.4%, 69.8%, 62.0% and 69.2%, respectively. The relatively low accuracy across these models could be attributed to the class imbalance observed between the healthy, unhealthy, CVDs, diabetic and CVD-diabetic groups. It has previously been shown that class-imbalances can significantly impact the value and meaning of accuracy across ML models (Chawla, 2005; Hossin and Sulaiman, 2015; Luque *et al.*, 2019). Consequently, models were likely to favour the majority class (healthy class) and bias was introduced (Ali, Shamsuddin and Ralescu, 2015). The minority classes were of the most interest in this project, thus it was imperative that classification models were trained with an equally distributed dataset. Hence, data-levelling was necessary to reduce the classes into 66 spectra each. Through under-sampling the majority class, discrepancies between the majority and minority classes were minimised (Ali, Shamsuddin and Ralescu, 2015).

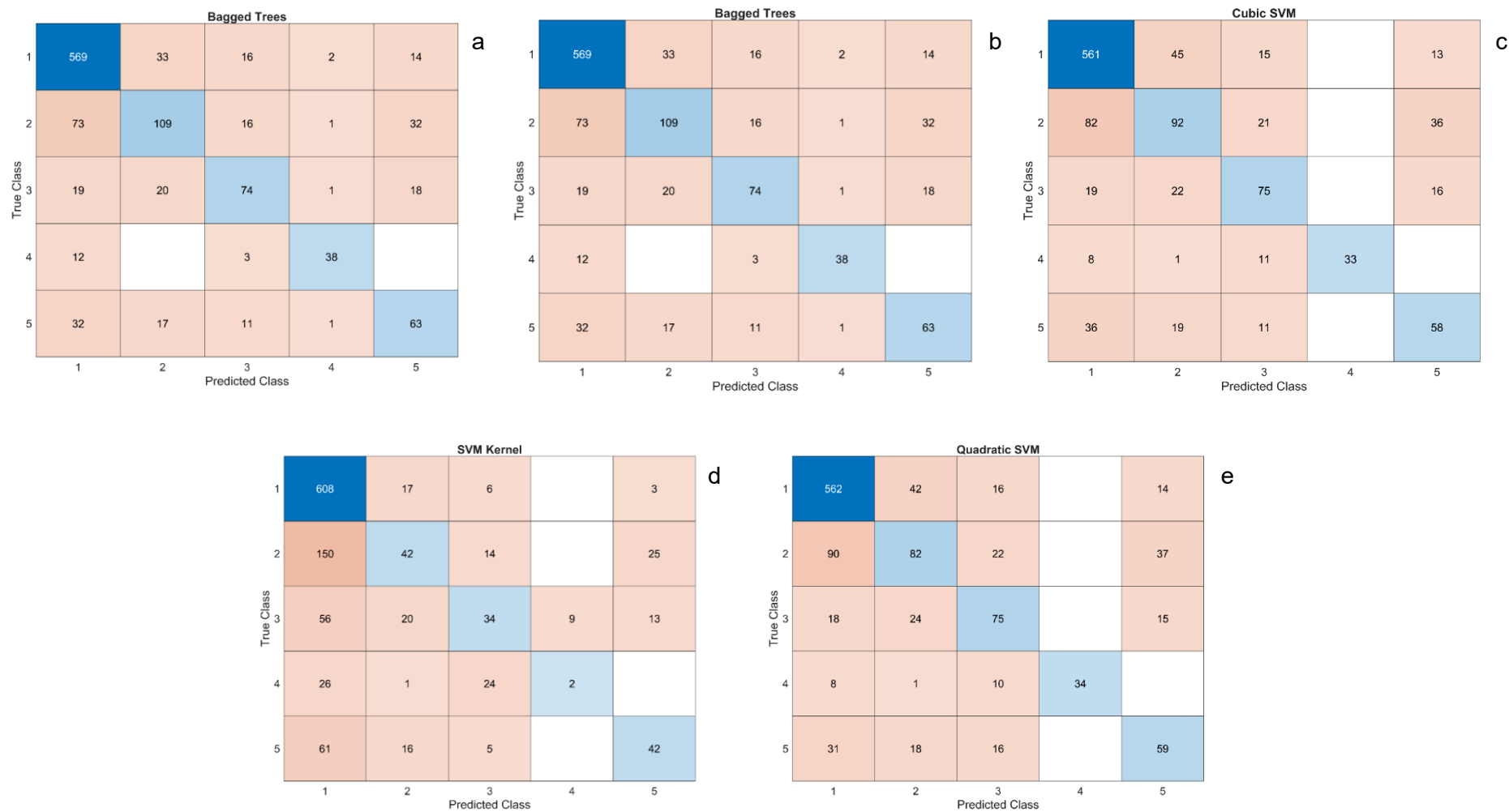


Figure 5.19. Unbalanced (a) bagged tree ensemble, (b) boosted tree ensemble, (c) cubic SVM, (d) SVM kernel and (e) quadratic SVM models of fingernails taken from healthy (1), unhealthy (2), CVD (3), diabetic (4) and CVD-diabetic (5) participants measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Models were balanced, with each group of interest consisting of 66 random spectra and were validated and tested using a 10-fold cross validation for the calculation of accuracy (Harper, 2023). Additionally, 20% of the data was withheld as a test set. After testing, the accuracy of the bagged tree ensemble and SVM kernel models improved, while the accuracy of the boosted tree ensemble, cubic SVM and quadratic SVM models decreased (Table 5.3). Nonetheless, the increased accuracies across four of the five models provided support to the notion that the class-imbalance observed in the previous models, negatively impacted the classification of disease. The improved accuracy of the SVM kernel model after testing, suggested that this model was most successful when handling unknown data. To determine the overall effectiveness of the classification models, the following parameters were employed: accuracy, AUC, FNR, FPR, F1-score, misclassification rate, precision, prevalence, specificity and recall (Table 5.4).

Table 5.3. Validation and training accuracy of the chosen ML models.

Model	Validation accuracy (95% CI) (%)	Training accuracy (%)
Bagged tree ensemble	79.2 (74.2-84.1)	81.8
Boosted tree ensemble	30.3 (24.7-35.8)	18.2
Cubic SVM	89.8 (86.8-93.9)	89.4
SVM kernel	78.4 (73.5-83.6)	80.3
Quadratic SVM	92.8 (89.8-96.4)	87.9

CI: Confidence intervals; SVM: Support vector machine.

Table 5.4. The accuracy, AUC, false positive rate, F1-score, misclassification rate, precision, prevalence, specificity and recall for each of the trained machine learning classification models.

Parameters	Bagged tree ensemble	Boosted tree ensemble	Cubic SVM	SVM Kernel	Quadratic SVM
FNR (%)	41.1	44.1	14.5	41.6	10.2
FPR (%)	13.1	77.1	8.91	13.4	6.34
Misclassification rate (%)	20.83	69.7	10.23	21.6	7.19
Prevalence (%)	79.9	79.9	79.9	79.9	79.9
Specificity (%)	58.9	79.7	85.5	58.4	89.9
Precision (%)	84.7	64.4	95.3	83.5	96.9
Recall (%)	86.9	22.9	91.1	86.6	93.7
F1-Score (%)	85.8	33.7	93.3	85.02	95.3
AUC	0.956	0.834	0.993	0.982	0.993

AUC: Area under the curve; FNR: False negative rate; FPR: False positive rate; SVM: Support vector machine.

The models which achieved the highest testing accuracy were cubic SVM and quadratic SVM with accuracies of 89.4% and 87.9%, respectively. Furthermore, the combination of NIR spectroscopy and SVM models in the literature showed success in the classification of cancer, endocrine diseases, neurological diseases and renal diseases (Vitorino *et al.*, 2023). In other studies, SVM models were considered the standard when working with NIR spectral data (Bedin *et al.*, 2021; Pasquini, 2003).

For the cubic SVM model, 27 misclassifications were seen, 12 of which were healthy spectra misclassified as unhealthy (n = 10) and CVD (n = 2) (Figure 5.20a). As shown in Figure 5.4, the presence of the highlighted components causes spectral differences between healthy and diabetic fingernails, which were accounted for within the classification models. It was interesting that no unhealthy or diseased spectra were misclassified as healthy spectra. The cubic SVM model showed FNR of 14.5%, FPR of 8.91%, specificity of 85.5%, recall of 91.1%, precision of 95.3% and F1-score of 93.3%. Hence, suggesting that the NIR spectrometer paired with the cubic SVM classification model was able to differentiate between healthy and diseased fingernails. Minor misclassification occurred between CVD and diabetic spectra, whereby two CVD spectra were misclassified as diabetic and four diabetic spectra were misclassified as CVD. The misclassification of CVD and diabetic spectra can be attributed to the sharing of biological mechanisms and inflammation between the two

NCDs, as well as their relationship as comorbidities. This was further supported through the misclassification of CVD-diabetic spectra as diabetic ($n = 2$), as well as CVD spectra ($n = 7$) misclassified as CVD-diabetic.

A similar trend was observed in the quadratic SVM model, which demonstrated 245 correct classifications and 19 misclassifications only (Figure 5.20b). Within this model, only eight healthy participants were misclassified as unhealthy ($n = 7$) and CVD ($n = 1$). From the CVD group, two participants were misclassified diabetic and four as CVD-diabetic. One diabetic spectrum was misclassified as CVD and four as CVD-diabetic. The quadratic SVM model achieved a misclassification rate of 7.19%, a FNR of 6.34%, a FPR of 10.2% and a specificity of 89.8%. For this model, a recall value of 93.7%, alongside a precision score of 96.9%. Thus, an F1-value of 95.3% was determined.

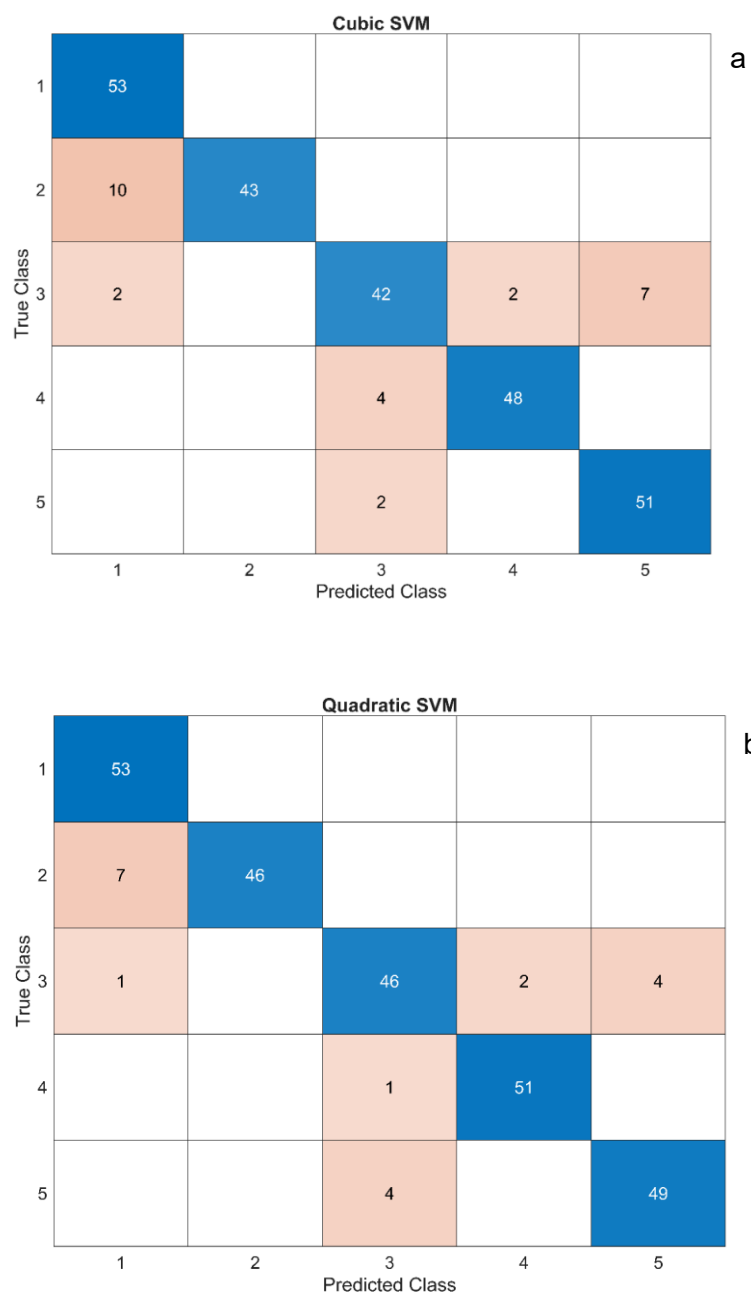


Figure 5.20. Balanced (a) cubic SVM and (b) quadratic SVM models of healthy (1), unhealthy (2), CVD (3), diabetic (4) and CVD-diabetic (5) fingernails measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Additional, binary classification models were trained and validated through the input of two classes, those being: healthy and CVD. As previously seen, an imbalance of data caused several misclassifications and reduced the overall accuracy of the model.

Therefore, a total of 66 random spectra were taken per class. Through binary classification, the accuracy of generated models was vastly improved. With several of the validated and trained models producing accuracies of > 90%. For example, DT models including fine tree (Figure 5.21a), medium tree (Figure 5.21b) and coarse tree (Figure 5.21c) demonstrated validation and testing accuracies of 95.3% and 92.3%, respectively (Table 5.5).

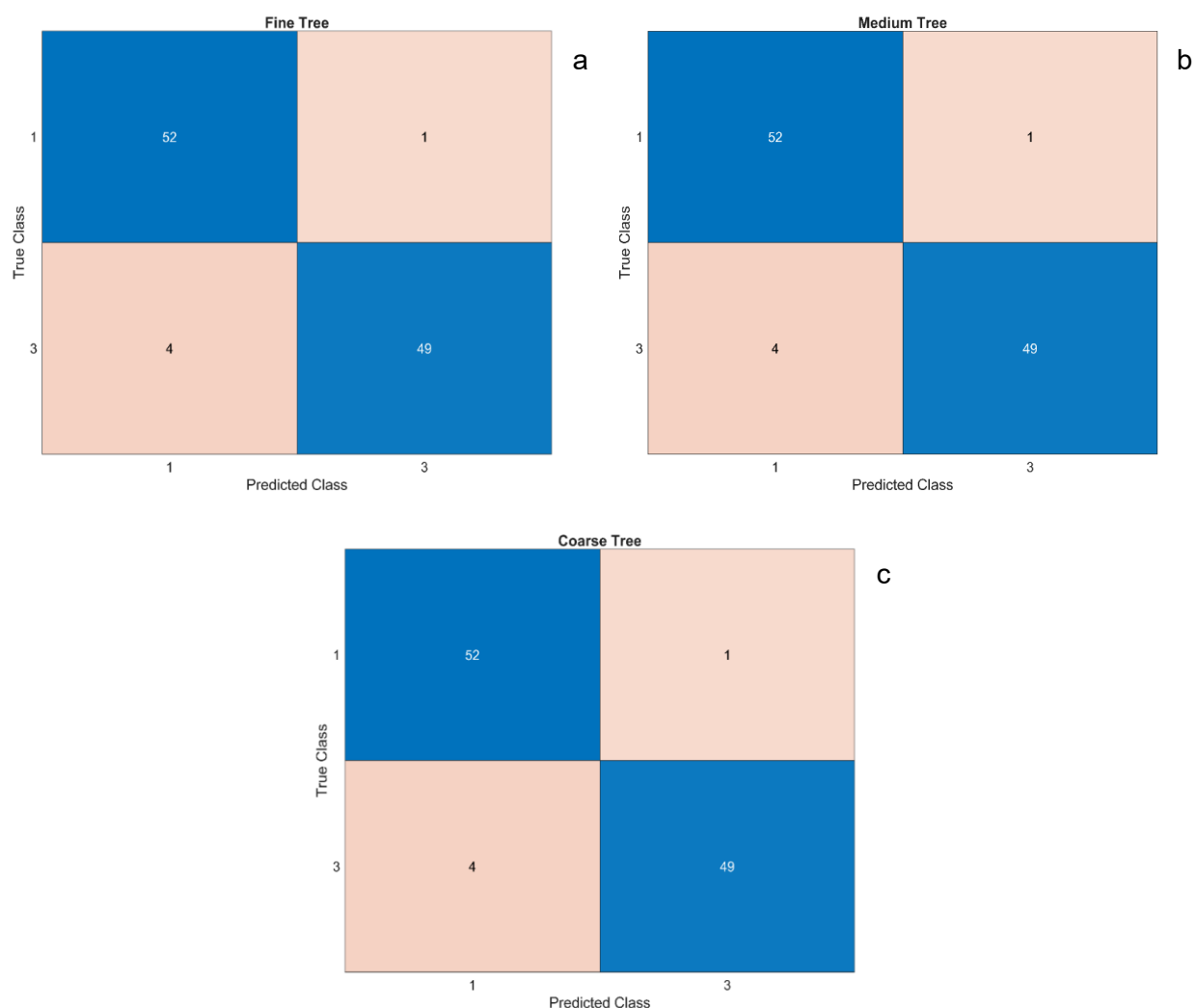


Figure 5.21. Balanced (a) medium (b) fine and (c) coarse tree models of healthy (1) and CVD (3) fingernails measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Table 5.5. Accuracy values of validation and test results for fine tree, medium tree and coarse tree models.

Model	Validation accuracy (95% CI) (%)	Training accuracy (%)
Fine Tree	95.3 (89.8-99.1)	92.3
Medium Tree	95.3 (89.8-99.1)	92.3
Coarse Tree	95.3 (89.8-99.1)	92.3

CI: Confidence intervals.

The AUC values for the aforementioned models were above 0.953 (Table 5.6). The misclassification across the tested DT model was very limited with only five misclassifications observed per model. Specifically, a healthy spectrum was misclassified as CVD and four CVD spectra were misclassified as healthy. Compared to previous studies, the accuracy obtained within this experiment is high. For example, work by Agbemade (2023) showed accuracies of 82% and 81% for the classification of patients with and without heart disease, respectively. However, this research did not highlight the ratio of cases to non-cases, therefore, the low accuracies seen in this research may be attributed to a class-imbalance. Moreover, this work utilised several attributes such as age, chest pain type, resting blood pressure, etc. unlike the researcher's experiment, which focused on NIR spectra of fingernails. In turn, the model developed by Agbemade (2023) increased in complexity, capturing noise and/or other irrelevant patterns within the training data. As a result, overfitting occurred, which indicated that the model could not be generalised to future detection events (Demšar and Zupan, 2021).

Table 5.6. Performance evaluation metrics for fine tree, medium tree and coarse tree models for the prediction of healthy and CVD NIR spectra.

Parameters	Fine Tree	Medium Tree	Coarse Tree
FNR (%)	15.4	15.4	15.4
FPR (%)	0	0	0
Misclassification rate (%)	7.69	7.69	7.69
Prevalence (%)	50	50	50
Specificity (%)	84.6	84.6	84.6
Precision (%)	86.7	86.7	86.7
Recall (%)	100	100	100
F1-Score (%)	92	92	92
AUC	0.953	0.953	0.953

AUC: Area under the curve; FNR: False negative rate; FPR: False positive rate.

Cubic and quadratic SVM models also showed success as binary-classifiers and not only multiclass classifiers as mentioned above. In this sense, the cubic and quadratic SVM models showed validation/training accuracies of 98.1% (CI 95% = 93.1-99.8%)/100% and 97.2% (95 CI = 92.1-100%) /100%, respectively. A total of two misclassifications were observed within the CVD cubic SVM model, whereby, two CVD spectra were misclassified as healthy (Figure 5.22a). Three misclassifications were shown within the CVD quadratic SVM model, those being one healthy spectrum as CVD and two CVD as healthy (Figure 5.22b). The false positive of one can be attributed to a type II error, whereby a spectrum of a healthy class has been misclassified as CVD. However, it was interesting to point out the two misclassified CVD spectra observed in Figure 5.22. The reoccurrence of this misclassification may not be an error of the classification models and could be related to common endogenous constituents between healthy and CVD fingernails' spectra.

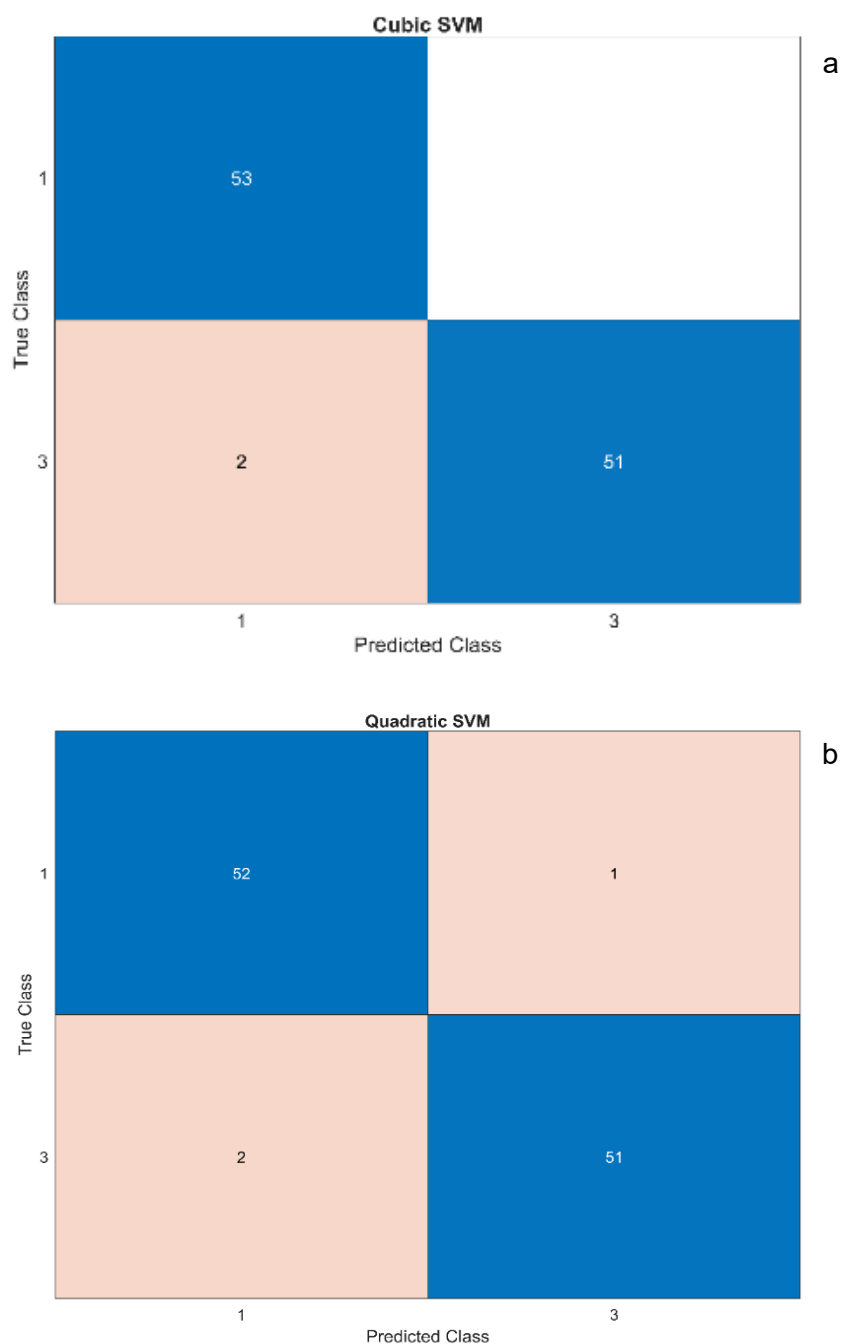


Figure 5.22. (a) Cubic SVM and (b) quadratic SVM models of healthy (1) and CVD (3) fingernails measured using the Perkin Elmer Spectrum Two N FT-NIR Spectrometer, equipped with NIRM.

Performance evaluation matrices were also applied to evaluate the performance of cubic and quadratic SVM models (Table 5.7). When compared directly to the multiclass

cubic and quadratic SVM models, the binary models possessed superior performance across all evaluation matrices. Therefore, suggesting that future detection of CVDs should be performed using binary models rather than multiclass models. The complexity of multiclass models creates overfitting within the model; hence, noise and/or other irrelevant patterns were misclassified. Furthermore, the increased number of misclassifications within the multiclass models could be attributed to the biological and pathological similarities between CVDs and DM, which caused similar structural and chemical changes to the fingernail.

Table 5.7. Performance evaluation metrics for cubic and quadratic SVM models for the prediction of healthy and CVD NIR spectra.

Parameters	Cubic SVM	Quadratic SVM
FNR (%)	0	1.87
FPR (%)	3.77	3.77
Misclassification rate (%)	1.89	2.78
Prevalence (%)	50	50
Specificity (%)	100	98.1
Precision (%)	100	98.1
Recall (%)	96.2	96.2
F1-Score (%)	98.1	97.1
AUC	0.985	0.987

AUC: Area under the curve; FNR: False negative rate; FPR: False positive rate

Binary classification models were also developed for the classification of healthy versus diabetic fingernails. For the classification of diabetic spectra, bilayered and trilayered neural networks produced the highest validation and training accuracies. For example, the bilayered neural network demonstrated a validation accuracy of 97.2%, which was then increased to 100% during training. Moreover, the trilayered neural network produced a validation accuracy of 96.2% and a training accuracy of 100%. The multilayered neural network models produced AUC values of 0.982 and 0.975, respectively, suggesting high levels of discrimination between healthy and diabetic spectra (Table 5.8). Thus, can be applied in future cases of DM detection.

While previous research has shown successful classification of DM, existing work focused on the application of DTs, K-NN, LR, random forest algorithms and SVM

(Iparraguirre-Villanueva *et al.*, 2023; Reza *et al.*, 2024). Cubic and quadratic SVM models, also produced high levels of accuracy for the prediction of DM. Firstly, the cubic SVM model produced a validation accuracy of 97.2% and a training accuracy of 100%. The AUC for this model was 0.982. The quadratic SVM produced a validation accuracy of 97.2% and a training accuracy of 100%. The AUC for this model was presented as 0.975.

Table 5.8. Performance evaluation metrics for bilayered and trilayered models for the prediction of healthy and diabetic NIR spectra.

Parameters	Bilayered neural network	Trilayered neural network
FNR (%)	3.77	5.66
FPR (%)	1.89	1.89
Misclassification rate (%)	2.83	3.77
Prevalence (%)	50	50
Specificity (%)	96.2	94.6
Precision (%)	96.3	94.6
Recall (%)	98.1	98.1
F1-Score (%)	97.2	96.3
AUC	0.982	0.975

AUC: Area under the curve, FPR: False positive rate, FNR: False negative rate.

5.4. Conclusions

This chapter investigated and evaluated the use of NIR spectroscopy for the detection of CVDs and DM using fingernails. Despite the presence of disease, all fingernails produced a strong ($n = 31$, 36.5%) or medium ($n = 54$, 63.5%) NIR activity with a moderately high number of NIR bands ($n = 7 - 17$). Spectral visualisation revealed that diabetic fingernails had the lowest absorbance across the five groups. This was attributed to the severe increase of glucose within the blood of diabetic patients that causes the displacement of glucose within the fingernail. Furthermore, extreme levels of glucose (hyperglycaemia) possess the ability to damage fingernail tissue. As a result, impairing the deposition of endogenous constituents into the fingernail and creating a lower level of NIR absorbance.

The overall quality and separation of broad NIR spectra was best improved through the application of MSC-D1, which produced strong NIR activity ($n = 85$, 100%), as well as a high number of bands ($n = 60 - 240$). MSC-D2 spectral pre-processing was also applied to the NIR spectra. While the aforementioned pre-processing technique did allow for the separation of NIR bands, a high level of spectral noise was introduced. To ensure that spectral noise was not misclassified or introduced irrelevant patterns within the data during the application of MLAs, NIR spectra were processed solely with MSC-D1.

Three areas of interest were identified through the comparison of healthy and diabetic spectra. The first region ($4,400 - 4,250 \text{ cm}^{-1}$) was attributed to C-H stretching, C-H bending and O-H bending of glucose. The broadness of bands present within this region highlighted the concentration of glucose within the fingernail. PCA models successfully classified healthy and diabetic scores. The most accurate clustering was observed when PCA was applied to spectra in the regions $7,100 - 6,000$ and $5,100 - 4,600 \text{ cm}^{-1}$. Yet, when PCA was applied to the fingernails' spectra of healthy, CVD and diabetic individuals, the accuracy of clustering decreased and this could be linked to the common endogenous constituents among individuals, as well as an imbalance of data. On the other hand, unsupervised classification ML models showed better classification than CWS and PCA with accuracies above 90%. This was seen for the cubic and quadratic SVM models when applied to balanced datasets. Therefore, ML models applied to NIR spectra of fingernails were better for classifying CVDs and DM than CWS, PCA and SOMs.

5.5. Key Messages

- Glycated proteins and AGEs were detected mainly at $4,864$ and $4,320 \text{ cm}^{-1}$ in the fingernails' NIR spectra.
- NIR spectroscopy paired with cubic and quadratic SVM classification models produced the highest accuracy of all ML models.
- Accuracy of detecting CVDs and DM was greatly improved through the application of binary classification models in comparison to multiclass models.

- Balanced and inclusive datasets are crucial for improving the accuracy of ML models.

Chapter six: Attenuated total reflectance-Fourier transform infrared spectroscopy for the detection of cardiovascular diseases and diabetes mellitus in fingernails

The aim of this chapter was to determine the feasibility of ATR-FTIR spectroscopy for detecting CVDs and/or DM in fingernails. Fingernails' ATR-FTIR spectra were measured using two portable spectrometers that were: (1) the Agilent 4500a and (2) the Agilent Cary 630 ATR-FTIR spectrometers. 117 and 126 fingernail sets were measured using the 4500a and Cary 630 spectrometers, respectively. In both cases, fingernails were separated into one of five groups: healthy, unhealthy, CVD, diabetic and CVD-diabetic. The findings showed that ATR-FTIR spectroscopy successfully identified key endogenous constituents such as amide I and II, amino acids, cholesterol and keratin, which varied in absorbance intensities. Further compounds detected in ATR-FTIR spectra were glucose, high-density lipoproteins (HDL) and homocysteine. The application of MLAs, such as CWS, PCA and SOMs showed accurate clustering between healthy and diseased fingernails.

6.1 Introduction

ATR-FTIR spectroscopy is widely employed for the identification of biological matrices such as blood, saliva, semen, sweat and urine (Gozdzialski *et al.*, 2023; Mistek and Lednev, 2018; Wilson *et al.*, 2023b). Previous research has utilised ATR-FTIR spectroscopy for detecting diseases such as acute myeloid leukaemia (Delrue *et al.*, 2023), Alzheimer's disease (Hahn *et al.*, 2010), anaemia (Delrue *et al.*, 2023), breast cancer (Depciuch *et al.*, 2016), leukaemia, thalassemia, lymphoma and myelodysplastic syndrome (Delrue *et al.*, 2023).

For DM detection, ATR-FTIR spectroscopy was successfully applied to human fingernail clippings by Coopman *et al.*, (2017), who had identified glycation at 1047 cm^{-1} in diabetic fingernails. Qiu (2024) also applied ATR-FTIR spectroscopy to fingernails and was able to determine factors including biological sex and geographic origins. As described in chapter four of the thesis (section 4.3.3), sex and ethnicity play an imperative role in the incorporation of endogenous constituents and disease related biomarkers into fingernails. Furthermore, the presence of diseases such as CVDs and/or DM also impacts the spectroscopic activity of endogenous compounds/biomarkers in fingernails. For example, systemic diseases possess the ability to gradually modify the inherent material properties and structural integrity of a range of tissues including fingernails. Furthermore, extended periods of hyperglycaemia (seen in diabetic patients) have resulted in tissue chronically deteriorating. Thus, it is imperative that the spectroscopic technique of choice has the ability to detect differences in the spectra of fingernails. This work aimed to explore the feasibility of ATR-FTIR spectroscopy for detecting CVDs and/or DM from fingernails. The objectives of this work were:

- To determine the ATR-FTIR activity of healthy, CVD and diabetic fingernails.
- To interpret key ATR-FTIR bands of CVD and DM biomarkers in fingernails.
- To evaluate the accuracy of MLAs for classification of CVD and DM from fingernails.

6.2 Method and Materials

6.2.1. Materials

For this project, 126 sets of fingernails ($n = 1191$ clippings) were collected and analysed. In total, 92 (73%) sets of fingernails were complete, 15 (12%) had nine clippings, eight (6%) had eight clippings, six (5%) had seven clippings and five (4%) had six clippings. Both female ($n = 76$, 60%) and male ($n = 50$, 40%) participants were recruited and were between 18 – 84 years old (Appendix B1, Tables 1-5).

Each clipping then received a label from A - J based on their size. For example, clippings labelled as A were the largest fingernail clipping in length and width. This clipping was often taken from the participant's thumb. Fingernail clipping J was the smallest fingernail clipping and was taken from the participant's little

finger. Measurements including length, depth and width were taken using the Digital Vernier caliper (0 – 150 mm) (Vernier Calipers, Nord, Italy) (Figure 6.1) (Appendix B2).

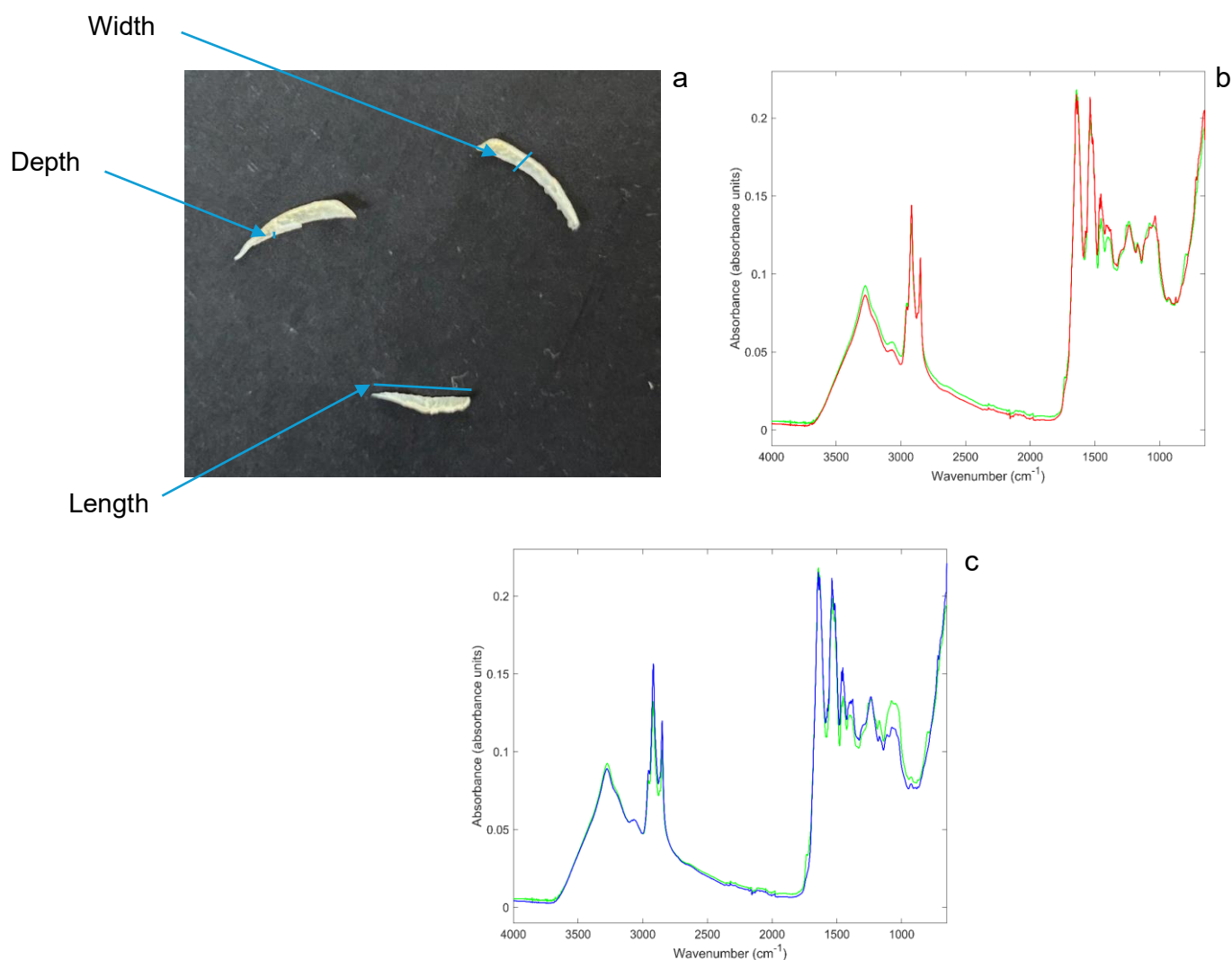


Figure 6.1. (a) Measurements taken from fingernail clippings including length, width and depth, (b) ATR-FTIR spectra of healthy (green) versus CVD (red) fingernails and (c) ATR-FTIR spectra of healthy (green) versus diabetic (blue) fingernails measured using the Agilent 4500a ATR-FTIR spectrometer equipped with an ATR diamond.

6.2.2. Instrumentation

ATR-FTIR spectra were collected using two ATR-FTIR spectrometers, those being: the Agilent 4500a and the Agilent Cary 630 ATR-FTIR spectrometers (Figure 6.2) (Agilent Technologies, Inc. Santa Clara, CA). Both spectrometers were equipped with

an ATR diamond holder. The spectral range varied in both systems such that the 4500a had a range of $4000 - 650 \text{ cm}^{-1}$ and the Cary 630 had a range of $4000 - 400 \text{ cm}^{-1}$. Each spectrum was the sum of 64 scans at a resolution of 4 cm^{-1} .

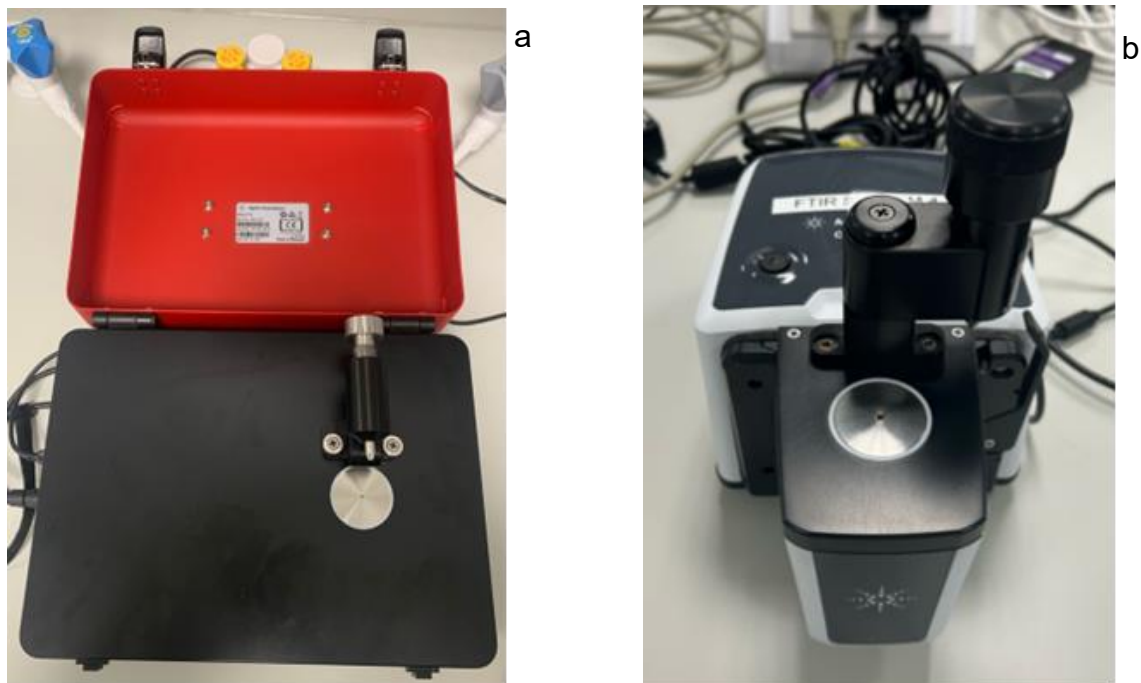


Figure 6.2. (a) The Agilent 4500a ATR-FTIR spectrometer equipped with an ATR diamond and diamond holder and (b) the Agilent 630 Cary ATR-FTIR spectrometer equipped with an ATR diamond and diamond holder.

6.2.3. Procedure

Fingernail clippings were removed from vials prior to analysis. Individual fingernail clippings were then placed directly onto the diamond holder. The contact between the fingernail clipping and diamond was made and maintained through the instruments' pressure clamps (Agilent Technologies, 2017). As highlighted in Figure 6.2, both of the ATR-FTIR instruments possessed a clamp sampling module, this module was rotated clockwise until resistance was felt and remained stationary throughout spectral collection.

A total of three spectra were taken from both sides of each fingernail clipping. Spectra were taken focussing on different sections of the fingernail clipping, as well as the dorsal and ventral layers. In cases where fingernail sets were incomplete (less than

ten clippings), additional spectra were collected. Between spectra, the fingernail was removed from the sampler and the diamond cleaned with soft lens tissues and isopropanol. A background was then taken for baseline corrections, which ensured the removal of background noise and accurate interpretation of spectral data. Polystyrene film was utilised as a reference spectrum for calibration and allowed the instruments to return to their original baseline. Across both instruments, spectra were exported as .csv files. Spectral interpretation was made through functional tables found within the literature as well as through reference spectra of standards.

6.2.4. Data Analysis

Pre-processing treatments were not applied to ATR-FTIR spectra, as they are inherently less affected by multiplicative scattering effects (Rinnan, 2010). As a result, scatter-correction techniques such as MSC and SNV, provided limited benefit for the interpretation of ATR-FTIR spectra and instead would have introduced noise and peak intensity distortion (Tsagkaris *et al.*, 2023). Spectral quality and interpretation assessments were made in MATLAB R2023a and Microsoft Excel 2019. Spectral quality parameters have previously been described in chapter four of the thesis (section 4.3.2). MLAs were applied to the ATR-FTIR spectra included CWS, PCA and SOMs.

6.3. Results and Discussion

ATR-FTIR spectroscopy was applied to a total of 126 fingernail sets. Spectral interpretation revealed key bands related to the fingernail's endogenous constituents and in several cases indicated the absence/presence of disease. The application of MLAs further demonstrated the detection capabilities of the ATR-FTIR spectroscopy for CVDs and/or DM in fingernails.

6.3.1. ATR-FTIR Activity of Fingernails

Spectral interpretation revealed bands featured at 3277, 3067, 1642, 1644-1642, 1582 – 1562, 1535, 1296 and 1249, 2960, 2920, 2851, 1452, 1738, 1398, 1115 and 1080

cm⁻¹. These bands were linked to proteins, lipids, amino acids and glucose, as well as amide groups (Table 6.1).

For proteins, the first band detected was at 3277 cm⁻¹ (symmetric N-H stretching) and was linked to amide A of keratin found within fingernails (Selvam and Gunasekaran, 2018). The band located at approximately 3067 cm⁻¹ corresponded to N-H stretching and was linked to amide A and B (Sharma *et al.*, 2020). This area has also been associated with the acyl stretching mode of CH₃ and lends itself to the presence of triglycerides (Selvam and Gunasekaran, 2018). At 1642 cm⁻¹ the presence of amide I was related to the fingernails' α -helix. This band has been attributed to C=O stretching coupled with in-plane bending of N-H and C-N stretching (Sundaram *et al.*, 2016). Gunasekaran *et al.*, (2010) made apparent the presence of Amide II and homocysteine between the region 1582 – 1562 cm⁻¹. The presence of this band lends itself to N-H in-plane bending vibration, strongly coupled to C-N stretching vibration of proteins and NH₂ scissoring (Gunasekaran *et al.*, 2010). Moreover, at 1535 cm⁻¹ (C-O stretching coupled with N-H bending deformation and C-H stretching) amide II was detected and was related to the proteins' backbones (Sundaram *et al.*, 2016). Finally, Sharma *et al.*, (2020) highlighted the presence of amide III at approximately 1296 and 1249 cm⁻¹. The aforementioned bands were attributed to N-H bending and N-H in plane bending paired with O=C-N bending and C-N stretching, respectively (Sharma *et al.*, 2020).

Regarding lipids, the first band identified was at 2960 cm⁻¹ and was attributed to C-H asymmetric stretching of CH₃ (Selvan *et al.*, 2018; Sundaram *et al.*, 2016). Similarly, lipids and proteins were detected at approximately 2920 cm⁻¹ (C-H symmetric stretching of CH₃), 2851 cm⁻¹ (C-H symmetric stretching of CH₂) and 1452 cm⁻¹ (CH₂ and CH₃ asymmetric bending modes) (Sharma *et al.*, 2020; Sundaram *et al.*, 2016). A band related to HDL was evident at 1738 cm⁻¹ and was attributed to C=O groups of cholesterol esters (Barton, 2004; Selvam and Gunasekaran, 2018).

Regarding amino acids, cysteine, cystine, tyrosine and tryptophan were detected at approximately 1398 cm⁻¹, which was associated with the symmetric mode of CH₃ (Bantignies *et al.*, 1998; Mitu *et al.*, 2023).

Glucose was detected at 1115 and 1080 cm⁻¹, which were attributed CO symmetric stretching (Selvam and Gunasekaran, 2018).

Table 6.1. Spectral interpretation of fingernails measured using the Agilent 4500a and Cary 630 ATR-FTIR spectrometers.

Band Position (cm ⁻¹)	Functional group	Association	Reference
3277	Symmetric N-H stretching	Amide A of protein	Selvam and Gunasekaran, 2018.
3067	N-H stretching	Amide A and B	Sharma <i>et al.</i> , 2020.
2960	C-H asymmetric stretching of CH ₃	Lipids and total cholesterol	Sharma <i>et al.</i> , 2020; Sundaram <i>et al.</i> , 2016.
2920	C-H symmetric stretching of CH ₃	Lipids and proteins	Sundaram <i>et al.</i> , 2016.
2851	C-H symmetric stretching in CH ₂	Lipids and proteins	Sundaram <i>et al.</i> , 2016.
1738	C=O groups of cholesterol esters	High-density lipoproteins	Barton, 2004; Selvam and Gunasekaran, 2018.
1644-1642	C=O stretching coupled with in-plane bending of N-H and C-N stretching	Amide I (α -helix)	Farhan, Sastry and Mandal, 2010; Fernández-Higuero <i>et al.</i> , 2014; Sundaram <i>et al.</i> , 2016.
1633	C=O stretching	Amide I	Sundaram <i>et al.</i> , 2016.
1582-1562	N-H in plane bending vibration strongly coupled to C-N stretching vibration protein, NH ₂ scissoring	Amide II, homocysteine	Gunasekaran <i>et al.</i> , 2010.
1535	C=O stretching coupled with C-N stretching and bending deformation of N-H	Amide II	Sundaram <i>et al.</i> , 2016.
1452	CH ₂ and CH ₃ asymmetric bending modes	Lipids and proteins	Sharma <i>et al.</i> , 2020; Sundaram <i>et al.</i> , 2016.
1447	Methyl symmetric deformation	Amino acids, triglycerides	Rakesh, Charmi and Rajesh, 2014.
1398	Symmetric mode of CH ₃	Amino acids	Bantignies <i>et al.</i> , 1998; Mitu <i>et al.</i> , 2023.
1296	N-H bending	Amide III	Sharma <i>et al.</i> , 2020.
1249	N-H in plane bending paired with O=C-N bending and C-N stretching	Amide III	Sharma <i>et al.</i> , 2020.
1115	CO symmetric stretching	Glucose	Selvam and Gunasekaran, 2018.
1080	CO symmetric stretching	Glucose	Selvam and Gunasekaran, 2018.

Variation was also observed from fingernails taken from the same finger but different hands. This was the case for fingernails A and B, which were both sampled from the thumb finger of each hand. At several ATR-FTIR bands, fingernail A demonstrated higher ATR-FTIR activity in comparison to fingernail B. For example, at band 3277 cm⁻¹, fingernail A showed an ATR-FTIR intensity of 0.1102 absorbance units, while fingernail B displayed an ATR-FTIR intensity of 0.0983 absorbance units. Therefore, suggesting that the endogenous constituent content was lower in fingernail B than A. The variation between two fingernails sampled from fingers sharing the same

proportions (length, width and depth of the finger) can be attributed to the handedness of the participant. Research by Griffiths and Reshad (1983) first suggested that left- and right-handedness played a role in the growth rate of each hand. This work identified that right-handed individuals displayed faster growth rates for fingernails on their right hand in comparison to their left hand. The same was observed for left-handed subjects, who demonstrated faster growth rates on their dominant left hand. Griffiths and Reshad (1983) believed that repeated minor trauma (e.g. scratching) to the fingernail plates found on their dominant hand, was the cause of increased growth rates. Trauma therefore acted as a proliferative stimulus and increased the overall rate of growth. However, the difference between growth rates of the right and left hand was not statistically significant (Griffiths and Reshad, 1983). This was also the case for Yaemsiri *et al.*, (2010), who did not detect a significant difference in the growth rates of right and left hands in relation to dominant hand use. Nonetheless, work by Jung *et al.*, (2015) implied that handedness played a role in fingernail curvature, with fingernails on the dominant hand demonstrating a larger radius of transverse fingernail curvature than those on the non-dominant hand.

6.3.1.1 Agilent 4500a ATR-FTIR Spectrometer

ATR-FTIR spectra showed differences in intensities and bands between 1500 and 650 cm^{-1} (Figure 6.3).

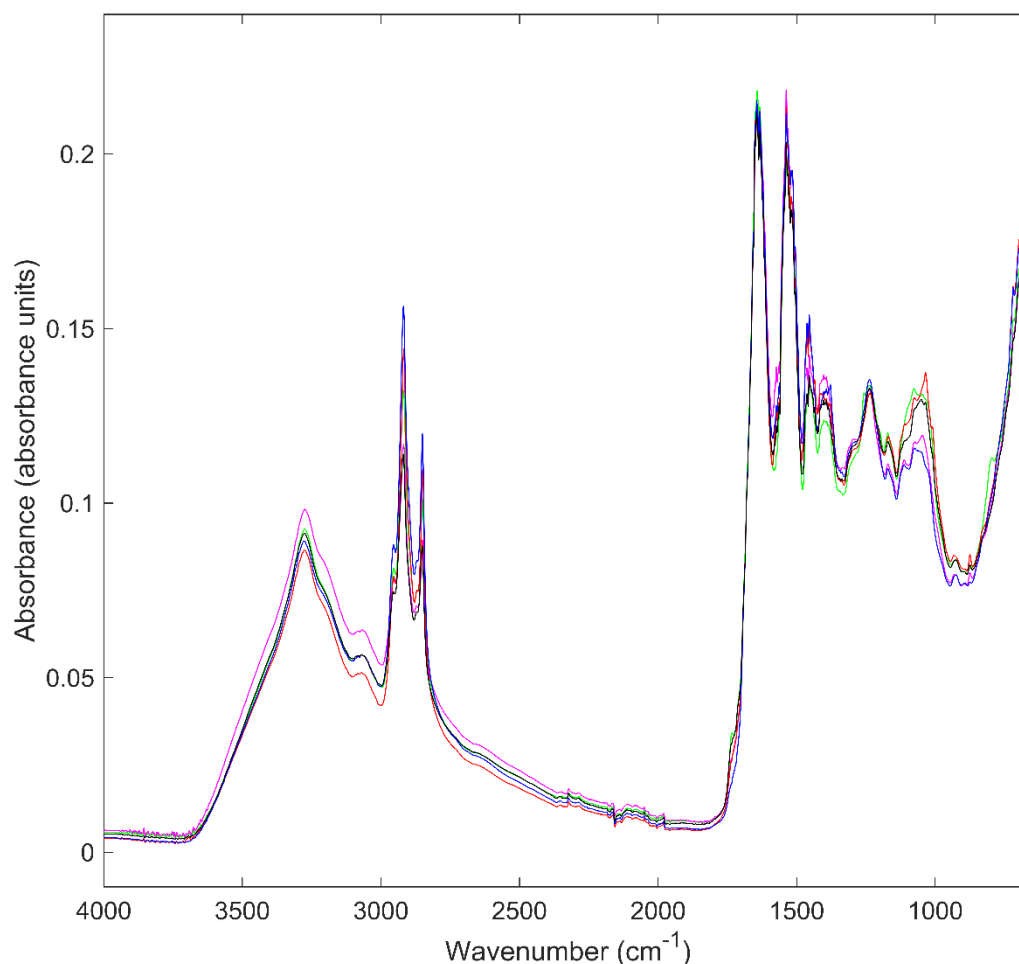


Figure 6.3. Average ATR-FTIR spectra of healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernails measured using the Agilent 4500a ATR-FTIR spectrometer equipped with an ATR diamond and holder.

Five regions of interest were examined to identify the variation between absorbance levels of the five groups. The first area of interest (3570 – 2999 cm^{-1}) was associated with amide groups (Selvam and Gunasekaran, 2018; Sharma *et al.*, 2020). Across this region the unhealthy participant showed the highest absorbance levels followed by healthy > CVD-diabetic > diabetic > CVD.

The second area of interest ($3000 - 2828 \text{ cm}^{-1}$) was attributed primarily to lipids and total cholesterol (Sharma *et al.*, 2020; Sundaram *et al.*, 2016). Within this region, diabetic fingernails demonstrated the highest absorbance and ATR-FTIR activity, followed by CVD > healthy > unhealthy > CVD-diabetic participants. The increased ATR-FTIR activity of lipids and cholesterol found in diabetic clipping was not uncommon (Vergès, 2020). A similar trend was observed in the region of $1482 - 1426 \text{ cm}^{-1}$, which was attributed to lipids, amino acids and triglycerides. Within this region, diabetic fingernails showed the highest ATR-FTIR activity followed by CVD > unhealthy > CVD-diabetic > healthy. Increased ATR-FTIR activity of amino acids was also observed in unhealthy > diabetic > CVD > CVD-diabetic > healthy fingernails within the region $1424 - 1362 \text{ cm}^{-1}$.

A third region of interest was selected as $1674 - 1588 \text{ cm}^{-1}$ and was associated with amide I, amide II and the fingernails' protein structure (Farhan, Sastry and Mandal, 2010; Fernández-Higuero *et al.*, 2014; Gunasekaran *et al.*, 2010; Sundaram *et al.*, 2016). Healthy clippings showed the highest ATR-FTIR activity within this area followed by DM > CVD > unhealthy > CVD-diabetic. This finding was supported by Sihota *et al.*, (2019), who investigated the amides I and II content of healthy controls, controlled and uncontrolled diabetic cases. This work showed that amides I and II content decreased in both controlled and uncontrolled diabetic cases in comparison to healthy controls. Therefore, indicating that the presence of DM resulted in a decreased activity of proteins and disruption to the fingernail's keratin structure. Within the fourth region ($1547 - 1521 \text{ cm}^{-1}$), amide II was also detected, with unhealthy participants showing the highest ATR-FTIR activity.

Following spectral interpretation, MLAs were applied and included: CWS, PCA and SOMs. The first MLA utilised was CWS, which indicated correlations between different fingernails. CWS was applied to 150 spectra (30 spectra per participant) of the following sets: MWS1, MWS46, MWS51, MWS57 and MWS98. Figure 6.4 demonstrated that all fingernail sets had an r value of +1 against themselves and indicated a positive match. Moreover, 12 spectra of fingernail set MWS1 had r values of +1, indicating matching spectra and sharing the same health status. This was further supported by the limited number ($n = 6$) of mismatches (false negatives) seen between healthy spectra against spectra of the same health status. However, false positives

were encountered between different categories e.g. DM and healthy and that introduced a limitation in applying CWS.

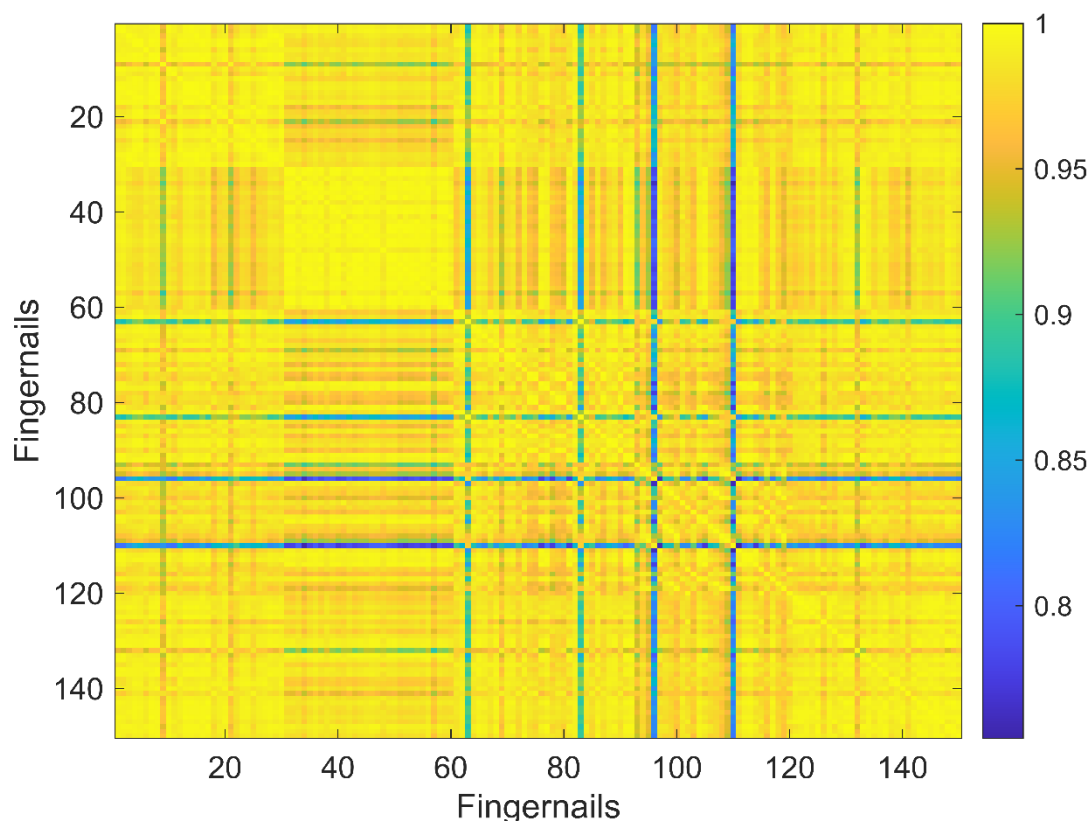
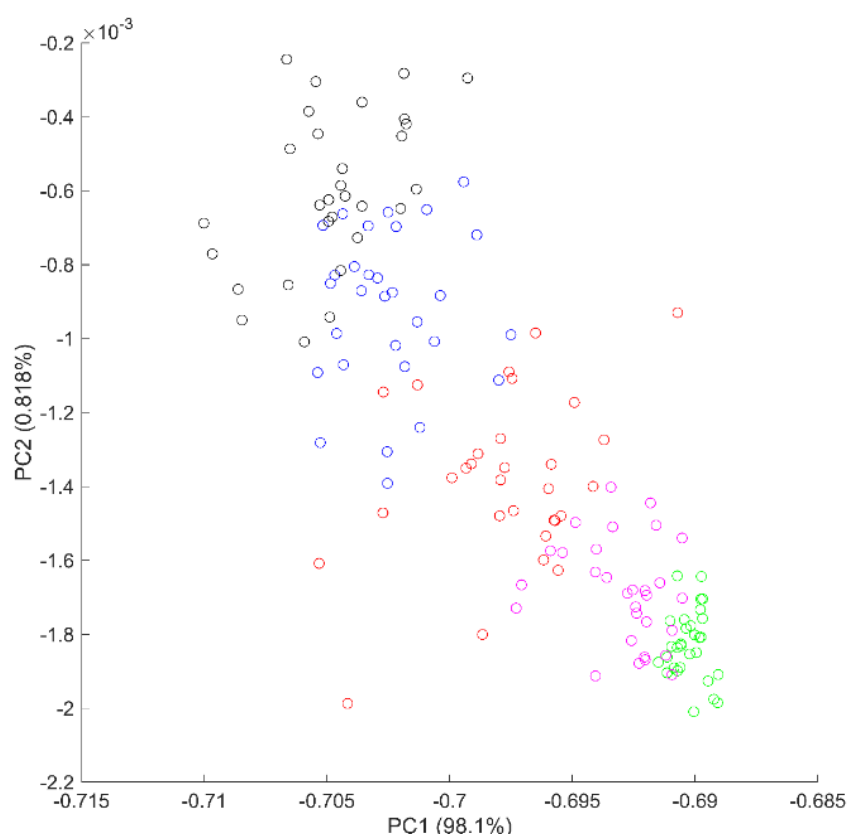


Figure 6.4. CWS of ATR-FTIR spectra taken from healthy (1-30), unhealthy (31-60), CVD (61-90), diabetic (91-120) and CVD-diabetic (121-150) participants measured using the Agilent 4500a ATR-FTIR spectrometer, equipped with an ATR diamond and holder. A dark blue colour represents a minimum r value of 0.75 and a bright yellow colour represents a maximum r value of 0.9999. In between, the r values 0.80 – 0.85 correspond to a blue colour, r value 0.85 – 0.90 correspond to a light blue colour, r value 0.90 – 0.95 correspond a green/yellow colour and r value 0.95 – 1.00 correspond to an orange/yellow colour.

PCA was then applied to the spectra of fingernail sets taken from participants MWS1 (healthy), MWS46 (CVD), MWS51 (DM), MWS57 (CVD-diabetic) and MWS98 (unhealthy). Figure 6.5 demonstrated the PC1 and PC2 score plots of fingernails' spectra that contributed to a total variance of 98.9%. Distinct clusters were observed in the scores plot of the five evaluated groups with the exception of DM and CVD/DM

that had one disease in common. Thus, diabetics fingernails' spectra influenced the model over other spectra. The remaining three categories were clustered accurately with healthy participants contributing to the highest variance in the model. This demonstrated the advantage of PCA over CWS in detecting CVDs and DM from fingernails.

Figure 6.5. PCA of ATR-FTIR spectra from healthy (green), unhealthy (magenta), CVD



(red), diabetic (blue) and CVD-diabetic (black) participants measured using the Agilent 4500a ATR-FTIR spectrometer, equipped with an ATR diamond and holder.

SOM neighbour weight distances (Figure 6.6) and SOM sample hits (Figure 6.7) supported the PCA findings. The SOM neighbour weight distances showed clusters of different groups in agreement with PCA.

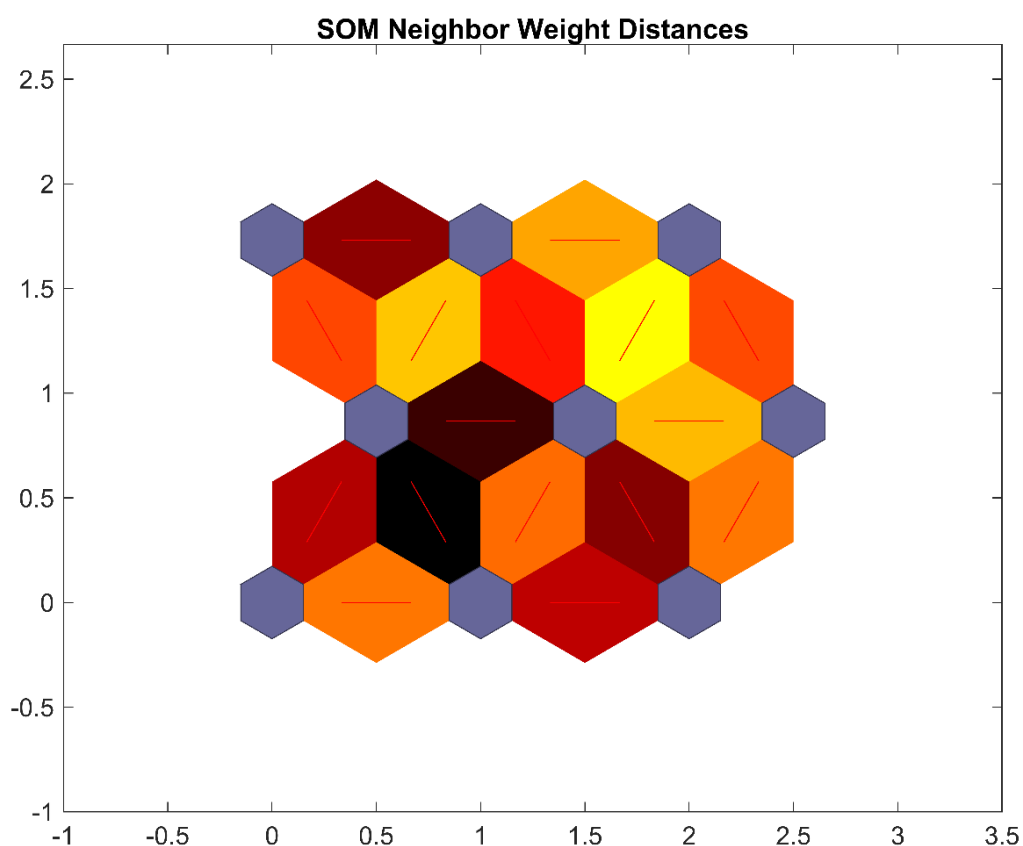


Figure 6.6. SOM neighbour weight distances of ATR-FTIR spectra taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent 4500a ATR-FTIR spectrometer, equipped with an ATR diamond and holder. Black and dark red represent small distances and closely related variables. Orange and light red correlate to medium distances and moderately related variables. Light orange and yellow are representative of larger distances and loosely related variables.

SOM sample hits were then explored to further highlight the grouping of fingernails (Figure 6.7). A total of nine groups were identified and consisted of 16, 24, 12, 24, 16, 32, 8, 4 and 14 elements.

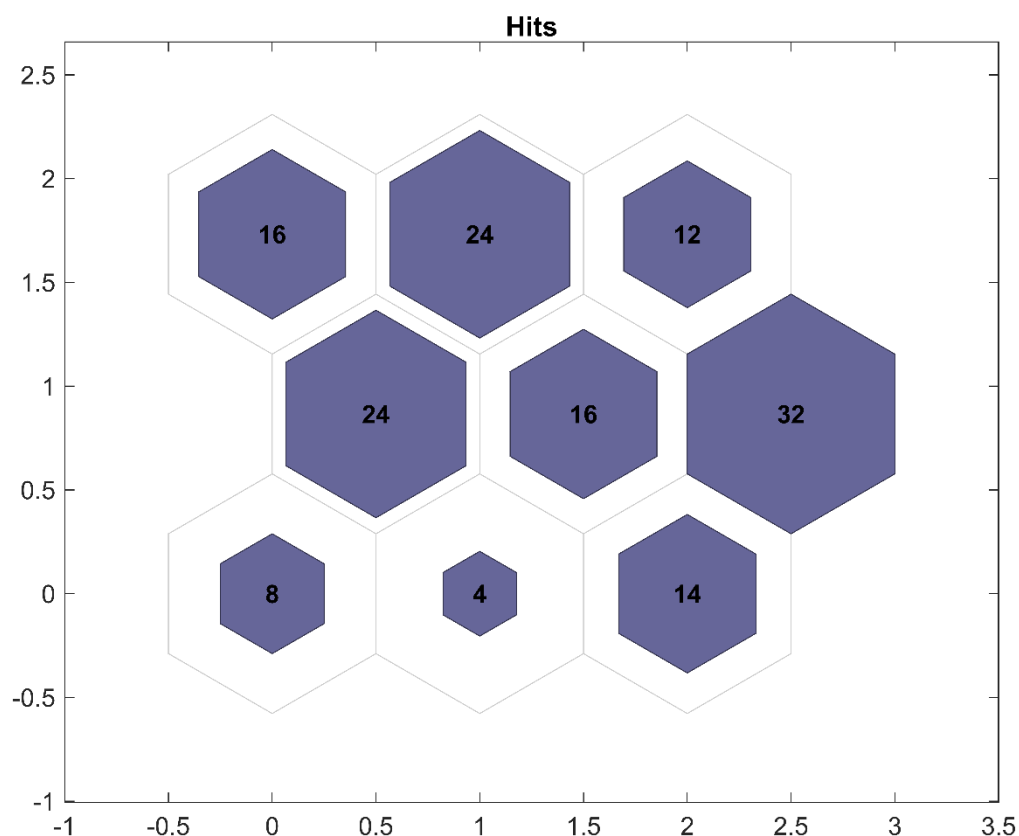


Figure 6.7. SOM sample hits of ATR-FTIR spectra taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent 4500a ATR-FTIR spectrometer, equipped with an ATR diamond and holder.

6.3.1.2. Agilent Cary 630 ATR-FTIR Spectrometer

The same spectral analysis approach was adapted for data obtained from the Cary 630 ATR-FTIR spectrometer for the five sets of fingernails including: MWS1, MWS46,

MWS51, MWS57 and MWS57. No difference was seen in terms of band positions or absorption of different fingernails (Figure 6.8).

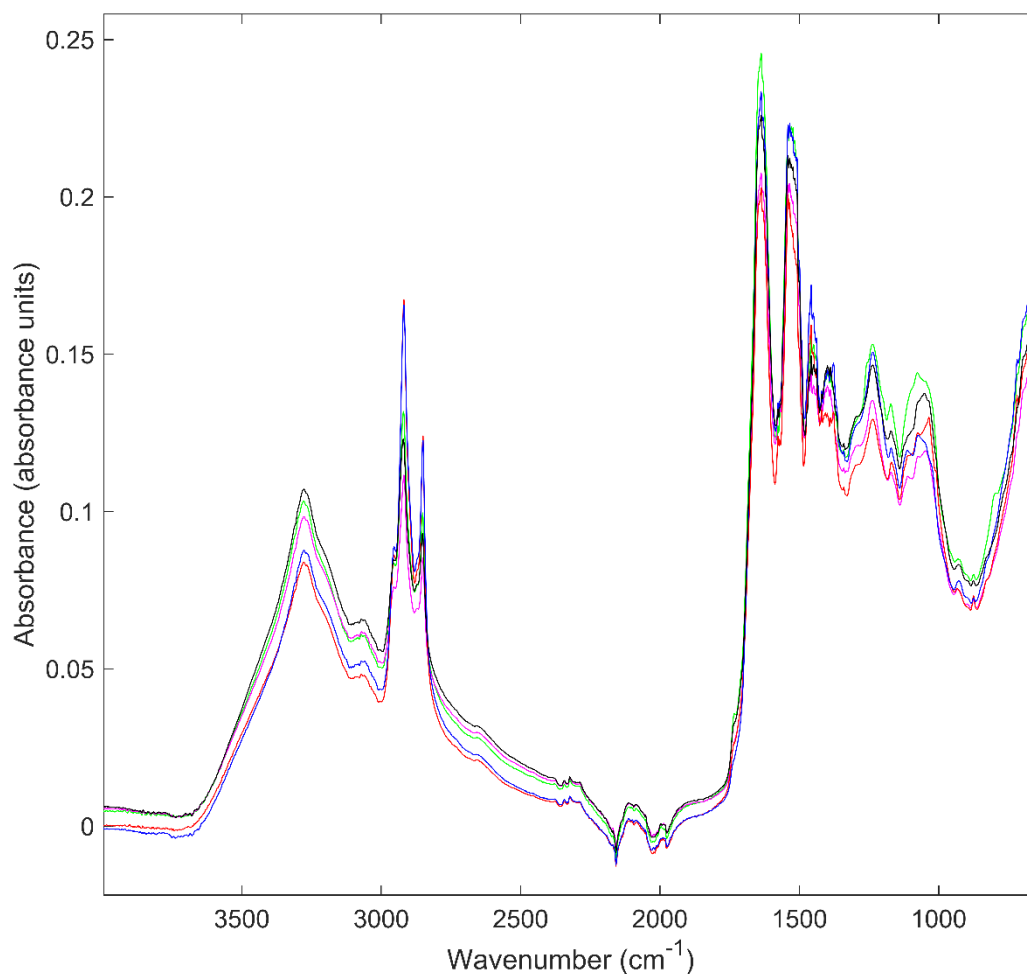


Figure 6.8. ATR-FTIR spectra of healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernails measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with an ATR diamond and holder.

CWS applied to the fingernails' spectra showed several false positives between different groups (Figure 6.9). Thus, CWS applied to Cary 630 data showed less accurate classification than the application to the 4500a data. Similarly, the PCA clusters were less accurate when constructed using spectra obtained from the Cary 630. This was visible in Figure 6.10 that showed overlap in the PC scores of CVD and

DM fingernails, as well as the crossover between scores of DM and CVD-DM. On the other hand, the remaining groups in this latter model were accurately classified.

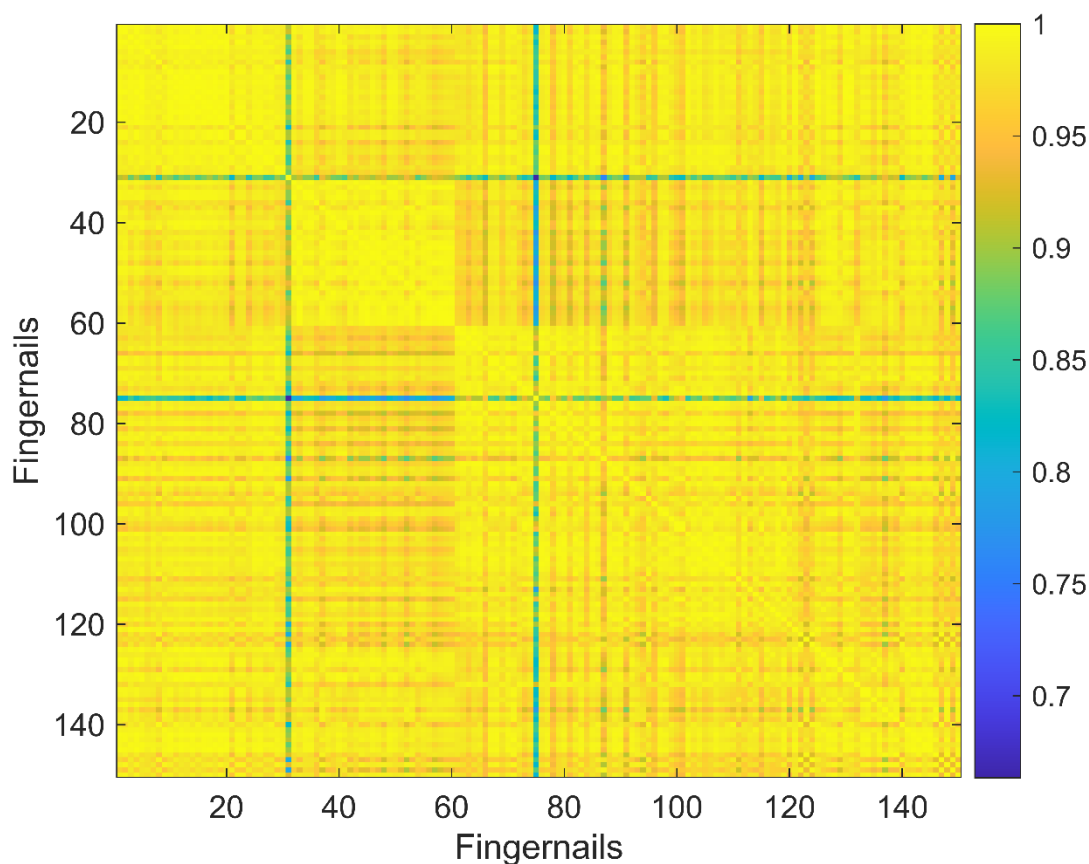


Figure 6.9. CWS of ATR-FTIR spectra taken from healthy (1-30), unhealthy (31-60), CVD (61-90), diabetic (91-120) and CVD-diabetic (121-150) participants measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with an ATR diamond and holder. A dark blue colour represents a minimum r value of 0.7 and a dark yellow colour represents a maximum r value of 0.9999. In between the r value of 0.7 – 0.75 correspond to a blue colour, r value of 0.75 – 0.80 correspond to a light blue colour, r value of 0.80 – 0.85 correspond to a teal colour, r value of 0.85 – 0.90 correspond to a green colour and r value of 0.90 – 0.95 correspond to an orange colour.

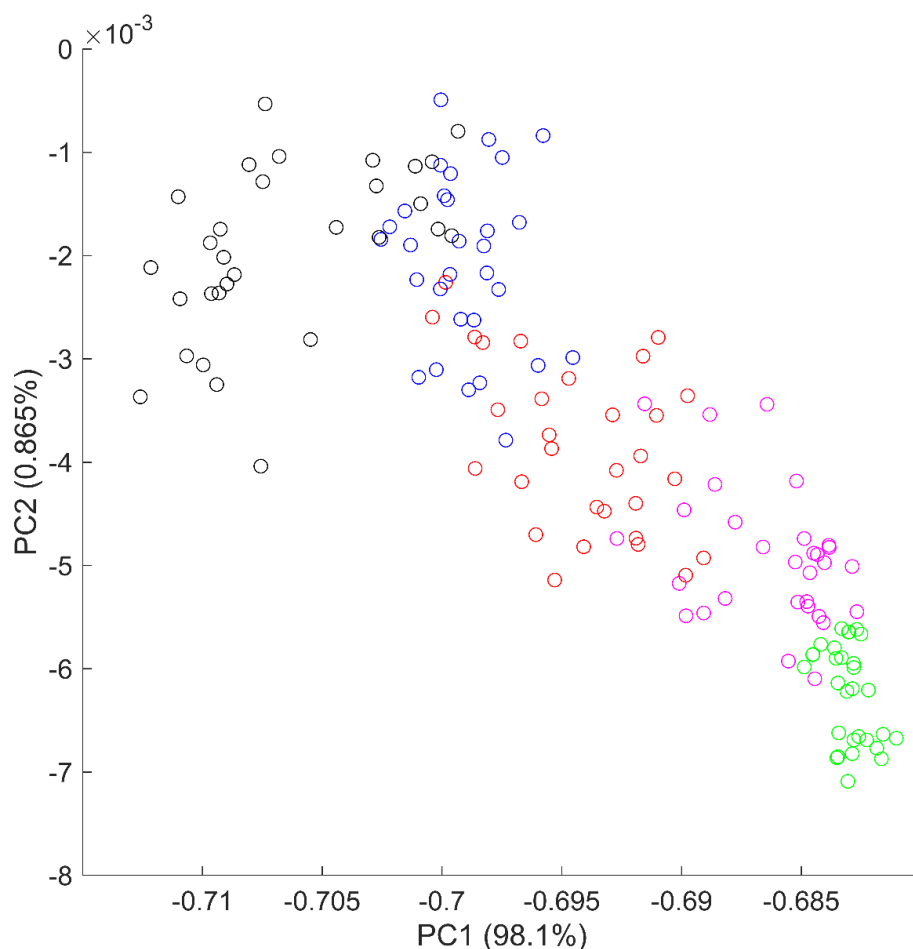


Figure 6.10. PCA of ATR-FTIR spectra taken from healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) participants measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with an ATR diamond and holder.

Likewise, SOMs applied to the Cary 630 spectra showed crossover between the five sets of fingernails in the neighbouring distance plot (Figure 6.11). The sample hits showed similar groups ($n = 9$) to the first instrument such that these groups had 20, 24, 20, 17, 18, 19, 13, 8 and 11 elements (Figure 6.12).

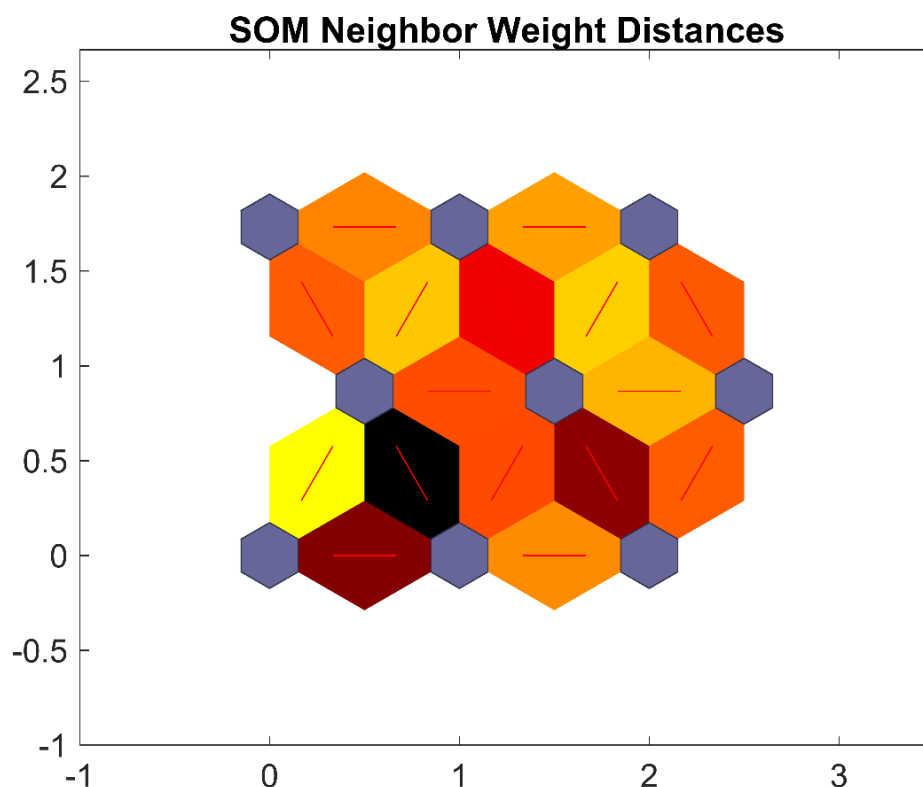


Figure 6.11. SOM neighbour weight distances of ATR-FTIR spectra taken from healthy, unhealthy, CVD, diabetic and CVD-diabetic participants measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with an ATR diamond and holder. Black and dark red represent small distances and closely related variables. Orange and light red correlate to medium distances and moderately related variables. Light orange and yellow are representative of larger distances and loosely related variables.

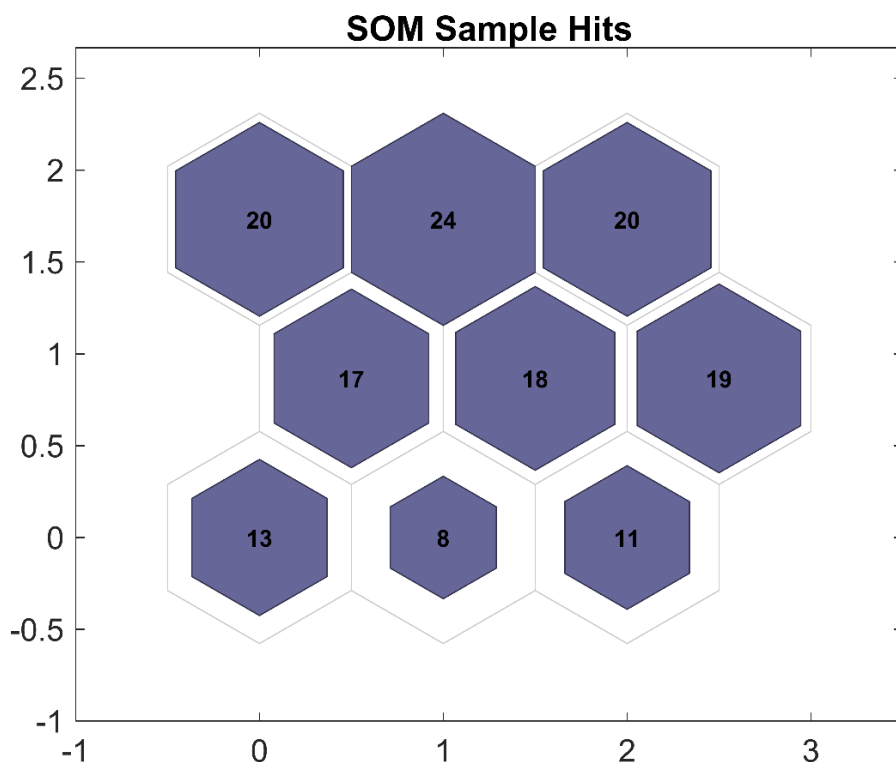


Figure 6.12. SOM sample hits of ATR-FTIR spectra taken from healthy, unhealthy, CVD, diabetic, CVD-diabetic participants measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with an ATR diamond and holder.

6.3.2. Spectral Quality of Fingernails

Spectral quality assessment applied to the 4500a spectra showed variable FTIR activity between different groups when number of bands, range, maximum intensity/position and S/N were applied (Table 6.2). The number of bands for fingernail sets (MWS1 – MWS117) ranged from 14 – 33 bands. The maximum band intensity of tested fingernails ranged from 0.135 – 0.342 absorbance units. The S/N ratio varied between fingernails and ranged from 21.6 – 68.7. Overall, the Agilent 4500a ATR-FTIR spectrometer produced spectra of medium (n = 6, 5.1%) and strong (n = 111, 94.9%) ATR-FTIR activity. Thus, produced high-quality spectra of fingernails regardless of their health or disease status. Moreover, spectral quality assessments demonstrated the feasibility of this instrument as a disease detection tool, such as its ability to detect bands attributed to disease-related compounds (glucose, HDL and

homocysteine), as well as differences in their ATR-FTIR activity between fingernails of varying classes (Table 6.3). Therefore, the Agilent 4500a ATR-FTIR spectrometer can be applied to future disease detection cases.

Table 6.2. Spectral quality of healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails measured using the Agilent 4500a ATR-FTIR Spectrometer equipped with an ATR diamond and holder.

Set	Number of bands	Maximum band Intensity (absorbance units)	Maximum band position (cm ⁻¹)	S/N	ATR-FTIR activity
MWS1	15	0.218	1644.34	30.0	M
MWS2	16	0.218	1642.47	30.0	M
MWS3	14	0.222	1644.34	29.5	M
MWS4	17	0.193	1642.47	22.6	M
MWS5	17	0.248	1644.34	31.4	M
MWS6	16	0.248	1644.34	33.0	M
MWS7	17	0.248	1644.34	31.4	S
MWS8	21	0.244	1644.34	36.3	S
MWS9	21	0.253	1644.34	49.2	S
MWS10	17	0.2026	1642.47	25.5	M
MWS11	22	0.246	1644.34	36.3	S
MWS12	18	0.2091	1644.34	36.1	S
MWS13	20	0.2071	1538.07	41.5	S
MWS14	20	0.261	1644.34	66.7	S
MWS15	22	0.2059	1644.34	67.8	S
MWS16	19	0.253	1644.34	31.4	S
MWS17	19	0.232	1644.34	32.1	S
MWS18	19	0.256	1644.34	33.4	S
MWS19	18	0.226	1642.47	31.6	S
MWS20	18	0.243	1644.34	35.3	S
MWS21	20	0.259	1644.34	49.0	S
MWS22	17	0.264	1644.34	38.2	S
MWS23	20	0.2073	1642.47	34.2	S
MWS24	24	0.135	1644.34	28.5	S
MWS25	23	0.2071	1644.34	29.5	S
MWS26	19	0.223	1644.34	29.0	M
MWS27	21	0.219	1644.34	31.9	S
MWS28	18	0.213	1644.34	38.7	S
MWS29	21	0.251	1644.34	52.7	S
MWS30	21	0.2304	1644.34	50.83	S
MWS31	20	0.224	1644.34	32.1	S
MWS32	21	0.224	1644.34	35.9	S
MWS33	17	0.225	1644.34	29.3	M

MWS34	22	0.249	1538.07	51.0	S
MWS35	21	0.2303	1644.34	67.1	S
MWS36	26	0.243	1644.34	68.7	S
MWS37	23	0.233	1644.34	32.6	S
MWS38	21	0.229	1644.34	31.1	S
MWS39	23	0.248	1644.34	38.8	S
MWS40	24	0.164	1644.34	26.3	S
MWS41	20	0.278	1644.34	36.3	S
MWS42	17	0.272	1644.34	45.0	S
MWS43	20	0.245	1644.34	32.6	S
MWS44	21	0.164	1644.34	28.9	S
MWS45	24	0.244	1536.21	30.53	S
MWS46	22	0.215	1644.34	30.0	S
MWS47	23	0.217	1633.15	26.8	S
MWS48	24	0.218	1644.34	26.3	S
MWS49	19	0.217	1644.34	31.7	S
MWS50	20	0.243	1644.34	32.5	S
MWS51	20	0.215	1644.34	30.0	S
MWS52	23	0.262	1538.07	30.53	S
MWS53	23	0.2081	1644.34	26.0	S
MWS54	24	0.219	1644.34	30.63	S
MWS55	25	0.191	1644.34	29.5	S
MWS56	24	0.20704	1644.34	25.3	S
MWS57	23	0.212	1644.34	28.9	S
MWS58	22	0.223	1642.47	26.8	S
MWS59	23	0.20501	1538.07	25.0	S
MWS60	26	0.235	1538.07	30.79	S
MWS61	22	0.246	1644.34	34.5	S
MWS62	23	0.246	1644.34	33.9	S
MWS63	21	0.246	1644.34	34.5	S
MWS64	23	0.218	1538.07	28.0	S
MWS65	24	0.216	1644.34	29.8	S
MWS66	22	0.216	1644.34	32.8	S
MWS67	25	0.231	1644.34	39.8	S
MWS68	26	0.195	1644.34	30.0	S
MWS69	24	0.2404	1644.34	33.0	S
MWS70	24	0.218	1644.34	30.53	S
MWS71	30	0.227	1644.34	31.1	S
MWS72	25	0.259	1644.34	29.3	S
MWS73	27	0.236	1644.34	34.7	S
MWS74	29	0.216	1642.47	30.37	S
MWS75	28	0.2085	1633.15	25.8	S
MWS76	28	0.2048	1644.36	21.6	S
MWS77	28	0.3104	1644.34	34.4	S
MWS78	29	0.2013	1644.34	25.7	S
MWS79	27	0.261	1644.34	32.4	S
MWS80	31	0.233	1644.34	33.9	S

MWS81	22	0.221	1642.47	28.9	S
MWS82	31	0.279	1644.34	31.3	S
MWS83	25	0.228	1644.34	27.5	S
MWS84	25	0.213	1644.34	31.8	S
MWS85	23	0.221	1644.34	30.5	S
MWS86	31	0.227	1644.34	36.9	S
MWS87	28	0.222	1644.34	31.6	S
MWS88	28	0.216	1644.34	27.9	S
MWS89	29	0.2093	1642.47	24.5	S
MWS90	28	0.2072	1642.47	26.3	S
MWS91	24	0.223	1644.34	25.3	S
MWS92	27	0.195	1644.34	30.50	S
MWS93	25	0.20063	1644.34	31.9	S
MWS94	27	0.2093	1644.34	24.9	S
MWS95	28	0.245	1644.34	30.45	S
MWS96	26	0.229	1642.47	30.0	S
MWS97	24	0.218	1538.07	29.4	S
MWS98	23	0.243	1644.34	31.0	S
MWS99	28	0.193	1644.34	27.5	S
MWS100	27	0.2032	1644.34	28.0	S
MWS101	26	0.197	1644.34	21.6	S
MWS102	23	0.2201	1642.47	29.0	S
MWS103	26	0.236	1644.34	30.59	S
MWS104	25	0.281	1644.34	34.1	S
MWS105	25	0.278	1644.34	37.2	S
MWS106	28	0.246	1644.34	44.6	S
MWS107	23	0.342	1644.34	51.5	S
MWS108	22	0.3053	1642.47	49.2	S
MWS109	33	0.255	1644.34	31.8	S
MWS110	29	0.255	1644.34	37.5	S
MWS111	26	0.258	1644.34	36.3	S
MWS112	28	0.282	1644.34	45.0	S
MWS113	24	0.272	1642.47	37.1	S
MWS114	24	0.272	1644.34	40.63	S
MWS115	24	0.264	1644.34	38.8	S
MWS116	27	0.278	1644.34	47.7	S
MWS117	26	0.259	1644.34	30.56	S
Median	23	0.227		31.4	
IQR1-IQR3	21-26	0.215-0.248		29.3-36.3	

IQR: Interquartile range; M: Medium; S: Strong; S/N: Signal-to-noise ratio.

Table 6.3. Frequency of ATR-FTIR bands present in healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernails measured using the Agilent 4500a ATR-FTIR spectrometer, equipped with ATR diamond and holder.

	Band position (cm ⁻¹)																		
Participant	3277	3067	2960	2920	2851	1738	1644	1642	1633	1582	1562	1535	1452	1447	1398	1296	1240	1115	1080
MWS1																			
MWS2																			
MWS3																			
MWS4																			
MWS5																			
MWS6																			
MWS7																			
MWS8																			
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MWS116																			
MWS117																			

Spectral quality assessment applied to the Agilent Cary 630 ATR-FTIR spectrometer showed similar performance to the 4500a spectra (Table 6.4). The number of ATR-FTIR bands per fingernail set ranged from 21 – 33. Fingernail sets MWS1 – MWS126 presented maximum band intensities of 0.116 – 0.357 absorbance units. The S/N ratio of average spectra was calculated and varied between 25.4 – 51.7.

While the overall average of ATR-FTIR activity was higher for spectra measured using the Agilent Cary 630 ATR-FTIR spectrometer versus the Agilent 4500a ATR-FTIR

spectrometer, both instruments produced a high number of bands, maximum band intensities/positions and S/N ratios. Therefore, are both feasible disease detection tools for CVDs and/or DM (Table 6.5).

Table 6.4. Spectral quality of healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with ATR diamond and holder.

Set	Number of bands	Maximum band intensity (absorbance units)	Maximum band position (cm ⁻¹)	S/N	ATR-FTIR activity
MWS1	24	0.246	1636.30	40.71	S
MWS2	23	0.2034	1636.30	37.7	S
MWS3	25	0.212	1638.16	31.4	S
MWS4	23	0.138	1636.30	28.3	S
MWS5	21	0.157	1636.30	33.1	S
MWS6	26	0.163	1636.30	41.7	S
MWS7	28	0.157	1636.30	40.0	S
MWS8	29	0.168	1638.16	47.0	S
MWS9	28	0.2019	1638.16	36.7	S
MWS10	23	0.2096	1638.16	32.9	S
MWS11	29	0.211	1636.30	30.0	S
MWS12	27	0.215	1636.30	32.1	S
MWS13	29	0.218	1638.16	32.1	S
MWS14	26	0.269	1636.30	43.2	S
MWS15	26	0.186	1636.30	37.7	S
MWS16	30	0.199	1638.16	38.5	S
MWS17	26	0.199	1636.30	33.3	S
MWS18	26	0.192	1638.16	36.9	S
MWS19	28	0.2085	1638.16	37.5	S
MWS20	30	0.226	1636.30	38.2	S
MWS21	26	0.2078	1636.30	36.7	S
MWS22	23	0.219	1636.30	36.3	S
MWS23	27	0.2012	1636.30	46.2	S
MWS24	30	0.116	1636.30	25.4	S
MWS25	25	0.153	1638.16	42.3	S
MWS26	23	0.181	1638.16	30.56	S
MWS27	27	0.224	1636.30	30.0	S
MWS28	24	0.178	1638.16	35.00	S
MWS29	22	0.196	1638.16	30.63	S
MWS30	29	0.219	1636.30	34.3	S

MWS31	25	0.211	1638.16	30.0	S
MWS32	26	0.235	1638.16	34.0	S
MWS33	25	0.189	1638.16	34.1	S
MWS34	31	0.182	1638.16	31.4	S
MWS35	25	0.2019	1636.30	32.3	S
MWS36	33	0.2056	1636.30	32.7	S
MWS37	29	0.256	1638.16	36.9	S
MWS38	28	0.211	1636.30	32.0	S
MWS39	27	0.213	1638.16	31.4	S
MWS40	31	0.181	1638.16	29.4	S
MWS41	26	0.227	1638.16	40.0	S
MWS42	26	0.2058	1638.16	31.4	S
MWS43	25	0.199	1636.30	37.2	S
MWS44	26	0.169	1638.16	30.33	S
MWS45	29	0.227	1535.66	28.6	S
MWS46	28	0.2029	1636.30	28.7	S
MWS47	26	0.181	1636.30	30.0	S
MWS48	26	0.217	1638.16	29.2	S
MWS49	26	0.2081	1636.30	31.7	S
MWS50	25	0.2054	1638.16	34.2	S
MWS51	23	0.233	1638.16	34.1	S
MWS52	29	0.2026	1636.30	31.5	S
MWS53	27	0.223	1638.16	32.1	S
MWS54	26	0.2092	1638.16	32.5	S
MWS55	30	0.2048	1636.30	31.5	S
MWS56	28	0.173	1636.30	35.2	S
MWS57	22	0.226	1638.16	34.6	S
MWS58	28	0.2029	1638.16	30.0	S
MWS59	29	0.177	1636.30	30.0	S
MWS60	29	0.217	1647.48	30.0	S
MWS61	28	0.232	1638.16	37.5	S
MWS62	26	0.194	1638.16	36.0	S
MWS63	24	0.20024	1636.30	30.78	S
MWS64	30	0.2075	1638.16	29.9	S
MWS65	28	0.226	1638.16	37.8	S
MWS66	25	0.212	1638.16	34.6	S
MWS67	29	0.234	1638.16	42.2	S
MWS68	30	0.191	1638.16	36.9	S
MWS69	28	0.218	1638.16	34.6	S
MWS70	29	0.211	1638.16	35.1	S
MWS71	25	0.2105	1636.30	27.4	S
MWS72	29	0.243	1638.16	43.8	S
MWS73	26	0.2032	1636.30	30.0	S

MWS74	31	0.212	1638.16	31.5	S
MWS75	24	0.175	1638.16	36.4	S
MWS76	27	0.182	1638.30	30.69	S
MWS77	29	0.249	1636.30	44.5	S
MWS78	26	0.175	1636.30	37.7	S
MWS79	29	0.221	1638.16	32.9	S
MWS80	27	0.211	1638.16	35.8	S
MWS81	28	0.2049	1638.16	32.2	S
MWS82	27	0.20028	1636.30	32.5	S
MWS83	27	0.223	1638.16	40.0	S
MWS84	26	0.234	1638.16	36.9	S
MWS85	32	0.215	1636.30	35.4	S
MWS86	27	0.228	1638.16	38.4	S
MWS87	27	0.212	1636.30	34.9	S
MWS88	31	0.193	1636.30	33.9	S
MWS89	29	0.2036	1636.30	34.2	S
MWS90	26	0.192	1638.16	36.1	S
MWS91	29	0.219	1636.30	34.6	S
MWS92	29	0.176	1638.16	39.7	S
MWS93	30	0.212	1638.16	34.2	S
MWS94	26	0.199	1636.30	36.2	S
MWS95	30	0.226	1638.16	37.7	S
MWS96	27	0.186	1636.30	35.4	S
MWS97	25	0.219	1638.16	35.8	S
MWS98	24	0.187	1638.16	30.0	S
MWS99	27	0.184	1638.16	34.2	S
MWS100	27	0.174	1636.30	33.8	S
MWS101	25	0.189	1638.16	36.1	S
MWS102	25	0.256	1638.16	40.67	S
MWS103	26	0.242	1638.16	45.0	S
MWS104	27	0.248	1638.16	46.7	S
MWS105	27	0.264	1638.16	47.3	S
MWS106	32	0.3095	1636.30	46.4	S
MWS107	26	0.239	1636.30	44.1	S
MWS108	28	0.215	1638.16	36.7	S
MWS109	27	0.2401	1638.16	50.09	S
MWS110	26	0.2204	1636.30	42.5	S
MWS111	26	0.228	1636.30	41.6	S
MWS112	27	0.259	1638.16	46.4	S
MWS113	28	0.275	1638.16	49.1	S
MWS114	27	0.254	1638.16	45.1	S
MWS115	30	0.252	1636.30	51.7	S
MWS116	28	0.2102	1638.16	33.3	S

MWS117	25	0.266	1636.30	47.4	S
MWS118	27	0.237	1638.16	42.5	S
MWS119	28	0.196	1638.16	41.8	S
MWS120	27	0.236	1636.30	42.7	S
MWS121	25	0.357	1636.30	45.5	S
MWS122	25	0.289	1636.30	50.82	S
MWS123	28	0.243	1638.16	43.3	S
MWS124	28	0.236	1638.16	45.4	S
MWS125	31	0.211	1636.30	40.63	S
MWS126	29	0.231	1638.16	48.1	S
Median	27	0.211		35.4	
IQR1-IQR3	26-29	0.196-0.227		32.1-40.47	

IQR: Interquartile range; S: Strong; S/N: Signal-to-noise ratio.

Table 6.5. Frequency of ATR-FTIR bands present in healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernails measured using the Agilent Cary 630 ATR-FTIR spectrometer, equipped with ATR diamond and holder.

Participant	Band position (cm ⁻¹)																
	3277	3067	2960	2920	2851	1738	1644	1642	1633	1582	1562	1535	1452	1447	1398	1296	1240
MWS1																	
MWS2																	
MWS3																	
MWS4																	
MWS5																	
MWS6																	
MWS7																	
MWS8																	
MWS9																	
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MWS126																			

6.4. Conclusions

This chapter explored the feasibility of ATR-FTIR spectroscopy for the detection of CVDs and DM from fingernails. Spectral interpretation allowed for the identification of key functional groups and their associated endogenous constituents such as amino acids, lipids and proteins, as well as the presence of amide I, II and III, which related to the fingernail's structural integrity and keratin. Additional constituents such as glucose, HDL and homocysteine were also detected during spectral interpretation. The presence of such constituents provided insight into the detection of diseases such as CVDs and DM.

Within the ATR-FTIR spectra, six areas of interest (3570 – 2999, 3000 – 2828, 1674 – 1588, 1547 – 1521, 1482 – 1426 and 1424 – 1362 cm^{-1}) were chosen to understand the deposition of endogenous constituents into fingernails. The absorbance of key ATR-FTIR bands provided information regarding the endogenous constituent content of fingernails in relation to their health/disease status.

MLAs further confirmed the ATR-FTIR spectrometers' ability to detect differences in fingernail's spectra of healthy versus CVD and diabetic individuals. Overall, PCA and SOMs offered more accurate clustering of fingernails' spectra with less false positives encountered.

To conclude, ATR-FTIR spectroscopy offered non-invasive and non-intrusive analysis of fingernails. The deposition of endogenous constituents into fingernails and their

corresponding ATR-FTIR activity revealed key information regarding the participants' disease status. Thus, the combination of ATR-FTIR spectroscopy paired with fingernails showed success in the detection of CVDs and DM.

6.5. Key Messages

- ATR-FTIR spectroscopy successfully detected the presence of key endogenous constituents in fingernails.
- CWS demonstrated high number of mismatches between fingernail status of varying health/disease status but also produced a high number of false positives.
- PCA and SOMs were more accurate in classifying fingernails of different disease status.

Chapter seven: Investigation of healthy, cardiovascular and diabetic fingernail quality using scanning electron microscopy.

This chapter investigated the quality of fingernails using SEM. To assess the fingernail quality, two factors were considered: elemental composition and surface topography (roughness). For this work the Environmental SEM-Quanta free electron gas (FEG)-250 was utilised. Two detectors were selected for this research, including: the EDX spectrometer and the STEM detector. The EDX spectrometer allowed the identification of the fingernails' elemental composition. In this respect, no significant differences in elemental composition were associated with age but significant difference was seen for biological sex, diet and disease presence. The STEM detector captured the visual manifestation of tissue damage caused by CVDs and/or DM. Tissue damage was characterised by a rough texture and was most prominent for diabetic fingernails, followed closely by CVD fingernails. The presence of hyperglycaemia and resulting accumulation of AGEs caused damage to the fingernail plate, envisioned as dry and brittle fingernails. The tissue damage observed in the CVD clippings was associated with impaired blood circulation caused by hypertension. Thus, this work demonstrated the capabilities of SEM to investigate tissue damage caused by CVDs and/or DM through elemental and topographical assessments.

7.1. Introduction

The presence of certain systemic diseases can cause alterations to the shape and size of fingernails, interrupting their natural growth patterns (Dhingra *et al.*, 2022). Research by Sihota *et al.*, (2019) indicated that in the presence of disease such as DM, the morphology of the fingernail is altered. For example, fingernails taken from diabetic cases demonstrated a higher level of roughness/texture to the fingernail plate in comparison to healthy controls (Sihota *et al.*, 2019). Hence, the application of fingernails and their surface texture is up in coming for the detection of pathological

conditions and diseases that occur elsewhere within the body (Beatty, Li and Anaekwe, 2024).

Similarly, the elemental composition of fingernails varies greatly between different populations and often indicated the presence of disease. Minerals such as calcium, copper, iron, magnesium, sodium and zinc are commonly identified within the fingernail plate (Muddasani *et al.*, 2021). In males, higher levels of zinc are often experienced, while females commonly demonstrate higher levels of magnesium (Muddasani *et al.*, 2021).

SEM offers the ability to identify differences between the topography of healthy versus diseased fingernails, as well as the impact of disease on key elements. Therefore, this chapter investigated the tissue quality of fingernails using SEM. To fulfil this aim, the following objectives were set:

- To explore the impact of disease on the fingernails' tissue quality and topography in terms of smoothness/roughness.
- To determine the elemental composition of fingernails in relation to factors such as age, biological sex and diet.
- To identify the elemental composition of fingernails in relation to the absence or presence of disease, particularly CVDs and/or DM.

7.2. Methods and Materials

7.2.1. Materials

The full sample size ($n = 126$ fingernail sets) was measured using the SEM. After flattening, one fingernail was taken per set. A small amount of the central section of the clipping was then taken and stored in a clear zip-locked bag. Each bag was labelled with the sample's corresponding participant code. Duplicate samples were prepared in cases of fingernails MWS3 (healthy), MW46 (CVD), MWS51 (diabetic), MWS57 (CVD-diabetic) and MWS98 (unhealthy). The initial set remained uncoated, while the duplicate set was coated in gold (Figure 7.1.).

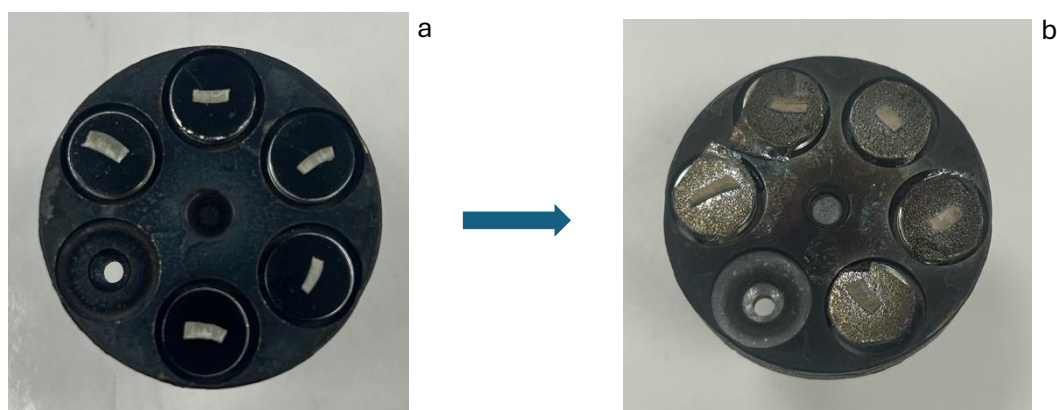


Figure 7.1. (a) Uncoated and (b) coated fingernail clippings.

7.2.2. Instrumentation

Measurements were taken using the Environmental SEM-Quanta FEG-250 (Elecmi, Madrid) (Figure 7.2.). The Quanta 250 was equipped with an FEG column, which allowed for a resolution of 1.4 nm at as low as 1 kV electron landing voltage. Furthermore, the SEM of choice was issued with several detectors including the Everhart-Thornley detector/Large Field detector, the gaseous analytical detect/low voltage high contrast (GAD/vCD) detector and the gaseous secondary electron detector/gaseous analytical (GSED/GAD) detector. After careful consideration, two detectors were selected for this experiment, those being: the EDX spectrometer and the scanning transmission electron microscopy (STEM) detector (Table 7.1.). The former detector was applied for elemental composition analysis, while the latter was utilised for STEM images.

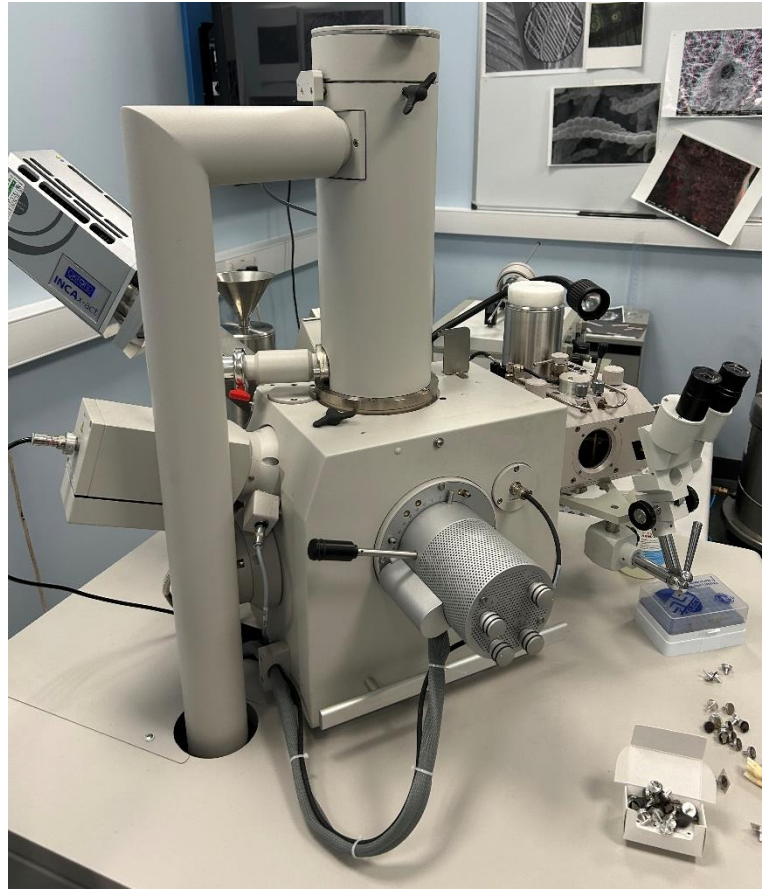


Figure 7.2. The Environmental SEM-Quanta FEG-250.

Table 7.1. Advantages and disadvantages of the Environmental SEM-Quanta FEG-250's detectors.

Detector	Advantages	Disadvantages	References
EDX	• Elemental identification, elements with an atomic number of ≥ 6 can be detected and identified. • Simultaneous detection allows for multiple elements to be detected simultaneously. • Simultaneous elemental mapping and capturing SEM images, hence is highly valuable for material characterisation. High spatial resolution is also produced when paired with SEM.	• Limited sensitivity to light elements (e.g. beryllium, boron, hydrogen and lithium). • Quantitative accuracy poor when elements are present in low concentrations. • Depth sensitivity, limited to a shallow region. However, this can be overcome by focussing on the highly proliferative area of the fingernail (the ventral layer).	Duma <i>et al.</i> , 2022; Scimeca <i>et al.</i> , 2018.
Everhart-Thornley/large field detector	• Sensitive to secondary electrons. Thus, produces high-resolution surface images. • Topographic imaging is clear, with high contrast and detailed surfaces. • Provides 3-dimensional images. • Works with both conductive and non-conductive materials.	• Limited depth information. Therefore, mainly captures the surface features. • Non-conductive samples often cause surface charging, which can create image distortion or a loss of signal. • When used independently, compositional analysis is challenging as it is difficult to distinguish between materials of similar secondary electron yields.	Lewczuk and Szyryńska, 2021.
GAD/vCD	• Works with non-conductive samples and biological samples without the need for coating. • High contrast at low voltages, which allows for fine surface feature observation and detection of surface morphology and topography.	• Setting up and maintaining a vCD or gas-assisted environment can become overly complex and costly. Thus, not suitable for LMICs. • Requires specialised sample preparation.	Frank <i>et al.</i> , 2012; Vlasov, Denisov and Verbeeck, 2023.
GSED/GAD	• Can be operated under low vacuum modes. Thus, does not require conductive coating. • Effectively reduces charges. • Surface sensitivity provides high contrast of the sample's surface features.	• Operating under low vacuum modes often creates lower image resolution, as well as noise. • Provides mostly surface information.	Vlasov, Denisov and Verbeeck, 2023; Zhu <i>et al.</i> , 2020.
STEM	• High spatial resolution commonly in the sub-nanometre range. • Possesses the ability to both scan and transmit electrons, offering information from the surface and subsurface areas of the sample. • When used in combination with other detectors such as EDX, can produce detailed composition analysis, elemental mapping and spectroscopy.	• Samples must be thin (typically 50 – 100 nm) for transmission. • Lower signal intensity, therefore, STEM imaging may require longer acquisition times or higher electron beam intensities.	Brodusch <i>et al.</i> , 2021.

EDX: Energy dispersive X-ray spectrometer; GAD/vCD: Gaseous analytical detect/low voltage high contrast; GSED/GAD: Gaseous secondary electron detector/gaseous analytical detector; STEM: Scanning transmission electron microscopy detector.

During the collection of STEM images, a spot size of 3 and a voltage of 10.00 kV was maintained. The aforementioned levels were increased during X-ray acquisition to 4 and 20.00 kV, respectively, as per LJMU's standard operating procedure (SOP). The increased voltage was to assure that a count rate of >1 was achieved. A working distance of 10 mm was maintained throughout the experiment. STEM images were collected over the range $\times 100 - \times 1000$ magnification. From each sample, one STEM image was taken, and three X-ray spectra. Parameters including the dwell time, horizontal width field (HFW) and vacuum mode went unchanged during the experiment. Standardised parameters included: 60 μs , 993 μm and high vacuum mode, respectively.

7.2.3. Procedure

Prior to SEM analysis, the fingernail was clipped so that the middle section of the fingernail could be placed comfortably onto the aluminium stub. To secure the fingernail, all aluminium stubs were fitted with carbon tabs or double-sided carbon tape. Not only did the carbon tabs/tape secure the clipping into place for analysis but also improved conductivity between the SEM and fingernail sample (Fischer *et al.*, 2013). Fingernails were fixed with the ventral layer facing upwards. Hence, was likely to demonstrate a highly representative spectra of the fingernail's elemental composition.

From fingernail sets MWS3 MWS46, MWS51, MWS57 and MWS98, two fingernails were taken. The first fingernail remained uncoated, while the second was coated with gold. The addition of gold coating was to identify whether the presence of a conductive coating was imperative for high-quality EDX spectra. The use of gold coating for SEM has shown previous success in improving the conductivity seen for non-conductive or low conductive materials. Furthermore, the addition of gold coating has previously prevented the charge-discharge phenomenon, also referred to as the charging effect (Endo *et al.*, 2010; He, Fu and Xue, 2018; Yamada *et al.*, 1997). This phenomenon is characterised by samples with poor conductivity. The poor conductivity of the sample's surface creates an electrostatic charge accumulation under the irradiation of the electron beam (He, Fu and Xue, 2018). Hence, impacting the electronic signal

transmission and generating image deformation and charged electron drifting (Kato *et al.*, 1975; Hutton *et al.*, 2013).

Due to the SEM stage, a total of six fingernail clippings were measured at a time. A STEM image was taken from each fingernail clipping through the software xT Microscope Control version 6.2.11, build 3381. Three EDX spectra were taken from each fingernail, focussing on three different areas of the ventral layer. This layer is in close contact with the fingernail bed, also known as the ventral matrix, which is considered the fingernail's transitional zone (Baswan *et al.*, 2017). Here, living cells are keratinised and integrate into the fingernail (De Berker, 2013; Kishabongo *et al.*, 2015). Not only is this layer highly proliferative, but unlike the dorsal layer (the primary barrier for nail permeation), is often unexposed to extrinsic contaminants. Between the sets of fingernails, the SEM was vacuumed to prevent electrical discharge in the gun assembling and to allow the electrons to move freely within the instrument. This also ensured that the instrument could return to baseline, preventing beam drift and fluctuations in current. The introduction of new samples required the SEM to be pumped, which created a low pressure for electrons to pass freely through the system without collisions with gas molecules.

7.2.4. Data Processing

Weight% values were calculated through the EDX's built-in software, based on the premise that the more abundant an element was, the more characteristic X-rays were detected (Garrido-Garica *et al.*, 2024). The EDX's software, INCA-FLOW, utilises equation 7.1. Therefore, the calculated weight% value was proportional to the number of X-rays each element produced. In this case, elements that were abundantly found within the fingernails such as oxygen demonstrated high weight% values, while less abundant elements such as bromine showed low weight% values (Table 7.2.)

Equation 7.1. Weight% values of elements detected in fingernails using EDX.

$$Weight\% = \frac{\text{element weight in sample}}{\text{weight of all elements in sample}} \times 100$$

Table 7.2. Elements and their corresponding thresholds detected using the Environmental SEM-Quanta FEG-250.

Element	Weight% (%)	Normal threshold	Role in fingernail	References
Al	0-5.83	0-7.83%	Trace element attributed to environmental and/or occupational exposure.	Álvarez <i>et al.</i> , 2021; Fulgenzi, Vietti and Ferrero, 2014; Hasan <i>et al.</i> , 2020; Hashem and Abed, 2007.
Br	0-4.14	1.1-49 µg/g (in hair)	Trace element attributed to environmental exposure.	Cuenca, Pories and Bray, 1988; Klančič, Gobec and Jakopin, 2022; Zwiener <i>et al.</i> , 2007.
Ca	0-14	0-0.75%	Plays a role in electrolyte balance, thus maintains the germinal matrix and nail bed. Signals keratinocytes.	Álvarez <i>et al.</i> , 2021; Clapham, 2007; Ko <i>et al.</i> , 2001; Park <i>et al.</i> , 2024; Siong and Ebihara, 2017; Wu <i>et al.</i> , 2012.
C	0-76.4	31.9-52.3%	Component of keratin thus plays a role in the formation and maintenance of the fingernail's structural integrity.	Álvarez <i>et al.</i> , 2021; Dittmar <i>et al.</i> , 2008; Schumacher, Dindorf and Dittmar, 2009.
Cl	0-7.99	0-0.74%	Found in trace amounts caused by environmental exposure.	Álvarez <i>et al.</i> , 2021.
Fe	0-15.4	10.2-41.5%	Involved in cellular metabolism and oxygen transport.	Briguglio <i>et al.</i> , 2020; Siong and Ebihara, 2017.
Mg	0-5.29	0-0.34%	Plays a role in physiological functions such as the production of adenosine triphosphate (ATP) and synthesis of keratin.	Álvarez <i>et al.</i> , 2021; Souza <i>et al.</i> , 2023.
O	39.6-83.4	22.8-40.98%	Associated with amino acids such as glutamic acid, serine, cysteine and threonine and peptide linkages, as well as disulfide bonding and structural integrity.	Álvarez <i>et al.</i> , 2021; Baden and Kvedar, 1993; Dittmar, Dindorf and Banerjee, 2008; Schumacher, Dindorf and Dittmar, 2009; Gupchup and Zatz, 1999; Laubé <i>et al.</i> , 2019.
P	0- 8.59	0-0.25%	Utilised in cellular activities and metabolic functions such as growth and maintenance of fingernails.	Álvarez <i>et al.</i> , 2021; Kritmetapak and Kumar, 2021.
K	0-4.47	0-0.59%	Involved in cellular functions such as maintaining fluid balance and osmolality of cells.	Álvarez <i>et al.</i> , 2021; D'Elia, 2024; Gumz, Rabinowitz and Wingo, 2015; Siong and Ebihara, 2017.
Si	0-22.9	0-0.14%	Stimulates collagen production.	Álvarez <i>et al.</i> , 2021; Price, Koval and Langford, 2013; Reffitt <i>et al.</i> , 2003; Saeedi, Shavandi and Meredith-Jones, 2018.
S	1.31-25.9	0.71-6.92%	Sulphur-rich amino acid cysteine, which is linked to disulfide bonds that crosslink keratin.	Álvarez <i>et al.</i> , 2021; Bulleid, 2012; Saeedi, Shavandi and Meredith-Jones, 2018; Siong and Ebihara, 2017.
Na	0-8.52	0-1.30%	Trace element in fingernails attributed to the body's electrolyte balance and natural circulation.	Álvarez <i>et al.</i> , 2021; Baswan <i>et al.</i> , 2017; Siong and Ebihara, 2017.
Ti	0 – 5.64	>1.1%	Biologically inert but present due to environmental exposure.	Decker, Daly and Scher, 2015; Lindenschmidt <i>et al.</i> , 1990; Ophus <i>et al.</i> , 1979; Skocaj <i>et al.</i> , 2011

Al: Aluminium; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; Fe: Iron; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

Prior to analysis, STEM images underwent quality assessments through visual inspection. Images were assessed based on several factors including charging, focus, presence of image artifacts and resolution. The presence of charging or image artifacts could skew topographical analysis and conceal key topographical details. Moreover, it was imperative that images possessed a high level of focus and resolution for maximised detail and analytical findings.

7.2.5. Pilot Study

A pilot study was conducted to develop and validate the preparation of fingernails for the application of SEM. For this experiment, five sets of fingernails were utilised, with three EDX spectra taken per fingernail. From each fingernail set, two clippings were taken, with one being coated with gold and the other remaining uncoated. Parameters including spot size, voltage and working space were investigated to ensure the collection of high-quality STEM images and X-ray spectra.

7.2.6. Qualitative Analysis

A combination of Microsoft Excel 2019 and SPSS vs29.0.2.0 were employed for the calculation of descriptive statistics. As the distribution of elements was skewed, the median and IQR were considered. Differences in the elemental composition of fingernails were considered in relation to age, biological sex, diet and disease presence. Based on the lack of multivariate normality, as well as the presence of five independent variables/groups, one-way ANOVA tests were performed to verify the null or alternative hypothesis. In the case of this work, the research hypothesis was described as a relationship between the elemental composition of fingernails and the presence/absence of disease. In contrast, the null hypothesis suggested no relationship between elemental composition and absence/presence of disease.

For each of the elements detected, an ANOVA test was performed in relation to the confounding variable (age, biological sex, diet and disease absence/presence). The application of the ANOVA tests allowed a comparison of the elemental means for each group of interest (e.g. healthy, unhealthy, CVD, diabetic and CVD-diabetic).

Participants were split into five age groups, those being: 18 – 24, 25 – 34, 35 – 39, 40 – 64 and 65 – 84 years old. Females and males were separated. Based on their provided data, participants were separated into one of three groups in relation to diet, which included extremely healthy, healthy and unhealthy. Participants who were classified as extremely healthy reported frequent/daily exercise and a well-balanced diet with little-to-no fast-food, high sugar, saturated or trans-fats food products. Healthy participants showed some aspects of a balanced diet, exercised occasionally and had very few fast-food or unhealthy food products. While unhealthy participants admitted to consuming multiple fast-food dishes weekly, as well as high sugar, saturated products, and reported little-to-no exercise. The alternative hypothesis for this ANOVA suggested a relationship between elemental composition of fingernails and the aforementioned factors (age, biological sex and diet), whereas the null hypothesis indicated no relationships.

7.3. Results and Discussion

EDX demonstrated the ability to detect and semi-quantify several elements within the fingernail matrix. Oxygen was classified as the most abundant element and occurred within all fingernail clippings, while bromine was the least abundant element, present in only four fingernail clippings. ANOVA results demonstrated that while the elements varied between fingernail clippings based on factors such as age, biological sex, diet and physical exercise, only calcium and sodium showed a statistically significant difference in weight% value for healthy versus diseased fingernails. Nonetheless, STEM images demonstrated differences in the topographical structure of fingernails. Fingernails taken from CVD and/or diabetic participants showed the rough, topographical fingernail plates, which indicated the presence of tissue damage. In contrast, clippings taken from healthy participants, showed smooth areas and indicated little tissue damage.

7.3.1. Pilot Study

In the pilot study, factors including sample presentation and instrumental parameters were considered.

7.3.1.1 Sample Presentation

To determine the presentation of fingernail samples, a comparison was carried out between the elemental composition of coated versus uncoated fingernails. As a key objective of this research was to identify key elements of fingernails in relation to the presence of disease, a high elemental yield was desirable. The findings of this pilot study revealed that not only did the coating of fingernails skew the elemental analysis but also did not improve elemental yield. Therefore, it was deduced that coating was not imperative for the elemental analysis of fingernails. This finding was confirmed by previous literature, which stated that the low water content of fingernails meant that fingernails could be analysed without coating and under high vacuum conditions (Álvarez *et al.*, 2021).

7.3.1.2 Instrumental Parameters

The pilot study also allowed the development of a standardised method, which was applied to the full sample size ($n = 126$). Firstly, all clippings were examined under high vacuum conditions, which ensured that the electron beam's intensity was maintained throughout the experiment (Williams, 2021). Parameters including the spot size, voltage and working space were also considered for the collection of high-quality STEM images and X-ray spectra. The latter three parameters had optimal values of 3-4, 10-20 kV and 10 mm. Parameters such as dwell time and HFW were not changed throughout the pilot study and included parameters of 60 μs and 993 μm , respectively.

7.3.1.3 Elemental Analysis

Across the five groups of interest, a total of 14 elements were identified and included aluminium, bromine, calcium, carbon, chlorine, iron, magnesium, oxygen, phosphorus, potassium, silicon, sulphur, sodium and titanium (Figure 7.3). In several cases, the aforementioned elements varied in presence and weight% value.

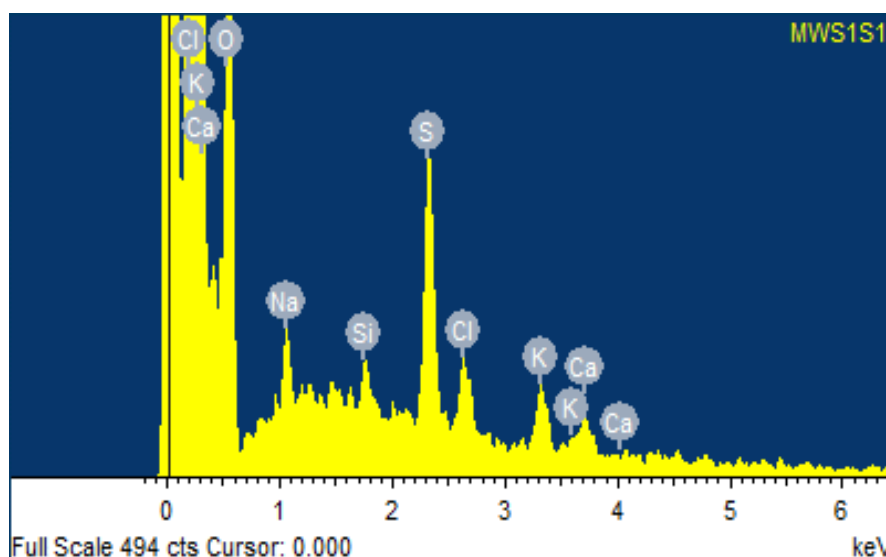


Figure 7.3. EDX spectra of fingernail set MWS1 measured using the Environmental SEM-Quanta FEG-250.

7.3.1.4. Morphological and Topographical Analysis

Through the examination of STEM images, alterations to the fingernail's surface topography (roughness) were observed and indicated tissue damage. The presence of such damages has previously indicated CVDs, hyperglycaemia and DM (Sihota *et al.*, 2019).

7.3.2. Sample of the Main Study

7.3.2.1. Elemental Analysis of Fingernails

Across the 126 fingernails, thirteen elements were detected and included: aluminium, bromine, calcium, carbon, chlorine, iron, magnesium, oxygen, phosphorus, potassium, silicon, sulphur, sodium and titanium (Figure 7.4. and Table 7.3.). The most abundant element detected was oxygen, which was identified within all 126 (100%) sets of fingernails. The weight% of this element ranged from 39.6 – 83.4% (median = 72.8%, IQR = 67.01 – 76.5%). In contrast, bromine was the least abundant element, identified only in four samples, with weight% values of 0 – 4.14% (median = 0%, IQR = 0 – 0%).

As demonstrated by Chapter three of the thesis (section 3.3.5), LR models showed close relationships between clinical factors including age, biological sex and diet in relation to the presence of CVDs and DM. Similarly, Chapter four (section 4.3.3) also made evident key relationships between the age, biological sex and ethnicity (strongly associated with diet/diet choices) and the fingernail's endogenous constituents. Therefore, to understand the presence of the detected elements, ANOVA tests were carried out. The aforementioned tests independently investigated the relationship between key clinical factors (age, biological sex and diet) and the element's presence and weight% value.

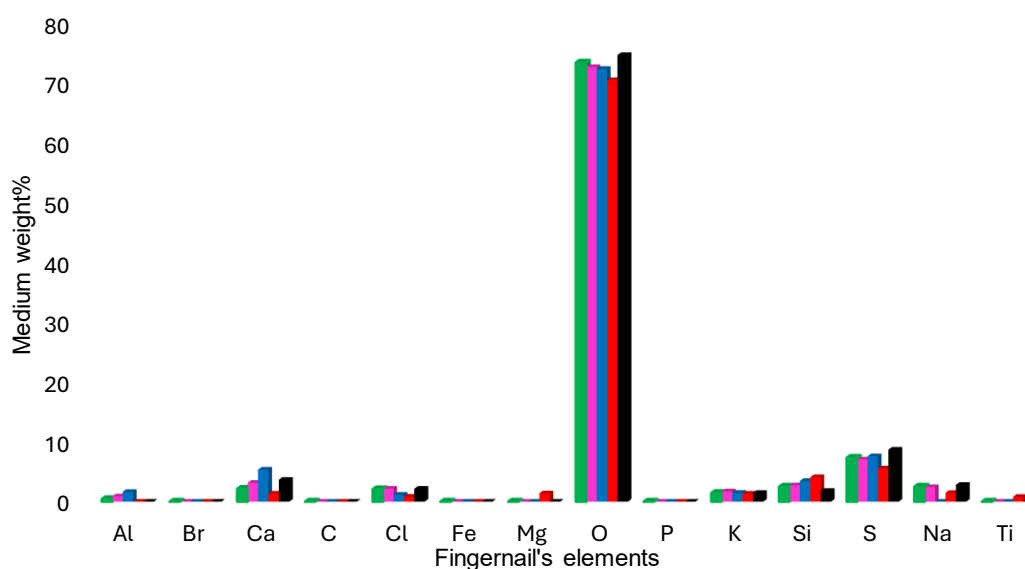


Figure 7.4. Median distribution of identified elements across healthy (green), unhealthy (magenta), CVD (red), diabetic (blue) and CVD-diabetic (black) fingernail clippings measured using the SEM Quanta FEG-250 EDX detector.

Table 7.3. Median weight% values of elements found in healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernail's ventral layer measured using the SEM Quanta FEG-250 (n = 126).

	Healthy	Unhealthy	CVD	Diabetic	CVD-diabetic
Element	Median (Q1-Q3) weight%				
Al	0.43 (0.00-2.05)	1.035 (0.00-1.85)	0.81 (0.00-2.03)	0.00 (0.00-1.51)	0.00 (0.00-1.05)
Br	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
Ca	2.17 (1.53-3.83)	3.19 (1.24-5.59)	4.88 (2.35-7.35)	1.35 (0.27-2.44)	3.66 (1.68-7.00)
C	0.00 (0.00-7.56)	0.00 (0.00-14.1)	0.00 (0.00-58.5)	0.00 (0.00-1.07)	0.00 (0.00-29.9)
Cl	2.08 (1.48-3.13)	2.17 (1.043-2.98)	1.51 (0.685-3.45)	0.85 (0.84-2.64)	2.16 (1.77-2.31)
Fe	0.00 (0.00-0.00)	0.00 (0.00-0.07)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
Mg	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.91)	1.39 (0.00-1.44)	0.00 (0.00-0.00)
O	73.3 (67.1-76.6)	72.5 (67.5-74.9)	72.9 (66.6-76.4)	70.31 (67.3-71.5)	74.5 (64.1-77.2)
P	0.00 (0.00-0.3025)	0.00 (0.00-0.15)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-1.27)
K	1.46 (0.878-2.25)	1.60 (1.29-6.98)	1.49 (1.02-2.005)	1.34 (1.27-1.54)	1.46 (0.89-2.05)
Si	2.51 (1.56-5.79)	2.91(1.28-6.98)	2.87 (1.47-5.76)	4.09 (2.21-6.71)	1.80 (0.665-2.55)
S	7.35 (5.26-10.95)	2.41(5.67-9.103)	7.77 (5.62-8.81)	5.56 (3.70-9.58)	8.70 (7.08-9.98)
Na	2.52 (1.88-3.67)	2.41(0.71-3.59)	0.00 (0.00-2.23)	1.46 (0.00-1.98)	2.81 (2.31-4.045)
Ti	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.80 (0.00-0.00)	0.00 (0.00-0.00)

Al: Aluminium; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; CVD: Cardiovascular diseases; Fe: Iron; IQR: Interquartile range; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

7.3.2.1.1. Elemental Analysis and Age

Previous research indicated a relationship between the elemental composition of fingernails and age. For example, Albucker, Conway and Lipner (2024) highlighted that sulphur content decreased in older individuals, which resulted in fewer disulfide bridges and impaired structural integrity of the keratin (Albucker, Conway and Lipner, 2024; Maddy and Tosti, 2018; Lubach, Cohrs and Wurzinger, 1986). EDX spectra revealed variation in the weight% of calcium, oxygen, potassium, silicon, sodium and sulphur in younger and older participants. However, differences were marginal and ANOVA results did not reveal statistically significant relationships between age and the elemental composition of fingernails (Table 7.4.).

Table 7.4. ANOVA results of elements found within fingernails in relation to age (n = 126).

Test of Homogeneity			ANOVA	
Levene's				
Element	Statistics	Sig.	F-value	Sig.
Al	1.84	0.125	1.055	0.382
Br	6.53	<0.001	1.56	0.187
Ca	1.62	0.173	0.119	0.975
C	3.062	0.019	0.54	0.707
Cl	1.025	0.397	0.161	0.958
Fe	3.57	0.009	0.826	0.511
Mg	0.186	0.945	0.048	0.996
O	1.8	0.133	1.039	0.39
P	3.042	0.02	0.97	0.427
K	1.19	0.316	0.2	0.938
Si	1.49	0.209	1.76	0.142
S	1.006	0.407	1.18	0.323
Na	5.077	<0.001	1.325	0.265
Ti	2.068	0.089	0.508	0.73

Al: Aluminium; ANOVA: Analysis of variance; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; Fe: Iron; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Sig.: Significance; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

7.3.2.1.2. Elemental Analysis and Biological Sex

ANOVA results determined a statistical relationship between biological sex and titanium. As demonstrated by Table 7.5, the weight% value of titanium significantly differed for female and male participants with a statistically significant p-value of 0.043. From the 19 clippings that demonstrated the presence of titanium, 14 (73.7%) were female. The presence of titanium in such cases could be related to the use of cosmetic products that contain titanium dioxide as an ingredient (Decker, Daly and Scher, 2015). Small variations between the weight% values of elements such as calcium, chlorine, silicon and sulphur were also noted. As expected, female and male participants shared similar weight% values for oxygen. In like manner, females and males demonstrated similar weight% values of potassium and sodium.

Table 7.5. ANOVA results of elements found within fingernails in relation to biological sex (n = 126).

Test of Homogeneity			ANOVA	
Levene's				
Element	Statistics	Significance	F-value	Sig.
Al	3.87	0.051	1.15	0.285
Br	4.49	0.036	1.15	0.286
Ca	0.492	0.484	0.001	0.975
C	0.162	0.688	0.055	0.815
Cl	1.86	0.175	0.001	0.979
Fe	2.67	0.105	0.782	0.378
Mg	0.049	0.826	0.003	0.959
O	2.39	0.124	0.037	0.848
P	1.89	0.171	0.409	0.524
K	0.825	0.365	0.109	0.742
Si	4.61	0.034	0.286	0.093
S	2.702	0.103	2.89	0.092
Na	1.73	0.191	0.794	0.375
Ti	17.6	<0.001	4.17	0.043

Al: Aluminium; ANOVA: Analysis of variance; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; Fe: Iron; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Sig.: Significance; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

7.3.2.1.3. Elemental Analysis and Diet

The ANOVA test showed a significant difference (p-value = 0.006) between the calcium weight% values of fingernails taken from extremely healthy, healthy and unhealthy participants (Table 7.6). Moreover, the post hoc test, revealed that there was also a significant difference (p-value = 0.038) in calcium weight% values for extremely healthy versus healthy participants. The highest weight% for calcium (median = 4.57%, IQR = 1.75 – 7.48%) was observed in extremely healthy participants, who frequently exercised, reported healthy or balanced diets and did not consume fast-food. In contrast, healthy participants, who reported some exercise and marginally healthy diets produced the lowest calcium weight% value (median = 2.17%, IQR = 1.39 – 3.49%). Participants who showed little to no calcium content (weight% value < 1.00%) included 10 healthy, four unhealthy and one extremely healthy. This could be linked to decreased intake of vitamins in the diet that in turn enhances calcium absorption (Khazai, Judd and Tangpricha, 2009; Michos *et al.*, 2021). Differences were also observed in the weight% values of chlorine, potassium, silicon, sulphur and sodium; yet this difference was not significant.

Table 7.6. ANOVA results of elements found within fingernails in relation to diet (n = 126).

Test of Homogeneity			ANOVA	
Levene's				
Element	Statistics	Significance	F-value	Sig.
Al	0.508	0.603	1.43	0.244
Br	4.95	0.009	1.29	0.279
Ca	5.97	0.003	5.28	0.006
C	1.38	0.256	0.271	0.763
Cl	1.74	0.18	0.815	0.445
Fe	4.58	0.012	1.38	0.257
Mg	2.81	0.064	0.858	0.426
O	1.2	0.305	0.261	0.771
P	0.638	0.53	0.351	0.795
K	2.59	0.079	1.29	0.277
Si	2.13	0.124	0.513	0.6
S	3.75	0.026	1.83	0.164
Na	11.2	<0.001	2.79	0.065
Ti	1.23	0.297	0.32	0.727

Al: Aluminium; ANOVA: Analysis of variance; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; Fe: Iron; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Sig.: Significance; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

7.3.2.1.4. Elemental Analysis and Disease

Elemental analysis did not yield independent biomarkers for the detection of CVDs and/or DM. However, variation in the weight% value of fingernails was seen between healthy and diseased fingernails and could be used as an indicator of disease (Table 7.7).

The ANOVA results revealed a statistically significant difference (p-value = 0.003) between the calcium weight% values of healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails. The highest weight% value (median = 4.88%, IQR = 2.35 – 7.35%) for calcium was observed in clippings taken from CVD participants. In contrast, the lowest weight% value for calcium (median = 135%, IQR = 0.27 – 2.44%) was observed from diabetic participants. Excessive circulating calcium has been linked to CVDs. The high weight% value of calcium (median = 3.66%, IQR = 1.68 – 7%) for CVD-diabetic

participants supported the relationship between calcium and CVDs. Yet, these participants reported the use of calcium supplements and vitamin D.

A significant difference in the weight% values of sodium was also identified through the ANOVA test (p-value = 0.018). Participants diagnosed with a CVD and DM, produced the highest weight% value (median = 2.81%, IQR = 2.31 – 4.045%), while CVD participants demonstrated the lowest weight% value (median = 0%, IQR = 0 – 2.23%).

Table 7.7. ANOVA results of elements found within fingernails in relation to the absence or presence of disease (n = 126).

Test of Homogeneity			Anova	
Levene's				
Element	Statistics	Significance	F-value	Sig.
Al	1.71	0.152	0.854	0.494
Br	10.74	<0.001	2.25	0.068
Ca	3.48	0.01	4.26	0.003
C	1.703	0.154	0.597	0.665
Cl	0.771	0.546	0.514	0.725
Fe	10.12	<0.001	2.41	0.053
Mg	1.46	0.218	0.416	0.796
O	0.351	0.843	0.269	0.897
P	1.17	0.329	0.224	0.925
K	0.371	0.829	0.651	0.627
Si	0.985	0.418	0.244	0.913
S	1.83	0.128	0.422	0.793
Na	14.4	<0.001	3.096	0.018
Ti	2.38	0.055	0.59	0.67

Al: Aluminium; ANOVA: Analysis of variance; Br: Bromine; Ca: Calcium; C: Carbon; Cl: Chlorine; Fe: Iron; Mg: Magnesium; O: Oxygen; P: Phosphorus; K: Potassium; Sig.: Significance; Si: Silicon; S: Sulphur; Na: Sodium; Ti: Titanium.

For observing patterns in elemental analysis of fingernails, PCA was applied to the EDX spectrometer results (Figure 7.5). A total variance of 95.97% was accounted for across the first three PCs scores. PC1, PC2 and PC3 contributed to 88.5%, 5.05% and 2.42% of the variance, respectively. The PC scores showed overlap among the

four groups (healthy, CVD, diabetic and CVD-diabetic), with no clear clustering between scores of healthy or diseased fingernails. This suggests that while the EDX spectrometer was able to detect differences in the elemental composition of fingernails, accurate classification could not be made. It is important to note that the inaccuracy of SEM paired with PCA could be in some part attributed to the imbalance of classes, whereby healthy scores were the majority.

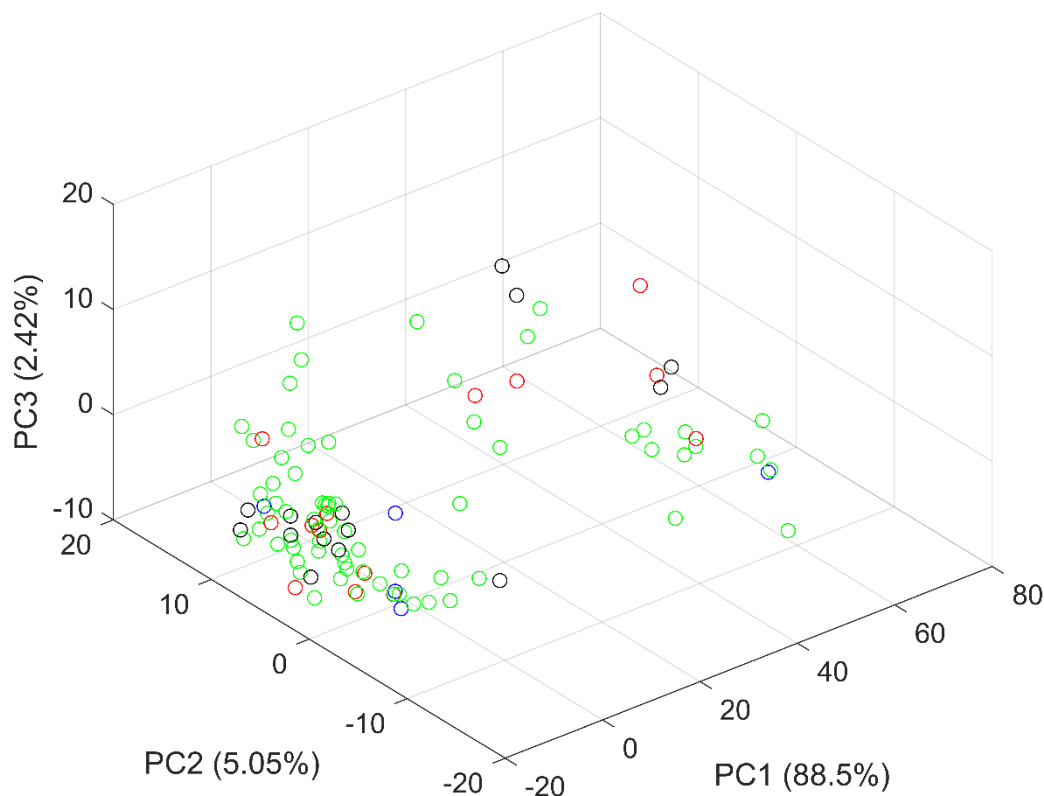


Figure 7.5. PCA of EDX spectra of fingernails taken from healthy (green), CVD (red), diabetic (blue) and CVD-diabetic (black) participants measured using the Environmental SEM-Quanta FEG-250.

7.3.2.2. Morphological and Topographical Analysis of Fingernails

To evaluate the fingernail plate's quality, surface morphology and roughness were assessed. The STEM images (Figures 7.6.) demonstrated the surface morphology of a clipped fingernail plate for healthy (Figure 7.6a.), unhealthy (Figure 7.6b.), CVD (Figure 7.6c.), diabetic (Figure 7.6d.) and CVD-diabetic (Figure 7.6e.) samples. Moderation to the fingernail plate was most noticeable for the diabetic clipping, taken

from participant MWS51, who was diagnosed with T1DM. The texturisation/roughness of the fingernail plate's surface was evidence of degradation caused by hyperglycaemia. In cases of prolonged hyperglycaemia, AGEs accumulate within the fingernail matrix and react irreversibly with amino acid residues of peptides and keratin, to form protein adducts (protein-AGE) or protein crosslinks (AGE-protein-AGE) (Gkogkolou and Böhm, 2012). Moreover, through continuous AGEs accumulation, prolonged endoplasmic reticulum stress can occur. As a result, proteins within the fingernail matrix become misfolded, calcium content depletes and the quantity of disulfide bonds reduces (Adamopoulos *et al.*, 2016; Eizirik, Cardozo and Cnop, 2008; Tanaka *et al.*, 2013). This could be confirmed through referring to the elemental analysis of diabetic clipping MWS51, which displayed weight% values of 0% for both calcium and sulphur. Through the alteration of disulfide content, the fingernail's structural integrity was impaired. Consequently, microstructural deformation occurred in the form of increased surface roughness (Sihota *et al.*, 2019). Moreover, smooth areas, with little texture were visualised for healthy clipping MWS3, which further supported the idea that tissue damage can be visualised in the form of rough textures.

Moderate tissue damage was also observed in CVD clipping MWS46 and was associated with impaired blood circulation caused by CVDs (Figure 7.6c). In this example, participant MWS46 has been previously diagnosed with hypertension. Fingernails require a viable blood supply and when impaired, the fingernail plate becomes thin and brittle (Hillson, 2017). As a result, the fingernail plate was likely to break, split and in severe cases, separate from the fingernail bed itself. An impaired circulation also prevents the deposition of key trace elements within the fingernail plate as shown by the previous elemental analysis. For example, CVD fingernails showed the lowest weight% value for sodium. Similar tissue damage was observed for unhealthy clipping MWS98, which was associated with high blood pressure. The chronic vascular inflammation and microvascular remodelling linked to blood pressure, likely impaired the circulation and blood flow/deposition to the fingernail. Clippings taken from participant MWS57, diagnosed with CAD, high blood pressure and T1DM, also demonstrated a rough texture and indicated the occurrence of keratin degradation and tissue damage (Figure 7.6e.).

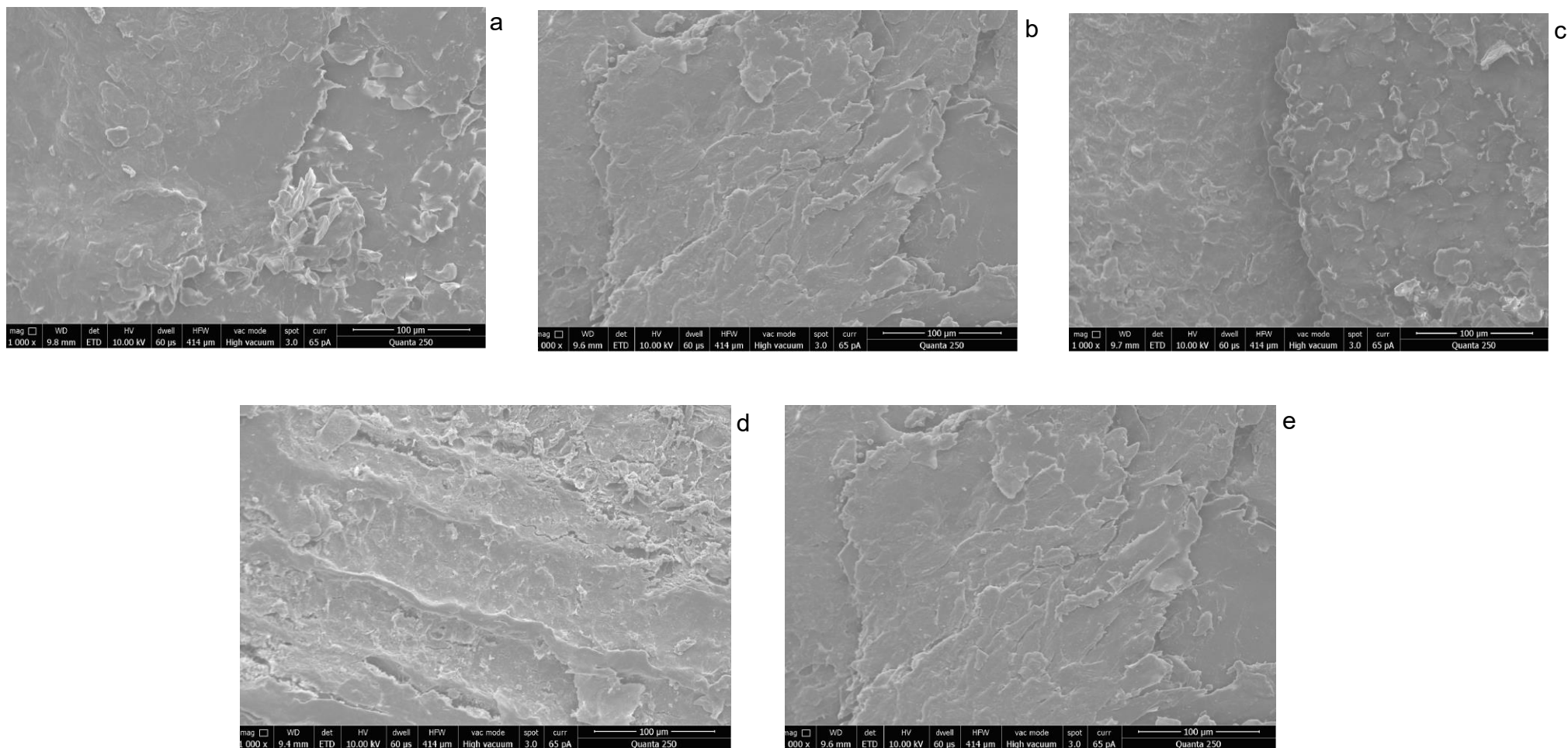


Figure 7.6. STEM images of (a) healthy, (b) unhealthy, (c) CVD, (d) diabetic and (e) CVD-diabetic fingernails using the SEM-Quanta FEG-250 under a x1000 magnification.

7.4. Conclusions

This chapter investigated the tissue quality of healthy, unhealthy, CVD, diabetic and CVD-diabetic fingernails using SEM, with the purpose of surface topography identification and elemental composition. Elemental analysis revealed a total of thirteen elements across the five groups of interest and included: aluminium, bromine, calcium, carbon, chlorine, iron, magnesium, oxygen, phosphorus, potassium, silicon, sulphur and sodium. The aforementioned elements were identified in fingernails taken from the five groups of interest, therefore did not provide independent disease detection. Nevertheless, the weight% values, revealed during the elemental analysis, provided insight into the fingernails' elemental composition. Factors including age, biological sex, diet and disease presence were examined closely in relation to elemental composition. Age did not have a significant impact on fingernails elemental composition in contrary to sex and diet. CVD and DM, on the other hand, influenced the weight% of calcium and sodium and the surface morphology. The presence of CVD and/or DM manifested as changes in the fingernails' topography. Diabetic clippings underwent the most severe topographical changes and in turn tissue damage. Tissue damage was also observed in the CVD clipping and was attributed to impaired blood circulation caused by hypertension.

7.5. Key Messages

- SEM successfully detected a range of elements within fingernails, including aluminium, bromine, calcium, carbon, chlorine, iron, magnesium, oxygen, phosphorus, potassium, silicon, sulphur and sodium.
- Age did not play a significant role in the elemental composition of fingernails.
- A significant difference in the weight% value of titanium was observed between female and male participants with females demonstrating the higher weight% value.
- The weight% value of calcium in fingernails significantly varied between individuals of different diet.

- Calcium and sodium significantly differed in fingernails of CVD and/or diabetic participants.
- Tissue damage to the fingernail was also observed for CVD and/or DM participants.

Chapter eight: Overall discussion

This thesis proposed an intelligent framework for detecting CVDs and/or DM in human fingernails through the main experimental studies. The first study was a systematic review of quantitative studies (chapter two), which identified biomarkers and metabolites utilised for detecting CVDs and/or DM. The systematic review also established techniques frequently employed for identifying such biomarkers/metabolites in a range of biological matrices. The review highlighted 302 biomarkers (78 for CVDs and 224 for DM) across seven biological matrices including blood, dried blood spots, fingernails, plasma, saliva, serum and urine. Moreover, key biomarkers identified in the systematic review included CRP and GDF-15 for CVDs and glucose for DM.

Techniques reported in the systematic review for detecting biomarkers included LC-MS/MS and vibrational spectroscopic techniques including mid-IR and NIR spectroscopy. Subsequently, the review's findings were considered in the following chapters. These involved using vibrational spectroscopy for measuring fingernails. Fingernail samples were collected from participants, alongside a questionnaire (chapter three). The outcomes of the questionnaire showed a strong relationship between risk factors and CVDs or DM. This was established by LR. The LR models showed OR values (0.00 – 11.3) that predicted the manifestation of disease in the presence of risk factors. Thus, creating an understanding of disease trends that complemented the spectroscopic data such as the spectral interpretation results.

Chapter four of the thesis explored the Raman signature of fingernails and their ability to act as an alternative biological matrix for disease detection. Spectral interpretation of fingernails confirmed the presence of endogenous constituents including amino acids, lipids and proteins, as well as disease-related biomarkers such as cytochrome C and glucose. The presence of such bands was confirmed in the literature (Birech *et al.*, 2017; Horiue *et al.*, 2020; Kourkoumelis *et al.*, 2018; Managò, Zito and De Luca, 2018; Olsztyńska-Janus *et al.*, 2012; Surmacki *et al.*, 2018; Towler *et al.*, 2007; Widjaja, Lim and An, 2008). Hence, this work demonstrated the capability of fingernails to accumulate CVD- and DM-related biomarkers.

Chapter five of the thesis complimented the Raman chapter and determined the physicochemical differences in fingernails based on disease status. NIR spectroscopy

was successful in capturing molecular fingerprints and characteristic frequencies, which could be utilised as diagnostic markers. Through comparing the NIR absorbing components between different fingernails, CVDs/DM were detected. This confirmed that NIR spectroscopy could be applied non-invasively to fingernails of varying health or disease status. Within the literature, a limited number of studies demonstrated the combination of NIR spectroscopy with fingernails (Monteyne *et al.*, 2018; Lazaro-Pacheco, Taday and Paldanius, 2024). NIR spectral interpretation revealed similarities in the overall shape and trend of NIR spectra, while absorbance intensities between fingernails of individuals varied. Among these individuals, diabetic fingernails' spectra showed the lowest absorbance values, whereas healthy fingernails showed the highest absorbance values.

The work undertaken in chapter six of the thesis demonstrated the feasibility of ATR-FTIR spectroscopy for detecting CVDs and DM from fingernails. As CVDs and DM are classified as a multifactorial disease, ATR-FTIR spectroscopy allowed the benefit of investigating several biomolecules simultaneously (Baker, Hughes and Hollywood, 2016; Paraskevaidi *et al.*, 2023). Thus, allowing for the generation of a biological signature, also known as a biological fingerprint, which includes information on the sample's endogenous constituents including amino acids, lipids, proteins and disease-related biomarkers such as glucose, HDL and homocysteine.

As fingernails share the same endogenous constituents, despite the absence or presence of disease, crossover was expected. This point was validated through several MLAs including CWS and PCA that showed clusters among different groups of individuals based on disease status.

Findings from chapter seven of the thesis confirmed the accumulation of AGEs and glycated proteins, as well as their subsequent damage to tissues. STEM images demonstrated the use of fingernails as a site for monitoring tissue damage, which manifested as a rough texturized surface, with overlapping and topographical plates. Results from the EDX spectrometer supported STEM findings, showing a decline in the weight% values of calcium and sodium in CVD and diabetic fingernail clippings.

8.1. Summary

What is already known regarding this topic:

- CVDs and DM represent major NCDs that contribute to morbidity and mortality on a global scale.
- CVDs and DM are comorbidities, with CVDs being the most prevalent morbidity and cause of mortality in diabetic patients.
- Vibrational spectroscopic techniques including IR, NIR and Raman spectroscopy have shown success in disease diagnosis previously.
- Fingernails are an accumulating matrix, which can offer a window into underlying systemic and localised diseases.

What this study adds:

- A mixed method approach allowed for a focused profile on the presence of CVDs and/or DM in fingernails, considering key risk factors and implementing them into spectral interpretation and understanding.
- Risk factors such as age and biological sex significantly increased the susceptibility of disease and should be considered simultaneously.
- The presence of disease can impair the deposition of endogenous constituents, which is reflected by the ATR-FTIR, NIR and Raman spectra of fingernails.
- Tissue damage caused by hyperglycaemia and/or poor blood pressure is reflected in the topography of the fingernail plate. A rough texturised surface is characteristic of tissue damage and poor keratin quality.
- Vibrational spectroscopic techniques can be utilised independently or in tandem to detect the presence of CVDs and/or DM in fingernails.
- MLAs including CWS, PCA and SOMs, alongside unsupervised classification models can be utilised for the detection and prediction of disease. But it is imperative that models are balanced, with an equal number of healthy, unhealthy and disease spectra.

8.2. Conclusion

Vibrational spectroscopic techniques offered rapid detection of CVDs and/or DM when applied to fingernails. Moreover, the application of pre-existing spectroscopic libraries,

would allow practitioners to compare spectra of suspected cases against confirmed cases, using key parameters such as absorbance/intensity, band presence and band shape. As a highly vascularised matrix, the fingernail, particularly its ventral layer, was influenced by and is the recipient of endogenous constituents and elements present in the circulation. Systemic diseases changed the activity of key endogenous constituents and elements and this change was reflected within the fingernail. During the application of fingernails as an alternative matrix, confounding variables related to the patient's individualised characteristics should be considered. Patient-related factors including age, biological sex, diet and smoking can not only influence the activity of key endogenous constituents but also cause tissue damage to the fingernail plate. Additionally, clinical-related factors such as drug/vitamin use and previous medical diagnosis are also impactful on the regulation/deposition of constituents into the fingernail. Therefore, it is imperative that healthcare practitioners take a holistic diagnostic approach considering factors including described symptoms, physical manifestations and spectral data. The combination of the aforementioned factors will improve the accuracy of diagnoses and allow for the dissemination of individualistic and efficient disease management strategies.

8.3. Implications on practice

- The findings of this study demonstrate the feasibility of several vibrational spectroscopic techniques including IR, NIR and Raman spectroscopy, that can be utilised by healthcare practitioners independently or simultaneously.
- Management of CVDs and/or DM is a multifactorial-process and requires patients to consider risk factors and behaviours such as diet, exercise and smoking habits, alongside medical management. The aforementioned factors are likely to increase the severity of disease, as well as the development of comorbidities such as chronic kidney disease, dyslipidaemia, inflammatory disorders and obesity.
- A holistic approach is paramount for accurate disease detection and should consider factors such as described symptoms, physical manifestations and spectral data. Risk factors such as age, biological sex and ethnicity provides explainability for spectral data and disease detection outcomes. Thus, these

factors must be considered during the analysis of spectra and potential detection of disease.

- Fingernails can be utilised as a non-invasive and non-intrusive alternative biological matrix, which can be sampled by the participant themselves and in the comfort of their own home. As a relatively stable matrix they also offer cost-related benefits in terms of storage and sample preparation.

8.4. Strength and limitations of the research

Table 8.1. Summary of strength and limitations of the work undertaken in this research.

Strengths	Comments:
The adoption of a social experiment provided justification for the prevalence of CVDs and DM and enhanced the research's validity and reliability.	The collection of participants' data, through questionnaires, offered real-life insight into the occurrence and management of disease. Thus, reiterating relationships between risk factors such as diet and smoking habits and disease prevalence. Furthermore, the application of participants' data to spectral data and STEM images provided justification for the presence of key bands and tissue damage.
A prospective study design was utilised.	The use of prospective study design allowed the principal investigator to investigate a wide range of risk factors and disease outcomes in the same population of participants.
Several vibrational spectroscopic techniques including ATR-FTIR, NIR and Raman spectroscopy were utilised.	The application of several spectroscopic techniques accounted for the principle of mutual exclusion, whereby vibrational modes that are active in one region are inactive in another. For example, ATR-FTIR spectroscopy favours hydrogen bonding, Raman spectroscopy, carbon-carbon bonding and NIR spectroscopy captures overtones and combination bands. The combination of such techniques ensured rich spectral data and maximised disease detection potential. This was demonstrated through the identification of diagnostic biomarkers such as cytochrome C, which was solely detected using the Raman spectrometer.
Participants' data and fingernails were taken from participants of varying backgrounds.	While samples were collected in the UK and Lebanon, participants varied in terms of age, biological sex and ethnicity. Participants were aged 18 – 84 years old and were taken from seven ethnic groups (Arab, Asian,

	Indian, Lebanese Arab, Middle Eastern Arab, Mixed and White). This ensured that findings could be generalised across a variety of different groups. Furthermore, the diversity observed within the sample population, allowed the PI to investigate the relationship between factors such as diet, ethnicity and religion and the presence of disease.
Demonstrated the use of fingernails as an alternative biological matrix for the detection of CVDs and/or DM.	This work validated the presence of key endogenous constituents and diagnostic biomarkers, as well as functional groups relating to the fingernail's structural integrity and tissue quality. The aforementioned molecules varied between healthy, unhealthy and diseased fingernails and can be utilised for future cases of CVD and/or DM detection. The accumulative nature of fingernails, as well as their cost-effective sampling, storage and treatment makes them a highly desirable matrix for LMICs. Moreover, participants are more likely to seek medical diagnosis or to adhere to diagnostic monitoring through the non-intrusive and non-invasive sampling of fingernail clippings rather than blood or saliva.
Weaknesses:	Comments:
A class imbalance was observed between the five groups of interest, whereby CVD and diabetic fingernails were underrepresented. The majority class was healthy with 68 participants, followed by unhealthy with 23 participants. In terms of cases, 15 were CVD, and 5 were diabetic. A crossover between the comorbidities was observed by 15 participants.	Several potential CVD and/or diabetic participants refused participation as they did not want to disclose personal information regarding medical diagnosis, medicine use and other lifestyle factors. It was likely that participants felt embarrassed about previous diagnosis or feared that spectral results would lead to further diagnoses. Nonetheless, the PI would like to reiterate that all potential participants received the participant information sheet, which disclaimed that the purpose of this work was not to diagnose but rather to detect existing diagnoses that had been made by a trained professional. This produced an imbalance in the data, with healthy participants being overrepresented. To account for this issue, the PI collected additional CVD and/or diabetic fingernails, ensuring a higher number of spectra were added to the pre-existing dataset. However, it is important to note that the refusal of the aforementioned potential participants prevented participant bias. This bias occurs when a participant changes their answers or behaviours to match what they think the researcher wants/requires. Furthermore,

	refusal also prevented the Hawthorn effect or social desirability bias, whereby the participant would have lied about the presence of medical conditions and medicine use to appear healthier. The occurrence of either bias would have skewed the participants' data and prevented accurate conclusions from being drawn. This class imbalance influenced model performance by biasing classification towards the majority class and reducing predictive reliability for underrepresented groups. The smaller class sizes limited the model's ability to capture within-class variability, resulting in reduced sensitivity and increased misclassification rates for disease classes. Despite this limitation, multiple performance metrics were examined to avoid over-reliance on overall accuracy alone. Moreover, additional models were trained with an equal number of spectra per class. This approach reduced classification bias towards the majority class and ensured that all classes contributed equally to the model. Although this balancing strategy improved the fairness in class representation, it also reduced the total number of spectra used for model training and in turn may have limited the representation of within-class variability. Therefore, the models' performances reflected a trade-off between minimising class bias and maintaining sample diversity.
Low response rates were observed for several of the questionnaire questions.	A low response rate was noted for several participants and questions. The questions related mostly to education levels, occupation and weight. The lack of responses can be attributed to participants feeling embarrassed and choosing not to answer questions. Nonetheless, all participants were made aware of the anonymity of questionnaires on receipt.
Risk of recall or recollection bias.	Recall bias may have been introduced into the research, whereby participants did not accurately remember past events/experiences and therefore failed to report them. However, data that was imperative for this research, including medical diagnosis and medicine use, are likely to be considered daily and therefore not forgotten during questionnaire completion. Moreover, the questionnaire was designed with key words/phrases to elicit key

	information and remind the participants of relevant events.
Participants' data relied on the quality of the participants' answers.	The quality of questionnaire answers and resulting participants' data was heavily reliant on the participants' quality of answers. For questions concerning diet and physical exercise, several participants provided base-level, simple answers. The low quality of these answers prevented the PI from drawing strong relationships and conclusions between diet, physical exercise and disease, despite the literature suggesting significant relationships. In some cases, poor quality answers also limited the quality and explainability of spectral interpretation.

CVDs: cardiovascular diseases; DM: diabetes mellitus; PI: principal investigator.

8.5. Recommendations

The presence of CVDs and/or DM create significant consequences for both the patient and healthcare systems, especially those living in LMICs. The aforementioned NCDs are common consequences of diagnostic errors whereby there is a lack of equipment, knowledge and training to make accurate diagnostic decisions. It is the responsibility of primary care and hospitals to improve diagnosis and disease-related outcomes. To reduce the appearance of diagnostic errors and in turn improve the patients' care, health outcomes and quality of life, healthcare systems should explore alternative techniques and biological matrices such as vibrational spectroscopic techniques and fingernails, respectively. Thus, recommendations that have been made based on this research include educational strategies and process improvements.

- Improvement in the early detection of CVDs and/or DM through the use of developed vibrational spectroscopic methods and libraries. Vibrational spectroscopic techniques such as IR, NIR and Raman spectroscopy also offer diagnostic monitoring over a period of six-to-nine months.
- Increased early disease detection and diagnostic compliance rates through the implantation of fingernails as an alternative biological matrix.
- Implement a holistic diagnostic approach whereby all aspects of the patients' well-being are considered.

8.6. Further Research

- Larger-scale studies including a greater number of participants with diverse backgrounds and characteristics should be carried out to further understand the relationship between clinical characteristics and the biological fingerprint of fingernails. These studies should ensure a higher number of CVD and/or diabetic participants are recruited for greater representation. A variety of CVDs should also be included such as aortic disease and PAD.
- In cases whereby classes are underrepresented such as CVD and/or diabetic, future work will explore the feasibility of synthetic data for the prediction of disease. Synthetic data can be applied in the support of medical and healthcare research, especially with small datasets.
- The work undertaken in this thesis demonstrated that handedness influenced the growth rate and deposition of endogenous constituents into fingernails. While the research showed variation in the activity of constituents in fingernails taken from the same finger but different hands, the participants did not declare which clipping came from which hand. Thus, future work will look at confirming handedness on sample collection and identifying the corresponding fingernail sets.
- As demonstrated by this research, AGEs and glycated proteins accumulate in the fingernail plate due to prolonged exposure of glucose (hyperglycaemia). The accumulation of such constituents allows for diagnostic monitoring over a timeframe of six-to-nine months. Within this study, the majority of participants did not declare the duration of diagnosis prior to sampling. Therefore, future work will look at the impact of disease duration on the biological composition of fingernails. Moreover, considering diagnostic monitoring via clippings once a month, will allow future research to assess disease management and the potential requirement for additional strategies.
- The research undertaken in this thesis, focussed primarily on the fingernail's ventral layer. This layer was chosen based on its affinity to the highly vascularised nail bed. However, future work should look at investigating the endogenous constituent content of the intermediate and dorsal layers.
- This research utilised ATR-FTIR, NIR and Raman spectroscopy. Future work will look at utilising diffuse reflectance infrared Fourier transform (DRIFT)

spectroscopy, which will allow deeper penetration of the fingernail, accounting for the chemical variation between the three layers of the fingernails. Moreover, future work will utilise additional Raman lasers such as 1064 nm, which has shown previous success analysing biological compounds and operates at a longer wavelength with lower energy.

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Appendices

Appendix A

Appendix A1 Ethical approval from Liverpool John Moores University

UREC opinion: Favourable ethical opinion with provisos
Research Governance Assessment: Approved – the study may commence.

UREC reference: 23/PBS/009A
Development of an Intelligent Framework for the Detection of Cardiovascular disease and Diabetic Biomarkers in Fingernails and Saliva using Handheld Spectroscopic Techniques

Appendix A2 Ethical approval from the Lebanese University

RAFIK HARIRI UNIVERSITY HOSPITAL  **مستشفى رفيق الحريري الجامعي**

Date: 25-Jan-2022
Reference: 2022-0104

To: Dr. Ismail Abbas
Professor of Physical, Inorganic and Environmental Chemistry
Faculty of Sciences
Lebanese University

From: Iyad Issa, MD
President of Institutional Review Board
Rafik Hariri University Hospital
Bir Hasan-Jnah, Lebanon

Research Title: Evaluating the Feasibility of Handheld Spectroscopic Techniques for Detection of Historic Endogenous Compounds, Drugs and Drug Metabolites in Human Nails

Thank you for submitting to the Institutional Review Board (IRB) the above entitled research and related documents for review:

- Submission letter
- University approval
- Research proposal
- Questionnaire with informed consent (English)
- Participant information sheet

The IRB reviewed the above documents and grants you the approval to the research entitled:

"Evaluating the Feasibility of Handheld Spectroscopic Techniques for Detection of Historic Endogenous Compounds, Drugs and Drug Metabolites in Human Nails"

The membership of this Institutional Review Board complies with the membership requirements in the US Code of Federal Regulations (21CFR56 and 45CFR46) of the Food and Drug Administration. In addition, the IRB operates in a manner consistent with Good Clinical Practices under the ICH guidelines, with FDA and applicable national/local regulations.

Sincerely,

Iyad Issa, MD
President of the IRB
Rafik Hariri University Hospital





Rafik Hariri University Hospital – Institutional Review Board Page 1 of 1



PARTICIPANT INFORMATION SHEET

Project Title: Development of an Intelligent Framework for Early Diagnosis of Cardiovascular Disease and Diabetes Mellitus in Fingernails using Handheld Spectroscopic Techniques

Principal Investigators:

- Megan Wilson, PhD candidate in Toxicology, Pharmaceuticals and Pharmacology, Liverpool John Moores University.
- Supervisors: Dr Sulaf Assi, Senior Lecturer in Forensic Science, Liverpool John Moores University; Dr Jason Birkett Subject Lead in Forensic and Biomolecular Sciences; Dr Iftikhar Khan Senior Lecturer in Pharmaceutics; Prof Dhiya Al-Jumeily OBE Professor in Artificial Intelligence

You are being invited to participate in a research study regarding early detection of cardiovascular diseases and diabetes mellitus in fingernails. Prior to making your decision on whether to participate in this research or not, it is crucial that you are made fully aware and understand why this research is being undertaken. It is also important that you understand what your participation will involve. Please carefully read the following information and ask questions regarding anything that is unclear and if you have any concerns or general questions about the research.

1. Purpose of the Research Study

Cardiovascular diseases (CVDs) and diabetes mellitus (DM) are the leading cause of death globally. The World Health Organisation (WHO) have reported that 20.5 million lives a year are lost to CVDs and 422 million people worldwide have DM. It is common for CVDs' symptoms such as chest pain to be misdiagnosed as gastrointestinal or stress-related, especially among women where CVDs are perceived as a 'man's disease'. Misdiagnosis often causes the symptoms to escalate to a life-threatening condition such as angina or heart failure. A similar trend is observed in DM misdiagnosis, which often escalates to diabetic ketoacidosis.

The most efficient way of dealing with the aforementioned life-threatening complications is early diagnosis, supporting the vision of 'preventing rather than treating'. Diagnosis is often expensive, time consuming and requires invasive procedures (e.g. blood or urine tests). However, alternative biological matrices such as fingernail clippings are non-invasive and can be easily collected by patients. Fingernails have key biomarkers and metabolites that change in CVDs and DM and provide an identification of disease prognosis. However, further research is still necessary to determine the ability of rapid and portable spectroscopic methods for monitoring disease diagnosis and prognosis in fingernails. Therefore, this study aims to use fingernail clippings *in vitro* as a biological matrix for the detection of CVDs and DM. In this respect, no intervention is required to participants in this research apart from obtaining the fingernail clippings that are measured after collection with handheld spectroscopic instruments.

2. Participation

As part of this study, participants will be asked to produce two sets of fingernail clippings, one taken from each hand. The fingernail clippings should be between 2-3 mm in length and should be taken from the fingernails overhang. This procedure is non-invasive and can be carried out by the participant in the comfort of their own home. Samples will be collected by the researcher (Megan Wilson) and stored securely. As a participant, you will be asked to complete a brief questionnaire providing some information regarding sociodemography, medicines and drugs' use and the presence

of any long-term illnesses. The questionnaire will help us interpret the spectroscopic data. The questions will not contain any personal details and all information provided will be anonymised and not shared outside the remit of the study.

3. Will I be photographed or video/audio recorded and how will the recorded media be used?

NA

4. Risks to the Participant

One risk to the participant by providing the sample of fingernail clipping is the release of the participant's name. This, however, has been prevented by providing participants with a unique identification code. There are no risks to the participant during the fingernail clipping procedure as this is a usual procedure conducted by individuals at home.

5. Are there any benefits in taking part?

The potential or hoped benefit of this study is for patients of CVDs and/or DM and healthcare providers in adopting fingernails as a biological matrix for understanding disease diagnosis and prognosis. Handheld spectroscopic instruments are rapid and of low cost when compared to traditional diagnostic instruments. Apart from the cost of the instrument, handheld spectroscopic instruments do not require extra costs associated with their use. Therefore, this study is economically effective for patient and beneficial to their health as early diagnosis prevents life-long complications of CVDs and DM.

6. Confidentiality

All participants will be given unique identification codes. Their donated samples will be referred to by this code. Information provided during the study such as age, sex, ethnicity, use of medication, long-term medical history and drug use will not identify the participant's name. The participation of volunteers will be kept confidential to the

extent permitted by law. All participants will receive a signed and dated copy of the consent form for their own records.

7. Costs for Participation:

7.1 Costs

There are no costs for the participants, who agree to take part in this research.

7.2 Rights

By signing the consent form, participants will not lose any legal rights.

8. Withdrawal

The participation of this research is completely voluntary. Participants are free to withdraw themselves before collection of the fingernail samples without giving any reason. Once fingernail samples are collected, it will be difficult to identify participants and remove the samples as the collection procedure is totally anonymous.

9. Further Questions or Concerns

At any point during the research, participants are free to ask questions or raise concerns/complaints. This should be addressed to researcher (Megan Wilson) or lead supervisor (Dr Sulaf Assi). You should expect a reply within 10 working days. If you remain unhappy or wish to make a formal complaint, please contact the Chair of the Research Ethics Committee at Liverpool John Moores University who will seek to resolve the matter as soon as possible: Chair, Liverpool John Moores University Research Ethics Committee; Email: FullReviewUREC@ljmu.ac.uk; Tel: 0151 231 2121; Research Innovation Services, Liverpool John Moores University, Exchange Station, Liverpool L2 2QP.

10. Data Protection

Liverpool John Moores University is the data controller with respect to your personal data. Information about your rights with respect to your personal data is available from

the link :<https://www.ljmu.ac.uk/legal/privacy-and-cookies/external-stakeholders-privacy-policy/research-participants-privacy-notice>

11. Contact details

Principal Investigator: *Megan Wilson*

LJMU PhD student

LJMU Email address: *[Redacted]*

LJMU School/faculty: *Pharmacy and Biomolecular Sciences*

LJMU Central telephone number: *[Redacted]*

Supervisor Name: *Dr Sulaf Assi*

LJMU Email address: *[Redacted]*

Questionnaire survey:

Please note that you may only participate in this survey if you are 18 years of age or over.

☐ I certify that I am 18 years of age or over

If you have read the information above and agree to participate with the understanding that the data (including any personal data) you submit will be processed accordingly, please tick the box below to start.

☐ Yes, I agree to take part

Appendix A4 Participant recruitment poster



PARTICIPANTS NEEDED FOR RESEARCH STUDY ON DIAGNOSIS OF DISEASE

Study for healthy, cardiovascular and diabetic adults

- We're looking for adults aged 18 or older who are healthy or have been diagnosed with a cardiovascular disease and/ or diabetes mellitus.
- Many cases of cardiovascular diseases and diabetes mellitus go undiagnosed, which leads to further complications. This research seeks to develop spectroscopic methods for the diagnosis of cardiovascular diseases and diabetes mellitus.

Location

Collection of samples will take place at the Byrom Street campus, Liverpool, in laboratory 655.

Eligibility

- 18 years or older
- Healthy individuals OR individuals who are diagnosed with a cardiovascular disease and/ or diabetes mellitus

Participants will be asked to provide:

- A set of fingernail clippings (10 fingernails clippings, taken from each finger)
- A saliva swab
- A short participant questionnaire

Please contact Megan Wilson via email for further information or enrolment

Appendix A5 Participant consent form



PARTICIPANT CONSENT FORM

Study title: Development of an Intelligent Framework for Early Diagnosis of Cardiovascular Disease and Diabetes Mellitus in Nails using Handheld Spectroscopic Techniques.

Research Ethics Committee Reference Number: 23/PBS/009A

Principal Investigator: *Megan Wilson*

LJMU Postgraduate researcher

LJMU Email address: *[Redacted]*

LJMU School/Faculty: *Pharmacy and Biomolecular Sciences*

LJMU Central telephone number: *[Redacted]*

Supervisor Names: Dr Sulaf Assi, Senior Lecturer in Forensic Science, Liverpool John Moores University; Dr Jason Birkett Subject Lead in Forensic and Biomolecular Sciences; Dr Iftikhar Khan Senior Lecturer in Pharmaceutics; Prof Dhiya Al-Jumeily OBE Professor in Artificial Intelligence

If you are happy to participate, please complete and sign the consent form below

		<i>Please initial</i>
1.	I confirm that I have read the information sheet dated.....for the above study, or it has been read to me. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand what taking part in the study involves	
3.	I consent voluntarily to be a participant in this study and understand that I can refuse to answer questions and I can withdraw from the study prior to donating fingernail samples, without giving a reason and without penalty or my legal rights being affected.	
4.	I have been advised about potential risks associated with taking part in this study and have taken these into consideration before consenting to participate	
5.	I give permission for the investigator to take possession of my fingernail clippings	
6.	I agree that the fingernail clippings will not be returned to me	
7.	I give permission to the investigator to take a copy of <i>the questionnaire</i> and to use the information in interpreting the data obtained upon measuring fingernails using spectroscopic techniques	
8.	I consent to my fingernail sample(s) being stored securely at LJMU <i>laboratory BS/811</i> until they have been analysed – and used for the research purposes outlined in the Participant Information Sheet.	
9.	I understand that no personal data will be collected from myself and sensitive data collected will remain confidential and that all efforts will be made to ensure they cannot be identified in reports or any further outputs	
10.	I agree to take part in this study	

Data Protection. Any personal information we collect and use to conduct this study will be processed in accordance with data protection law as explained in the Participant Information Sheet and the [Privacy Notice for Research Participants](#).

Name of Participant _ _ _ _ _ Date _ _ _ _ _ Signature _ _ _ _ _

For participants unable to sign their name, mark the box instead of signing

☐

I have witnessed the accurate reading of the consent form with the potential participant and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Investigator _ _ _ _ _ . Date _ _ _ _ _ . Signature _ _ _ _ _

Name of Person taking consent _ _ _ _ _ . Date _ _ _ _ _ . Signature _ _ _ _ _

(if different from investigator)

Appendix A6 Participant questionnaire



PARTICIPANT QUESTIONNAIRE

Introduction:

Diagnostic error remains a significant concern for the health sector and its users, especially as they subsequently lead to negative implications such as psychological distress, increased financial costs, inappropriate treatment and further life-threatening implications. Recent figures showed that diagnostic errors significantly affected cardiovascular and diabetic patients, with several patients receiving late or incorrect diagnoses. To prevent diagnostic error, this work aims to develop an intelligent framework for the early diagnosis of cardiovascular diseases and diabetes mellitus. Furthermore, this work aims to look at the detection of disease using novel biological matrices such as fingernails, which are non-invasive, non-intrusive and have a simple collection process.

During this survey you will be asked to fill in some background information regarding your clinical history in terms of diagnosis and medicinal use and will focus primarily on cardiovascular diseases and diabetes mellitus. This survey will also look at factors that may influence the spectroscopic signature of fingernails including: sociodemography, long-term conditions, medicinal and cosmetic use.

Please answer these questions honestly and to the best of your ability. This should take about 20 minutes.

Your participation is entirely voluntary and you are free to withdraw at any point prior to the collection of fingernails, after which all data and samples will be completely anonymised. Data collected will be referred to via an anonymised code, thus your participation will not be disclosed. Data will be stored and handled following the Data Protection Act 2018. If you do wish to withdraw from the study, please contact the researcher using the provided contact information and the unique code you were provided with.

If you do not wish to answer a particular question, then please leave it blank. There are no right or wrong answers, so please answer as you deem appropriate and using your own personal point of view.

If you have any further questions or inquiries, please contact the principal investigator using the email provided.

Part I: Demographic

What is your age?

What is your gender?

Male ☐ Female ☐ Prefer not to say ☐

What is your ethnic background?

White ☐ Black ☐ Asian ☐

Mixed ☐ Other ☐

Do you practice religion, if so, which religion do you practice?

Yes ☐ No ☐

If yes, please specify.

What is your marital status?

What is your education level?

What is your occupation?

How many people live at your home?

Part II: Clinical Factors

Do you have any medical conditions?

Yes ☐ No ☐

If yes, please list all the conditions.

Do you have diabetes mellitus? Including Type 1 and Type 2 diabetes.

Yes ☐ No ☐

If yes, please specify the diagnosis duration.

Do you take any medicines for diabetes mellitus?

Yes ☐ No ☐

If yes, please mention all.

Have you been to A&E or seen an emergency doctor because of diabetes mellitus?

Yes ☐ No ☐

Do you have any cardiovascular disease(s)?

Yes ☐ No ☐

If yes, please list all.

Do you take any medicines for any cardiovascular disease(s)?

Yes ☐ No ☐

If yes, please list all.

If you have hypertension/hypotension, does your blood pressure fluctuate?

Yes ☐ No ☐

Do you have a family history of any disease(s)?

Yes ☐ No ☐

If yes, please specify.

Do you take any medicines for other medical condition(s)?

Yes ☐ No ☐

If yes, please list all.

How many medicines do you take?

How frequently do you take your medicines?

Do you skip your medicines intake for any reason?

Yes ☐ No ☐

If yes, please specify.

Are you happy with the management of your condition(s)?

Yes ☐ No ☐

Please specify.

Part III: Life-style Factors

Are you a smoker?

Yes ☐ No, ex-smoker ☐

No, never smoked ☐

If yes, please specify how frequently.

If yes, what do you smoke?

Cigarettes ☐ Vapes ☐ Sheesha ☐

What is your weight? Do you suffer from overweight?

Yes ☐ No ☐

If yes, did the doctor advise you to lose weight?

What is your height?

How active do you consider your life-style? E.g. carrying out exercises/activities that make your heart rate beat faster.

What kind of exercise do you participate in?

Do you work?

Yes ☐ No ☐

If yes, please specify.

How many meals do you have a day?

How healthy do you consider your diet? What does a normal mealtime consist of?

How many drinks/snacks do you have a day? What do they consist of?

Do you eat fast food?

Yes ☐ No ☐

If yes, how frequently do you eat fast food?

Appendix A7 Fingernail collection standard operating procedure



STANDARD OPERATING PROCEDURE

SAMPLING PROCEDURE FOR FINGERNAILS CLIPPINGS

The purpose of this standard operating procedure (SOP) is to outline the sampling procedure for clipping fingernails. This SOP is aimed at participants involved in the donation of fingernails for the detection of cardiovascular diseases and/or diabetes mellitus in fingernails. Participants should follow the SOP to the best of their ability. Queries and further questions can be asked by making contact with the principal investigator (Megan Wilson).

1. Cleaning

- **IF APPLICABLE:** Remove all nail polish by soaking in acetone.
- Wash hands thoroughly with water and soap. Remove dirt and other contaminants from beneath the fingernail overhang using a fingernail brush.
- Dry hands and begin the sampling procedure immediately.

2. Sampling

- Using fingernail clippers, clip approximately 2 – 3 mm of the fingernail overhang. As seen in Figure 1, the fingernail overhang is the excess growth of the fingernail plate.

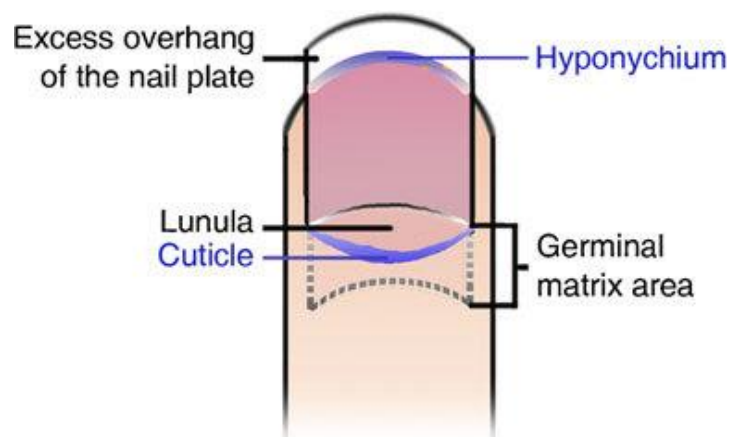


Figure 1. Schematic diagram of the anatomical structure of a fingernail (Hang, Xiang and Shen, 2013).

- Repeat for each finger for a complete set of ten fingernail clippings.
- Fingernail clippings should be stored in a clear zip lock/food bag that can be found in the participant's home.

3. Donation

- Participants are to bring their completed questionnaire and set of fingernails to laboratory BS/655.
- No further participation.

References:

Hang, C., Ping, X. and Min, S. (2013) 'Long-term follow-up analysis of Zolpidem in fingernails after a single oral dose', *Analytical and Bioanalytical Chemistry*, 405(23), pp. 7281–7289. doi:10.1007/s00216-013-7188-3.

Appendix A8 ATR-FTIR standard operating procedure

Agilent 4500a ATR-FTIR Spectrometer

Daily Memos:

- Record room temperature
- Record room humidity
- Perform a performance verification test and save results daily. You must not start analysis before carrying out a performance verification test. A successful test will be indicated by a green circle at the top left of the software screen; this shows that analysis can begin. A yellow or red circle at the top left of the software screen is an indication that the instrument is operating outside of the factory defined parameters and must be altered.
- Results can be validated by running a known sample with a sample specific method.
- Performance validation tests should be carried out on an annual basis and demonstrate whether the instrument is performing within its specification.
- If in doubt on any procedure read the Agilent 4500a ATR-FTIR Spectrometer operation user manual.
- Data must be backed up every day.
- ATR diamond holder must be cleaned before and after using isopropanol (CAS 67-63-0). You must never assume that the ATR diamond is cleaned.

Turn on Computer:

- Agilent computer
- **Computer Login:** Agilent
- No password.

MicroLab PC:

- Once logged on to the computer, double click on the MicroLab PC link on the computer's desktop.
- A box will appear with the software information. Print screen when using it for the first time for future reference.

ATR-FTIR Spectra

- The instrument must have a warmup period of five minutes before reliable analysis begins.
- Before analysis begins, a background spectrum should be carried out as this provides a baseline profile of the systems conditions with no sample loaded. The collection of a background before analysis, ensures that changes in the environment do not have a negative effect on the sample.
- X-axis: Wavenumber 650-4000 cm^{-1}
- Y-axis: Absorbance
- ATR-FTIR is specifically used for molecular compound identification and quantification. Absorbance of light creates a unique spectral fingerprint, which can be used to identify the sample's molecular structure and its exact quantity.

MicroLab Screen Guide:

- **Acquire:** Button on the bottom left of the spectral screen. This is used for acquiring spectral readings.
- **MicroLab PC Browser:** The column at the top of the screen, which highlights the spectra being analysed.
- **Top Right:** Demonstrates the red/orange or green circle, which highlights whether the system is working within the correct factory parameters.
- **Bottom Left:** Sends you back to the home page of the MicroLab PC software. Here you can alter and activate your chosen method and its parameters.

What to do if the top left circle is red/orange:

- Double click on the red/orange circle, it will open and demonstrate the instrument's status window.
- Elements highlighted in green are working correctly and should not be altered.
- If the ATR diamond is highlighted in red and labelled as failed, the diamond should be cleaned with isopropanol (CAS: 67-63-0) and a soft tissue.
- Double click on the ATR diamond button that is highlighted in red and run a performance verification test
- Once the test has been carried out, a window will open with the status of the performance verification test. This document should be saved. This document will highlight in green writing, if the procedure was successful or not.

What the Performance Validation test analyse:

- **Energy Level:** measures the energy level of the instrument based on the interferogram voltage or height.

- **Laser Frequency Calibration Test:** Measures the frequency and accuracy. This test requires, running a spectrum of polystyrene film, which is pressed firmly against the diamond holder during sample collection. Absorbance frequencies from this spectrum are compared to frequencies set by NIST SRM 1921 polystyrene film. The software allows administrative-level users to use the test results to set the laser calibration.

- **Performance (Signal-to-noise) Test:** Measures the signal-to-noise (S/N) level at two regions of the IR spectrum, 2500 cm^{-1} and 1000 cm^{-1} , respectively. The signal-to-noise is defined as the reciprocal of the root mean squared (RMS) noise the defined region for a blank sample, which is measured with a blank background. The background and sample are measured at a resolution of 4 cm^{-1} with a one-minute collection time. Tests take two minutes per test, but 10 tests should be collected to produce an accurate picture of the performance.

- **Stability Test:** Measures the short-term stability at two regions of the spectrum 3000 cm^{-1} and 1000 cm^{-1} . Stability is a measurement of the baseline differences, which are observed over the selected time period. This test measures a background at the beginning, then one sample every minute for the duration of the test as specified in the 'Number of minutes' field in the software. Test results are expressed as %transmittance of maximum deviation during the stability test.

How to Perform an ATR-FTIR Spectral Reading:

- Make sure the ATR diamond holder is cleaned using isopropanol (CAS: 67-63-0) and a soft tissue.

- Click on the acquire button on the bottom right corner.

- Click on advanced test on the left-hand side of the screen, this will open a window.

- Go to the advanced tab:

1. Change path to save the spectra to the wanted file by clicking on the three dots next the path box and choosing the desired file destination. It is recommended that you create and select your own independent folder.

2. Write the sample name in the appropriate box at the top of the screen.

3. The same scan time should be changed to 64 scans.

4. Background scans time should be saved to 64 scans.

5. Data should be saved from $4,000\text{ cm}^{-1}$ to 650 cm^{-1} .

6. Resolution should be set up to four for powders or two for liquids.

7. Method should be saved.

- Go back to the first tab and make sure the ATR diamond is fully open.
- Click the run button and wait for the background scan to be complete.
- Name the sample using the MicroLab PC browser column.
- Once the background scan has been collected and sample named, click on the run button. The previously open window will close, and the spectral graph area will appear.
- Click on the mode tab and chose the wanted mode to save the spectra as.

How to Measure the S/N Ratio:

- Print the screen with the spectra wanted, this should be carried out once for every sample batch.
- Paste pressing ctrl+V into Word. The image can be zoomed into if necessary.
- Lines should be drawn at the top and bottom on the part of the spectrum, which is noise (noise power). Measure the distance between these lines using a ruler (N)
- Draw a stop and bottom line on the highest band (signal power) and measure the distance between lines with a ruler (S).
- Divide S by N and calculate the ratio between the two. This should be a unitless number.

Prepared by Megan Wilson

18/02/2022

Appendix B

Appendix B1 Participant's data

Table 1. Characteristics of healthy participants (n = 68) (part one).

Participants	Age Range	Biological sex	Ethnicity	Religious belief	Marital status	Education level	Occupation	Household	Medical Condition	Medicines
MWS1	40-64	F	Arab	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn	2-8 cigarettes daily	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS2	40-64	M	Arab	No	diclofenac, occasionally	E-cigarettes frequently shisha occasionally tobacco occasionally	No	Four	No	diclofenac, occasionally
MWS3	40-64	M	White	No	No	No	No	Two	No	No
MWS4	18-24	F	White	No	No	No	No	Five	No	No
MWS5	40-64	F	Arab	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily	2-8 cigarettes daily	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn

					paracetamol prn					
MWS6	18-24	F	Asian	No	Single	BSc	Student and teaching support officer	One	No	paracetamol, occasionally
MWS7	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS8	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS9	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	diclofenac gel ibuprofen prn ipratropium bromide prn mebeverine HCl daily omeprazole daily paracetamol prn
MWS10	40-64	M	Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	diclofenac
MWS11	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	diclofenac gel ibuprofen prn ipratropium bromide prn mebeverine HCl daily omeprazole daily paracetamol prn
MWS12	18-24	F	White	No	Cohabiting	BSc	Student and teaching support officer	Two	No	Microgynon 30 (levonorgestrel/ethinylestradiol) daily
MWS13	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn

										loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS14	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS17	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS18	40-64	M	Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	diclofenac occasionally
MWS19	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS20	40-64	M	Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	diclofenac occasionally
MWS21	18-24	F	Arab	Islam	Single	College qualifications	Student	Four	No	aspirin (75 mg) during Winter
MWS22	18-24	F	Arab	Islam	Single	College qualifications	Student	Three	No	Roaccutane (isotretinoin)
MWS23	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	loratadine mebeverine HCl omeprazole
MWS24	18-24	F	White	No	Single	A-levels	Students	Four	No	Microgynon 30 (levonorgestrel/ethinylestradiol) daily

MWS25	40-64	M	White	No	Divorced	No formal qualifications	Mechanic and auto electrician	Two	No	No
MWS26	40-64	M	White	No	Divorced	No formal qualifications	Mechanic and auto electrician	Two	No	No
MWS27	40-64	M	White	No	Divorced	No formal qualifications	Mechanic and auto electrician	Two	No	No
MWS28	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	loratadine mebeverine HCl omeprazole
MWS30	18-24	M	White	No	0	0	0	0	No	No
MWS31	18-24	M	White	No	0	0	0	0	No	No
MWS33	40-64	M	Arab	0	0	0	0	0	No	paracetamol, occasionally
MWS34	18-24	M	Asian	0	0	0	0	0	No	No
MWS36	40-64	M	White	0	0	0	0	0	No	No
MWS37	40-64	F	Lebanese Arab	0	0	0	0	0	No	diclofenac occasionally ipratropium bromide occasionally loratadine daily mebeverine HCl daily omeprazole daily
MWS38	40-64	F	Lebanese Arab	0	0	0	0	0	No	diclofenac occasionally ipratropium bromide occasionally loratadine daily mebeverine HCl daily omeprazole daily
MWS39	40-64	M	Arab	0	0	0	0	0	No	ibuprofen occasionally paracetamol occasionally
MWS40	18-24	F	White	No	Single	A-levels	Student	Five	No	desogestrel (75 mcg)
MWS42	25-34	M	White	No	Cohabiting	BEng	Engineer	Two	No	paracetamol, occasionally
MWS45	35-39	F	Arab	0	0	0	0	0	No	Dulcolax (bisacodyl)
MWS53	40-64	F	Arab	0	0	0	0	0	No	No
MWS54	40-64	M	Arab	0	0	0	0	0	No	Ibuprofen paracetamol tramadol HCl

MWS67	40-64	F	Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	loratadine mebeverine HCl omeprazole
MWS68	40-64	M	Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	ibuprofen occasionally paracetamol occasionally
MWS70	40-64	F	White	No	Cohabiting	BSc, MSc and PhD	Senior lecturer	Two	No	Dymista nasal spray (azelastine HCl and fluticasone) fexofenadine HCl
MWS72	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	diclofenac occasionally ipratropium bromide occasionally loratadine daily mebeverine HCl daily omeprazole daily
MWS74	40-64	M	Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	ibuprofen occasionally paracetamol occasionally
MWS75	18-24	M	Asian	No	Single	Postgraduate	Cloud architect	Three	No	No
MWS80	25-34	F	Asian	Hinduism	Married	MSc	0	Six	No	No
MWS81	25-34	F	Asian	Hinduism	Single	MSc	Data analyst	Five	No	No
MWS82	25-34	M	Indian	Hinduism	Single	MSc	Senior data scientist	Four	No	No
MWS83	35-39	M	Arab	Islam	Married	PhD	Researcher	Six	No	No
MWS85	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	diclofenac occasionally ipratropium bromide occasionally loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS89	18-24	F	Mixed	0	0	0	0	0	No	isotretinoin (20 mg) daily for six months
MWS93	18-24	F	Asian	0	0	0	0	0	No	No
MWS94	18-24	F	White	0	0	0	0	0	No	No
MWS102	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily

										mebeverine HCl daily omeprazole daily paracetamol prn
MWS103	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS104	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS106	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS107	40-64	M	Middle Eastern Arab	Islam	Married	PhD	Professor	Four	No	ibuprofen and paracetamol occasionally
MWS108	18-24	M	White	No	Cohabiting	BEng	Marine design engineer	Two	No	paracetamol, occasionally
MWS109	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS111	40-64	M	White	No	Engaged	No formal qualifications	Pathology laboratory assistant	Two	No	No
MWS112	40-64	F	White	No	Married	BSc, MSc and PhD	Senior lecturer	Two	No	dymista nasal spray (azelastine and fluticasone)

										fexofenadine HCl Hay fever medication daily
MWS113	40-64	F	White	No	Married	BSc, MSc and PhD	Senior lecturer	Two	No	dymista nasal spray (azelastine and fluticasone) fexofenadine HCl Hay fever medication daily
MWS114	40-64	F	White	No	Married	BSc, MSc and PhD	Senior lecturer	Two	No	dymista nasal spray (azelastine and fluticasone) fexofenadine HCl Hay fever medication daily
MWS115	40-64	F	Lebanese Arab	Islam	Single	BSc, MSc and PhD	Senior lecturer	One	No	ibuprofen prn ipratropium bromide prn loratadine daily mebeverine HCl daily omeprazole daily paracetamol prn
MWS116	40-64	M	Middle Eastern Arab	Islam	Married	BSc, MSc, MPhil and PhD	Professor	Four	No	ibuprofen and paracetamol occasionally
MWS122	40-64	M	White	No	Engaged	No formal qualifications	Pathology laboratory assistant	Two	No	No
MWS123	40-64	M	White	No	Engaged	No formal qualifications	Pathology laboratory assistant	Two	No	No

Table 1. Characteristics of healthy participants (n = 68) (Part two).

Skip medication	Family history of disease	Vitamins	Happy disease management	Smoke	Weight	Height	Exercise	Meals a day	Diet
Medicine taken when required	No	Biotin collagen hyaluronic acid vitamin C vitamin D	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	E-cigarettes frequently Shisha and tobacco occasionally	0	0	Exercise and active, attends the gym	Three	No specific diet type
NA	No	No	NA	No	12 st	5'11	Occasionally practices weights	Three	No specific diet type
NA	No	No	NA	Yes, vape daily and smoke 1-2 cigarettes daily	0	5'8	Occasionally go running	Two	Not extremely healthy, some snacks like crisps and biscuits
Medicine taken when required	No	Biotin collagen hyaluronic acid vitamin C vitamin D	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	No	0	0	Occasional gym and cardio	Three	No specific diet type
Medicine taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	Biotin Collagen Hyaluronic acid	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant

		Vitamin C daily Vitamin D daily							
Medicine taken when required	No	Biotin Collagen drinks Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	E-cigarettes frequently Shisha and tobacco occasionally	0	0	Exercise and active, attends the gym	Three	No specific diet type
Medicine taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
No	Stroke	Vitamin C and D occasionally	NA	No	10 st, 13 lbs	5'10	Not active	Three	High amounts of carbohydrates
Medicine taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Some medicine taken when necessary	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	E-cigarettes frequently Shisha and tobacco occasionally	0	0	Exercise and active, attends the gym	Three	No specific diet type

Medicine taken when required	No	Biotin Collagen drinks Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	E-cigarettes frequently Shisha and tobacco occasionally	0	0	Exercise and active, attends the gym	Three	No specific diet type
Taken seasonally	No	Cod liver oil with omega 3 (410 mg) Vitamin A Vitamin D Perfectil	NA	No	0	0	Not active	Three	No specific diet type
No	No	No	NA	No	0	0	Not active	Three	No specific diet type
Taken daily	No	Vitamins	NA	Yes, daily	0	0	Active, gym and cardio	Three	No specific diet type
No	No	Biotin Multivitamins	NA	No	0	6'0	Not active	Two	No specific diet type
NA	No	No	NA	No	12 st	5'11	Not active	Three	No specific diet type
NA	No	No	NA	No	12 st	5'11	Not active	Three	No specific diet type
NA	No	No	NA	No	12 st	5'11	Not active	Three	No specific diet type
Taken daily	No	Vitamins	NA	Yes, daily	0	0	Active, gym and cardio	Three	No specific diet type
NA	No	Creatine monohydrate	NA	No	0	0	Yes, four times a week	Three	No specific diet type
NA	No	Creatine monohydrate	NA	No	0	0	Yes, four times a week	Three	No specific diet type
Medicine taken when required	0	Vitamin C and D	0	E-cigarettes occasionally	0	0	Not active	0	No specific diet type
NA	0	No	NA	No	0	0	No	0	No specific diet type
NA	0	No	NA	No	0	0	Frequently exercise	0	No specific diet type
diclofenac and ipratropium bromide taken when needed	0	Biotin Collagen drinks Vitamin C daily Vitamin D daily	0	Cigarettes and E-cigarettes daily	0	0	No active	0	Halal meat

diclofenac and ipratropium bromide taken when needed	0	Biotin Collagen drinks Vitamin C daily Vitamin D daily	0	Cigarettes and E-cigarettes daily	0	0	Occasional exercise	0	Halal meat Lactose intolerance Mostly vegetarian
0	0	Vitamin C and D	0	No	0	0	Yes	0	Halal meat
Taken daily	No	No	NA	E-cigarettes	0	0	Frequently exercise, gym multiple times a week	Three	High protein diet
Medicine taken when required	No	No	NA	No	0	0	Frequent exercise, gym multiples times a week	Two	High protein diet
0	0	Multivitamin daily Ten herbs Vitamin D once a week	0	No	0	0	Not active	0	No specific diet type
NA	0	No	0	Sheesha once a week	0	0	Yes, frequently	0	No specific diet type
NA	0	No	0	Yes, daily	0	0	No	0	Halal meat
No	No	Vitamins	NA	Yes, daily	0	0	Active, gym and cardio	Three	Halal meat
Medicine taken when required	No	No	NA	No	0	0	Active and exercise	Three	Halal meat
Medicine taken when required	No	Calcium Vitamin D Zinc supplements	NA	No	0	0	Not active	Three	No specific diet type
diclofenac and ipratropium bromide taken when needed	No	Biotin Collagen drinks Vitamin C Vitamin D	NA	Yes, daily	0	0	Active, gym and cardio	Three	Halal meat
Medicine taken when required	No	Vitamin D	NA	No	0	0	Active and attend the gym	Three	Halal meat
NA	No	No	NA	No	0	79 cm	Pretty active, 10k steps a day, takes part in football and swimming once a month and jogging once a week	Two or three	Very healthy

NA	No	No	NA	No	0	5'6 ft	Quite active, walking, hikes and some sports such as badminton	Three	Quite healthy, lots of proteins
NA	DM Hypertension	No	NA	No	70 kg	5'8 ft	Not very active, occasionally goes walking	Three	Healthy balanced diet
NA	No	No	NA	No	75 kg	5.47 ft	Yes, 2-3 times a week including, cardio, running and walking	Three	Balanced diet including pasta, rice vegetables and dairy products. Occasionally have fast food
NA	No	No	NA	No	72 kg	173 cm	Gym occasionally	Three	Fine
diclofenac and ipratropium bromide taken when needed	No	Biotin Collagen drinks Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Active, gym and cardio	Three	Lactose intolerant
Taken daily	0	No	0	No	0	0	No	0	No specific diet type
NA	0	No	0	No	0	0	Yes, daily	0	No specific diet type
NA	0	No	0	No	0	0	Three times a week	0	No specific diet type
ibuprofen and ipratropium bromide taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
ibuprofen and ipratropium bromide taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
ibuprofen and ipratropium bromide taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
ibuprofen and ipratropium	No	Biotin Collagen Hyaluronic acid	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant

bromide taken when required		Vitamin C daily Vitamin D daily							
Forgetful	Cancer	Vitamin C and D	NA	Yes, e-cigarettes and sheesha occasionally	70 kg	166 cm	Very active, exercise frequently including boxing, gym, swimming and running	Three	Healthy diet, occasional snacking
Medicine taken when required	No	No	NA	Ex-smoker of cigarettes and vapes	100 kg	5'11 ft	Exercise at least three times a week at the gym, gold and walking	Two	Beef, chicken and pasta eaten frequently
ibuprofen and ipratropium bromide taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
No	No	No	NA	No	0	0	Used to run frequently, job requires to be on feet majority of the day	Two	Occasionally skips meals, eats snacks of chocolate, biscuits and sweets
No	No	Calcium Vitamin D Zinc supplements	NA	No	0	0	Occasionally exercise, walking	Three	Lactose intolerant
No	No	Calcium Vitamin D Zinc supplements	NA	No	0	0	Occasionally exercise, walking	Three	Lactose intolerant
No	No	Calcium Vitamin D Zinc supplements	NA	No	0	0	Occasionally exercise, walking	Three	Lactose intolerant
ibuprofen and ipratropium bromide taken when required	No	Biotin Collagen Hyaluronic acid Vitamin C daily Vitamin D daily	NA	Yes, 2-8 cigarettes daily	0	0	Exercise and active, attends the gym	Two or three	Lactose intolerant
Medicine taken when required	No	No	NA	Occasionally smoke	0	0	Exercise frequently with boxing, gym, swimming and running	Three	Healthy diet

				e-cigarettes and sheesha					
No	No	No	NA	No	0	0	Used to run frequently, job requires to be on feet majority of the day	Two	Occasionally skips meals, eats snacks of chocolate, biscuits and sweets
No	No	No	NA	No	0	0	Used to run frequently, job requires to be on feet majority of the day	Two	Occasionally skips meals, eats snacks of chocolate, biscuits and sweets

Table 2. Characteristics of unhealthy participants (n = 23) (Part one).

Participants	Age Range	Biological sex	Ethnicity	Religious belief	Marital status	Education level	Occupation	Household	Medical Condition	Medicines
Androgenetic alopecia										
MWS76	25-34	M	Indian	No	Single	MSc	Full stack developer	Four	Androgenetic alopecia (ED70.0)	finasteride (2-3 daily)
Anxiety										
MWS16	40-64	F	White	No	Engaged	No formal qualifications	Care assistant	Two	Anxiety (MB24.3)	CBD oil
MWS32	40-64	F	White	No	Engaged	No formal qualifications	Care assistant	Two	Anxiety (MB24.3) Asthma (CA23)	CBD oil Fostair inhaler (beclometasone with formoterol) paracetamol
MWS35	40-64	F	White	No	Engaged	No formal qualifications	Care assistant	Two	Anxiety (MB24.3) Asthma (CA23)	CBD oil Fostair inhaler (beclometasone with formoterol) paracetamol
MWS41	25-34	F	White	No	Married	BSc	Laboratory technician	Two	Anxiety (MB24.3) High cholesterol (5C80.0Z) IBS (DD91.0Z)	Buscopan (hyoscine butylbromide) citalopram
MWS69	25-34	F	White	No	Married	BSc	Laboratory technician	Two	Anxiety (MB24.3) High cholesterol (5C80.0Z) IBS (DD91.0Z)	Buscopan (hyoscine butylbromide) citalopram
MWS84	35-39	M	White	No	0	PhD	Research assistant	Two	Anxiety (MB24.3) Chronic sinusitis (CA0A.Z)	propranolol HCl (10 - 40 mg)

MWS105	25-34	M	White	No	Cohabiting	BSc, MSc and PhD	Senior lecturer	Two	Depression (6A7Z) Anxiety (MB24.3) Chronic sinusitis (CA0A.Z) Depression (6A7Z)	propranolol HCl (10 - 40 mg)
Asthma										
MWS110	40-64	F	White	No	Engaged	No formal qualifications	Care assistant	Two	Asthma (CA23) Anxiety (MB24.3)	Fostair inhaler (beclometasone/formoterol) daily paracetamol occasionally
MWS121	40-64	F	White	No	Engaged	No formal qualifications	Care assistant	Two	Asthma (CA23) Anxiety (MB24.3)	Fostair inhaler (beclometasone/formoterol) daily paracetamol occasionally
Bipolar										
MWS77	18-24	M	Asian	Islam	Single	Undergraduate	Software engineer	Three	Bipolar (6A6Z)	Three tablets taken, unspecified
BPH										
MWS49	65-84	M	Lebanese Arab	0	0	0	0	0	BPH (GA90) High cholesterol (5C80.0Z) Meniere's disease (AB31.0) Parkinson's disease (8A00.0Z)	aspirin (81 mg) cinnarizine Betaserc (betahistine dihydrochloride) (16 mg) Sinemet (carbidopa/levodopa) (25mg/100mg)
Breast cancer										
MWS15	40-64	F	White	No	Married	BSc and MSc	Forensic accountant	Four	Breast cancer (2C6Z)	letrozole daily Taxotere (docetaxel)
Coeliac disease										
MWS61	35-39	F	Arab	Islam	0	0	0	0	Coeliac disease (DA95) Crohn's disease (DD70.Z)	Gastropan (pantoprazole) Mephigastryl (bismuth subsalicylate) Salofalk (mesalazine) Soflax (docusate sodium)

COPD										
MWS117	65-84	M	White	No	0	0	0	0	COPD (CA22.Z) Low blood pressure (BA2Z)	sotalol HCl
Depression										
MWS99	35-39	F	Lebanese Arab	0	0	0	0	0	Depression (6A7Z) Tensional headaches (8A81.Z)	paroxetine (20 mg)
FMF										
MWS50	18-24	F	Lebanese Arab	0	0	0	0	0	FMF (4A60.0)	colchicine
High blood pressure										
MWS98	25-34	F	White	0	0	0	0	0	High blood pressure (MC80.0Z)	Aldomet (methyldopa) (250 mg)
Hyperthyroidism										
MWS78	25-34	M	Asian	No	Married	University degree	Salaried occupation	Two	Hyperthyroidism (5A00.Z)	levothyroxine
MWS90	40-64	F	Asian	0	0	0	0	0	Hyperthyroidism (7 years diagnosis) (5A02.Z)	Euthyrox (levothyroxine) (100 mg) Probetol (propranolol HCl) (10 mg) Tarden (atorvastatin) (10 mg)
Phenylketonuria										
MWS29	18-24	M	White	No	Single	A-levels	Student	Four	Phenylketonuria (5C50.0Z)	No
Photosensitive epilepsy										
MWS96	18-24	M	Arab	0	0	0	0	0	Photosensitive epilepsy (8A61.40)	Briviact (brivaracetam) Convulex retard (valproic acid) Petnidan (ethosuximide)
Rhinitis										

MWS101	18-24	F	Arab	0	0	0	0	0	Rhinitis (CA09.0)	Isosupra (isotretinoin) (16 mg) Zyrtec (cetirizine HCl)

BPH: Benign prostatic hyperplasia; COPD: Chronic obstructive pulmonary disease; FMF: Familial mediterranean fever.

Table 2. Characteristics of unhealthy participants (n = 23) (Part two).

Skip medication	Family history of disease	Vitamins	Happy disease management	Smoke	Weight	Height	Exercise	Meals a day	Diet	Fast-food
Androgenetic alopecia										
No	No	No	Yes	No	70 kg	5'4 ft	Active, takes part in cycling, dance, swimming and running	Two	Healthy diet	Yes
Anxiety										
Medicine taken when required	Stroke	No	Yes	No	0	5'9 ft	Not active	Three	No specific diet type	No
CBD oil and paracetamol taken when required	Stroke	No	Yes	No	0	5'9 ft	Not active	Three	No specific diet type	No
CBD oil taken when required	Stroke	No	Yes	No	0	0	Not active	Three	No specific diet type	No
Buscopan taken when needed	No	No	Yes	No	0	0	No active	Three	No specific diet type	No
No	No	No	Yes	No	0	0	Not active	Three	No specific diet type	No
Infrequently when needed	No	No	Yes	No	81.2 kg	6 ft	Some exercise focussing on cardio	Three	Reasonably healthy diet, meals consist mostly of either rice, pasta or bread and meat or cheese.	Yes

Medicine taken when required	No	No	Yes	Infrequently smoke cigarettes	0	0	Gym occasionally, mainly cardio	Two	Balanced diet including pasta, rice, vegetables and dairy products. Occasionally have fast food	Yes
Asthma										
paracetamol taken when required	Stroke	Vitamin D	Yes	No	0	0	Job is extremely active and requires moving and handling	Three	Varied diet	Yes
paracetamol taken when required	Stroke	Vitamin D	Yes	No	0	0	Job is extremely active and requires moving and handling	Three	Varied diet	Yes
Bipolar										
No	DM	No	Yes	No	61 kg	165 cm	Exercise includes jumping jacks and pullups	Three	Healthy diet	Yes
BPH										
0	0	No	0	No	0	0	Yes, walking daily	0	No specific diet type	No
Breast cancer										
No	Stroke	No	Yes	No	0	5'9 ft	Not since September 2021	Three	No specific diet type	No
Coeliac disease										
No	No	Vitamin D	Yes	No	0	0	Not active	0	Gluten free	No
COPD										
No	No	No	Yes	Yes, twice daily	0	0	Gardening and table tennis	Three	Good diet, balanced with meat and	No

										vegetables. Mostly fresh food	
Depression											
0	0	No	0	20-25 cigarettes daily	0	0	Not active	0	No specific diet type	No	
FMF											
0	0	Vitamin C and D	0	No	0	0	No	0	No specific diet type	No	
High blood pressure											
0	0	Vitamin D	0	No	0	0	Not active	0	No specific diet type	No	
Hyperthyroidism											
No	DM	No	Yes	No	Overweight	178 cm	Gym twice a week	Three	Very healthy	No	
0	0	Vitamin D	0	No	0	0	No	0	No specific diet type	No	
Phenylketonuria											
NA	No	75 Phlexy- 10 tablets daily 6 Phlexy vitamins daily.	Yes	No	0	0	Yes, six times a week	Three	Low protein diet, most protein comes from supplementation	No	
Photosensitive epilepsy											
0	0	Vitamin D	0	Passive smoker about three times a week	0	0	Twice a week	0	No specific diet type	No	
Rhinitis											
Taken daily	0	Iron (every period) Vitamin D (5 times a week)	0	No	0	0	Not active	0	No specific diet type	No	

Table 3. Characteristics of participants with CVDs (n = 15) (Part one).

Participants	Age Range	Biological sex	Ethnicity	Religious belief	Marital status	Education level	Occupation	Household	Medical Condition	Medicines
Atrial fibrillation										
MWS44	40-64	M	White	0	0	0	0	0	Atrial fibrillation (BC81.3Z)	sotalol HCl (80 mg)
Heart disease										
MWS48	65-84	F	Lebanese Arab	0	0	0	0	0	Asthma (CA23) Heart disease (BC4Z) Kidney disease (GC2Z)	Concor (bisoprolol fumarate) HandiHaler inhaler (tiotropium bromide) HISTamed (pseudoephedrine and triprolidine) Lasix (furosemide) Regula (triaryl methane) Spiriva (tiotropium bromide) Tapazole (methimazole)
MWS86	40-64	M	White	Yes	Married	No formal qualifications	Cleaner	Three	Heart disease (BC4Z) High blood pressure (MC80.0Z)	aspirin (75 mg) bendroflumethiazide (2.5 mg) irbesartan (300 mg) Monomil XL (isosorbide mononitrate) (60 mg) Tildiem LA (diltiazem HCl) (200 mg) All taken daily
Hypertension										
MWS46	40-64	F	Asian	0	0	0	0	0	Hypertension (BA00.Z) Hyperthyroidism (5A02.Z)	Amlor (amlodipine besylate) Concor (bisoprolol fumarate) Deanxit (flupentixol/melitracen) Euthyrox (levothyroxine) NanoFib (fenofibrate) Zyloric (allopurinol)

MWS47	65-84	F	Lebanese Arab	0	0	0	0	0	Hypertension (BA00.Z) Hyperthyroidism (5A02.Z) Neurological conditions (QA0A.Y)	Eltroxin (levothyroxine) fluoxetine risperidone Vesicare (solifenacin succinate) Xanax (alprazolam)
MWS52	40-64	F	Lebanese Arab	0	0	0	0	0	Hypertension (BA00.Z)	Micardis (telmisartan)
MWS55	65-84	F	Lebanese Arab	0	0	0	0	0	Hypertension (BA00.Z) Thyroid removed (5A50.02)	Amlor (amlodipine besylate) (5 mg) Co-Diovan (valsartan) (80/12.5 mg) Euthyrox (levothyroxine) (75 mg) Lexotanil (bromazepam) (1.5 mg) Protac (fluoxetine) (20 mg) Risperdal (risperidone) (1 mg) Vessel Due F (sulodexide)
MWS56	65-84	F	Lebanese Arab	0	0	0	0	0	Hypertension (BA00.Z) Thyroid removed (5A50.02)	Amlor (amlodipine besylate) (5 mg) Co-Diovan (valsartan) (80/12.5 mg) Euthyrox (levothyroxine) (75 mg) Lexotanil (bromazepam) (1.5 mg) Prozac (fluoxetine) (20 mg) Risperdal (risperidone) (1 mg) Vessel Due F (sulodexide)

MWS63	65-84	F	Lebanese Arab	Islam	0	0	0	0	Hypertension (BA00.Z) High cholesterol (5C80.0Z)	Amlor (amlodipine besylate) Bisacor (bisoprolol fumarate) furosemide Lorine (loratadine)
MWS64	65-84	M	Lebanese Arab	Islam	0	0	0	0	Hypertension (BA00.Z)	aspirin Concor (bisoprolol fumarate) ramipril
MWS66	40-64	F	White	0	0	0	0	0	Hypertension (BA00.Z)	bendroflumethiazide (2.5 mg) perindopril (4 mg) verapamil HCl (240 mg)
MWS71	40-64	F	Lebanese Arab	Islam	0	0	0	0	Hypertension (BA00.Z) Thyroid removed (5A50.02)	Amlor (amlodipine besylate) (5 mg) Co-Diovan (valsartan) (80/12.5 mg) Euthyrox (levothyroxine) (75 mg) Lexotanil (bromazepam) (1.5 mg) Prozac (fluoxetine) (20 mg) Risperdal (risperidone) (1 mg) Vessel Due F (sulodexide)
MWS91	40-64	F	Arab	0	0	0	0	0	Hypertension (11 years diagnosis) (BA00.Z) Hyperthyroidism (29 years diagnosis) (5A02.Z)	diclofenac levothyroxine (200 mg) (daily) Nebilet (nebivolol) (5 mg) half a pill daily
MWS95	40-64	M	Asian	0	0	0	0	0	Hypertension (BA00.Z)	Concor (bisoprolol fumarate) (5 mg)

MWS100	40-64	F	Middle Eastern Arab	0	0	0	0	0	Hypertension (BA00.Z) High cholesterol (5C80.0Z)	Aspicot (aspirin) Lipitor (atorvastatin) Micardis (telmisartan) (80 mg)

F: female, HCl: Hydrochloride; M: male, T2DM: Type 2 diabetes mellitus.

Table 3. Characteristics of participants with CVDs (n = 15) (Part two).

Skip medication	Family history of disease	Vitamins	Happy disease management	Smoke	Weight	Height	Exercise	Meals a day	Diet	Fast-food
Atrial fibrillation										
0	0	Omega-3 fish oil (1000 mg)	0	No	0	0	Exercise daily	0	No specific diet type	No
Heart disease										
0	0	Maxi-D	0	No	0	0	Not active	0	No specific diet type	No
No	Angina	No	Yes	Ex-smoker	15 ½ st	5'7 ft	Active job, walk and bike to work	Two	Average diet	Yes
Hypertension										
Taken daily	0	Magne B6 Vitamin D	0	No	0	0	Not active	0	No specific diet type	No
0	0	Calcium Vitamin D 10000	0	Yes, two packets a day	0	0	Not active	0	Low salt diet	No
0	0	Vitamin B, C and D complex	0	No	0	0	No	0	No specific diet type	No
0	0	Vitamin C and D, occasionally	0	Yes frequently, one box a day	0	0	No	0	No specific diet type	No
0	0	Vitamin C and D, occasionally	0	Yes frequently, one box a day	0	0	No	0	No specific diet type	No
No	No	No	Yes	No	0	0	Not active	Three	No specific diet type	No
No	No	No	Yes	No	0	0	Not active	Three	No specific diet type	No
0	0	Berocca Vitamin B and C	0	No	0	0	Walking daily	0	No specific diet type	No
No	No	Vitamin C and D occasionally	Yes	Yes, daily	0	0	Not active	0	No specific diet type	No

0	0	Multivitamins daily	0	Sheesha three times a week	0	0	No	0	Lactose intolerant but consumes some dairy products	No
0	0	No	0	Twice a day	0	0	Not active	0	No specific diet type	No
0	0	Centrum Vitamin D	0	No	0	0	Not active	0	No specific diet type	No

Table 4. Characteristics of participants with DM (n = 5) (Part one).

Participants	Age Range	Biological sex	Ethnicity	Religious belief	Marital status	Education level	Occupation	Household	Medical Condition	Medicines
T1DM										
MWS51	18-24	F	Lebanese Arab	0	0	0	0	0	T1DM (5A10)	Apidra (insulin glulisine) Lantus (insulin glargine)
T2DM										
MWS43	40-64	M	White	0	0	0	0	0	T2DM (5A11)	Meteforme (metformin HCl) twice a day
MWS79	25-34	M	Asian	Hinduism	Married	MSc	Data scientist	Two	T2DM (2 years) (5A11)	Melmet-500 SR (metformin HCl)
MWS87	65-84	F	White	Catholicism	Married	College qualifications	Retired	Two	T2DM (29 years) (5A11)	atorvastatin (80 mg) pm duloxetine (60 mg) daily empagliflozin (10 mg) daily Evacal D3 (calcium with colecalciferol) (1500 mg/400 units) twice daily levothyroxine sodium (100 mg) daily omeprazole (20 mg) daily paracetamol (500 mg) 1-2 tablets four times daily risedronate sodium (35 mg) (weekly) solifenacin succinate (10 mg) daily tramadol HCl (50 mg) once or twice daily
MWS88	65-84	M	Arab	0	0	0	0	0	T2DM (5A11)	Aprovel (irbesartan) diameril Glucophage (metformin HCl) Vesicare (solifenacin succinate)

F: Female, HCl: Hydrochloride, M: Male, T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

Table 4. Characteristics of participants with DM (n = 5) (Part two).

Skip medication	Family history of disease	Vitamins	Happy disease management	Smoke	Weight	Height	Exercise	Meals a day	Diet	Fast-food
T1DM										
No	0	Neurobion Vitamin D	0	No	0	0	Yes, six times a week	0	No specific diet type	No
T2DM										
Fish oil	NA	No	0	0	No active	Three	No specific diet type	No	Fish oil	0
No	Yes	No	60 kg	159 cm	Exercises two times a week including walking and yoga	Three	Good diet	Yes	No	Yes
No	Yes	No	Overweight	5'1 ft	Low level of exercise, recently had a total knee replacement and is awaiting another one.	Three	Frequent consumption of porridge or bran flakes, low calorie slimming world means and selection of fresh fruit. Snacks include kiwi, sugar biscuit and fruit. Drinks tea twice a day, coffee and hot chocolate once a day.	Yes	No	Yes

Vitamin D	0	No	0	0	No	0	No specific diet type	No	Vitamin D	0

T1DM: Type 1 diabetes mellitus, T2DM: Type 2 diabetes mellitus.

Table 5. Characteristics of participants with CVD and DM (n = 15) (Part one).

Participants	Age Range	Biological sex	Ethnicity	Religious belief	Marital status	Education level	Occupation	Household	Medical Condition	Medicines
MWS57	65-84	M	Arab	Islam	0	0	0	0	Alzheimer's disease (8A20) CAD (BA8Z) T1DM (5A10) Hypertension (BA00.Z)	Aldactone (spironolactone) (25 mg) Cardura (doxazosin) (4 mg) clopidogrel (75 mg) furosemide (40 mg) Glurenor (gliquidone) (30 mg) Praxilene (naftidrofuryl oxalate) (200 mg) Procoralan (ivabradine) (7.5 mg) simvastatin (20 mg) Vastarel (trimetazidine) (35 mg)
MWS58	65-84	F	Arab	Islam	0	0	0	0	T1DM (5A10) Hypertension (BA00.Z) Osteoporosis (FB83.1Z)	Amaryl (glimepiride) (2 mg) aspirin B-Cor (bisoprolol fumarate) (5 mg) Betaserc (betahistine dihydrochloride) (16 mg) candesartan cilexetil (8 mg) clopidogrel (75 mg) furosemide (40 mg) Janumet (sitagliptin and metformin HCl) (50/100 mg) Jardiance (empagliflozin) (25 mg) Tresiba (insulin degludec)
MWS59	65-84	F	Arab	Islam	0	0	0	0	T2DM (5A11) Hypertension (BA00.Z) High cholesterol (5C80.0Z)	Aspicot (aspirin) Atorec-20 (atorvastatin) Diovan-160 (valsartan) Galvus Met (vildagliptin)
MWS60	65-84	F	Arab	Islam	0	0	0	0	T2DM (5A11) Hypertension (BA00.Z)	Glucophage (metformin HCl) Normoten (losartan potassium) ramipril

MWS62	40-64	M	Arab	Islam	0	0	0	0	T2DM (5A11) Hypertension (BA00.Z)	Coveram (perindopril and amlodipine) Glucophage (metformin HCl) Nanofib145 (fenofibrate)
MWS65	40-64	M	Arab	Islam	0	0	0	0	T2DM (5A11) Hypertension (BA00.Z) High cholesterol (5C80.0Z)	Atorvex (atorvastatin) (20 mg) MicroFibrate (fenofibrate) (200 mg) NOVELDO-MET (metformin HCl) (1050 mg) ZIOREL Plus (hydrochlorothiazide and irbesartan) (150/12.5 mg)
MWS73	65-84	M	White	Methodist	Married	HNC	Retired	Two	Ex-cancer (2D4Z) T2DM (40 years) (5A11) Poor left ventricle (BD11.Z)	atorvastatin (10 mg) bisoprolol (5 mg) bisoprolol fumarate (2.5 mg) empagliflozin (10 mg) metformin HCl (500 mg) montelukast (10 mg) omeprazole (20 mg) ramipril (2.5 mg) salbutamol spironolactone (25 mg) sumatriptan (50 mg) Trelegy inhaler (fluticasone, umeclidinium and vilanterol)
MWS92	40-64	M	Mixed	0	0	0	0	0	T2DM (5A11) Weakness of heart muscle (BC43.Z)	Burinex (bumetanide) Galvus Met (vildagliptin and metformin) Concor (bisoprolol fumarate) Lipanthyl (fenofibrate) Lipitor (atorvastatin)
MWS97	40-64	M	Asian	0	0	0	0	0	T2DM (5A11) Hypertension (BZ00.Z)	Janumet (metformin HCl) (50/1000 mg) Tarka forte (trandolapril and verapamil HCl) (240/4 mg)
MWS118	40-64	M	White	No	Cohabiting	High school	Clerical officer	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril

									Hypertension (BZ00.Z)	
MWS119	65-84	F	White	No	Single	High school	Retired	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z) Hypertension (BZ00.Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril
MWS120	65-84	F	White	No	Single	High school	Retired	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z) Hypertension (BZ00.Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril
MWS124	65-84	F	White	No	Single	High school	Retired	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z) Hypertension (BZ00.Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril
MWS125	65-84	F	White	No	Single	High school	Retired	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z) Hypertension (BZ00.Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril
MWS126	65-84	F	White	No	Single	High school	Retired	Two	T2DM (8 years) (5A11) High cholesterol (5C80.0Z) Hypertension (BZ00.Z)	atorvastatin Meterforme (metformin HCl) twice daily ramipril

CAD: Coronary artery disease, F: Female, HCl: Hydrochloride, M: Male, T2DM: Type 2 diabetes mellitus.

Table 5. Characteristics of participants with CVD and DM (n = 15) (Part two).

Skip medication	Family history of disease	Vitamins	Happy disease management	Smoke	Weight	Height	Exercise	Meals a day	Diet	Fast-food
No	No	Fish oil	0	No	0	0	Not active	Three	No specific diet type	No
No	No	Magnesium Vitamin D	Yes	Yes, daily	0	0	Not active	Three	No specific diet type	No
No	No	Vitamin D-10000 Multivitamins Omega-3	Yes	No	0	0	Not active	Three	No specific diet type	No
No	No	No	Yes	No	0	0	Not active	Three	No specific diet type	No
No	No	Omega-3	Yes	Yes, daily for 20 years	0	0	Not active	0	No specific diet type	No
No	0	No	Yes	Yes, 40 cigarettes a day	0	0	Not active	0	No specific diet type	No
No	No	No	Yes	No	71.2 kg	5'7 ft	Some walking and gardening, table tennis once a week	Three	Good diet	No
0	0	Vitamin B12 and D	0	No	0	0	No	0	No specific diet type	No
0	0	No	0	30 cigarettes daily	0	0	Not active	0	No specific diet type	No
No	No	No	Yes	No	0	5'8 ft	Not very active, occasionally goes walking	Three	Reasonably healthy diet	No
No	No	No	Yes	E-cigarettes	0	5'3 ft	Regularly goes walking	Three	Reasonably healthy diet	No
No	No	No	Yes	E-cigarettes	0	5'3 ft	Regularly goes walking	Three	Reasonably healthy diet	No
No	No	No	Yes	E-cigarettes	0	5'3 ft	Regularly goes walking	Three	Reasonably healthy diet	No

No	No	No	Yes	E-cigarettes	0	5'3 ft	Regularly goes walking	Three	Reasonably healthy diet	No
No	No	No	Yes	E-cigarettes	0	5'3 ft	Regularly goes walking	Three	Reasonably healthy diet	No

Appendix B2 Details of collected fingernails

Fingernails												
Dimensions (mm)	MWS1										Mean (mm)	RSD (%)
	A	B	C	D	E	F	G	H	I	J		
Length	12.9	12.7	10.93	10.92	7.91	7.91	6.13	5.98			9.43	29.7
Depth	0.49	0.43	0.29	0.33	0.39	0.28	0.24	0.25			0.338	26.8
Width	2.14	1.71	1.91	1.93	1.33	1.18	1.14	1.13			1.56	26.3
MWS2												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	14.9	14.7	14.4	12.5	12.4	10.97	10.26			13.1	14.2
Depth	0.38	0.35	0.3	0.33	0.34	0.31	0.32	0.27			0.325	10.27
Width	2.27	1.96	2.12	1.93	2.42	3.11	2.62	1.47			2.24	22.1
MWS3												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	14.2	13.9	12.7	12.3	11.9	11.6	11.4	7.24	6.91	11.7	23.1
Depth	0.21	0.21	0.23	0.22	0.21	0.25	0.24	0.24	0.2	0.22	0.223	7.34
Width	1.41	1.36	1.24	1.26	1.01	1.98	1.12	1.13	1.63	1.12	1.33	21.9
MWS4												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.3	15.2	15.2	13.5	9.81	7.99	7.62	7.43	6.83	6.49	10.64	37.2
Depth	1.27	1.04	0.86	0.81	0.71	0.48	0.89	1.24	0.75	1.12	0.917	27.3
Width	2.15	2.12	1.91	2.45	1.92	1.61	1.33	0.94	1.18	2.14	1.78	27.6
MWS5												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)

Length	19.2	18.9	14.9	13.9	13.8	13.6	12.5	8.91			14.5	23.3
Depth	0.3	0.27	0.17	0.19	0.17	0.28	0.39	0.21			0.248	31.1
Width	1.23	0.94	1.64	1.84	2.13	1.24	1.83	1.26			1.51	26.8
MWS6												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.7	10.74	9.98	9.81	8.52	8.28	7.43	7.31	6.49		9.25	26.8
Depth	0.3	0.28	0.18	0.18	0.15	0.28	0.43	0.2	0.15		0.239	38.6
Width	1.14	2.13	2.42	0.91	1.93	1.11	1.24	1.68	0.92		1.49	37.3
MWS7												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.8	13.3	10.81	10.31	10.28	9.43	9.29	8.82	7.81	7.65	10.15	20.43
Depth	0.75	0.53	0.13	0.61	0.51	0.1	0.2	0.38	0.13	0.25	0.359	64.2
Width	1.92	1.14	1.84	1.35	2.27	1.94	1.23	1.35	1.12	1.39	1.56	25.8
MWS8												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.6	15.4	10.41	10.29	9.82	9.68	8.36	8.23	7.82	7.79	10.35	28.1
Depth	0.36	0.18	0.18	0.18	0.15	0.08	0.1	0.05	0.05	0.05	0.138	69.5
Width	1.12	1.23	1.37	0.91	1.14	0.94	1.17	0.97	1.34	1.45	1.16	16.1
MWS9												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	13.7	12.1	11.2	10.11	9.88	9.79	7.64	6.87		10.59	23.1
Depth	0.56	0.36	0.61	0.58	0.41	0.43	0.33	0.36	0.3		0.438	26.6
Width	1.94	1.78	2.37	1.68	0.94	1.94	1.97	1.92	1.96		1.83	20.93
MWS10												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.2	13.5	10.91	10.68	10.42	10.11	10.01	9.98	9.97	9.79	10.96	14.4

Depth	0.53	0.48	0.48	0.53	0.51	0.48	0.46	0.43	0.38	0.38	0.466	11.7
Width	2.43	3.66	2.65	4.12	2.77	3.93	3.37	1.91	2.94	3.22	3.1	22.4
MWS11												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.9	10.37	9.94	9.89	9.72	9.59	9.34	9.29	7.81	7.27	9.61	15.7
Depth	0.51	0.38	0.56	0.36	0.41	0.38	0.41	0.3	0.33	0.28	0.392	22.3
Width	2.82	2.11	1.92	1.84	2.96	2.24	2.14	2.14	1.93	1.86	2.19	17.8
MWS12												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	8.23	8.18	7.32	7.19	6.92	6.79					7.44	8.38
Depth	0.42	0.38	0.38	0.17	0.24	0.3					0.315	30.57
Width	6.82	3.11	2.93	3.95	3.94	5.81					4.43	35.1
MWS13												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.22	9.89	9.33	9.28	8.93	8.85	8.73	8.71	7.94	7.79	8.97	8.5004
Depth	0.34	0.37	0.34	0.32	0.41	0.28	0.29	0.32	0.43	0.3	0.34	14.7
Width	0.94	1.66	1.74	1.13	1.15	1.34	1.65	0.98	1.92	1.54	1.41	24.5
MWS14												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.5	12.3	10.31	10.29	9.82	9.58	8.21	8.18	7.31	7.28	5.096	44.7
Depth	0.61	0.35	0.35	0.39	0.56	0.37	0.39	0.43	0.34	0.35	1.14	8.29
Width	2.13	1.93	1.96	1.44	1.88	1.95	1.34	1.84	2.16	2.11	1.87	14.8
MWS15												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.4	10.19	9.62	9.49	7.61	7.58	7.23	7.14			8.79	18.3
Depth	0.16	0.43	0.21	0.37	0.32	0.24	0.24	0.42			0.299	33.8

Width	1.78	3.36	2.15	2.24	0.92	1.11	1.75	1.43			1.84	41.7
MWS16												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.4	12.8	12.8	11.6	11.5	9.93	8.99	8.25	8.23	7.98	10.56	20.14
Depth	0.49	0.25	0.28	0.28	0.21	0.33	0.21	0.37	0.36	0.32	0.31	27.3
Width	2.33	2.23	2.15	1.94	1.91	2.14	2.81	1.85	1.73	2.33	2.14	14.6
MWS17												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.1	11.9	11.4	11.4	10.92	9.99	9.87	8.98	7.34	6.99	10.099	18.2
Depth	0.48	0.29	0.22	0.31	0.29	0.35	0.33	0.44	0.32	0.45	0.348	23.8
Width	2.93	2.74	3.95	3.12	2.23	3.12	2.64	2.91	2.42	2.51	2.86	16.9
MWS18												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.4	15.5	15.1	14.9	13.4	13.3	12.9	10.97	9.81	9.46	13.2	18.4
Depth	0.58	0.56	0.37	0.52	0.47	0.41	0.42	0.45	0.43	0.43	0.464	14.7
Width	1.89	1.57	1.2	1.95	1.44	1.61	1.68	1.97	1.93	1.24	1.65	17.5
MWS19												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.1	13.9	10.82	9.92	9.91	9.85	9.79	7.91	7.88	6.89	10.097	23.6
Depth	0.46	0.41	0.54	0.44	0.36	0.42	0.38	0.38	0.38	0.31	0.408	15.4
Width	2.19	2.21	2.33	1.63	1.92	1.43	1.32	1.44	2.12	2.33	1.89	21.1
MWS20												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.9	11.7	11.5	11.3	11.2	10.99	10.11	9.98	9.14	8.98	10.78	11.3
Depth	0.43	0.33	0.32	0.26	0.29	0.21	0.38	0.28	0.21	0.31	0.302	22.9
Width	4.12	4.63	3.91	5.82	4.33	4.22	3.53	4.92	3.98	3.82	4.33	15.2

MWS21												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	8.92	8.68	8.43	8.28	8.11	7.46	7.31	7.28	6.86		7.93	9.055
Depth	0.39	0.31	0.31	0.33	0.31	0.26	0.31	0.16	0.32		0.3	20.75
Width	1.34	1.33	1.72	1.24	1.82	1.19	1.86	1.22	1.2		1.44	19.5
MWS22												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	6.82	6.79	6.73	6.33	6.29	5.82	5.68	5.43	5.42	5.28	6.059	9.99
Depth	0.31	0.23	0.21	0.23	0.09	0.17	0.23	0.19	0.24	0.22	0.212	26.6
Width	1.11	1.02	1.82	1.48	1.46	1.93	1.48	1.34	1.35	1.29	1.43	19.7
MWS23												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.2	9.82	9.79	8.91	8.68	7.15	7.13	4.58			8.66	29.2
Depth	0.44	0.28	0.23	0.27	0.32	0.29	0.29	0.33			0.3063	20.27
Width	1.58	1.26	1.36	1.49	2.14	1.27	1.44	1.12			1.46	21.4
MWS24												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.51	10.37	8.22	7.89	7.21	7.06					8.54	17.9
Depth	0.36	0.28	0.28	0.29	0.29	0.31					0.3017	10.15
Width	2.33	1.96	1.94	2.01	1.18	1.13					1.76	27.8
MWS25												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.1	12.9	11.5	10.97	10.62	10.19	8.92	8.79	8.43	8.28	10.68	22.8
Depth	0.45	0.55	0.43	0.36	0.56	0.41	0.34	0.37	0.36	0.33	0.416	19.9
Width	2.34	3.13	1.95	2.53	1.98	1.96	1.43	1.56	1.92	1.91	2.071	23.7
MWS26												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.9	16.9	12.6	12.5	11.4	11.4	11.3	9.29	9.11	8.97	12.05	23.9
Depth	0.54	0.64	0.35	0.46	0.49	0.32	0.52	0.46	0.39	0.35	0.452	22.3
Width	2.17	3.15	2.35	2.12	2.93	2.15	2.12	2.13	2.12	1.9	2.31	17.3
MWS27												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.9	12.9	12.1	11.8	11.4	11.4	9.22	8.98	8.82		11.4	19.8
Depth	0.55	0.55	0.49	0.37	0.41	0.37	0.42	0.37	0.46		0.443	16.5
Width	3.12	1.82	1.93	2.12	1.43	1.93	1.87	1.91	1.74		1.99	23.4
MWS28												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.01	10.49	10.22	10.2	9.91	9.77	9.21	8.79	6.81	6.26	9.27	16.9
Depth	0.32	0.17	0.15	0.33	0.23	0.43	0.32	0.22	0.41	0.42	0.3	34.4
Width	0.69	0.76	0.84	0.77	0.94	0.82	0.98	1.13	0.98	0.63	0.854	17.9
MWS29												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.1	14.9	9.91	8.98	8.74	7.99	7.81	6.96	6.52	6.18	9.41	36.3
Depth	0.22	0.22	0.34	0.32	0.31	0.32	0.31	0.22	0.31	0.34	0.291	17.3
Width	2.63	1.12	0.96	1.16	1.01	0.92	1.14	0.93	0.91	1.12	1.19	43.3
MWS30												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.1	10.12	9.29	9.02	8.92	8.78	8.51	8.18	8.09		9.34	16.5
Depth	0.55	0.41	0.38	0.37	0.33	0.22	0.41	0.44	0.29		0.378	24.9
Width	2.11	2.02	1.98	1.91	2.34	2.31	2.43	2.14	3.02		2.25	14.9
MWS31												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.4	10.88	9.82	9.78	9.71	8.99	8.22	7.48	7.22	6.89	9.54	28.8
Depth	0.45	0.43	0.36	0.36	0.51	0.39	0.38	0.32	0.31	0.31	0.382	17.1
Width	1.91	1.73	1.19	1.41	1.92	1.14	1.32	1.24	1.12	0.99	1.39	24.2
MWS32												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.2	11.3	9.93	9.92	9.31	9.19	9.12	7.89	7.43	7.39	9.47	18.9
Depth	0.45	0.43	0.36	0.36	0.51	0.39	0.38	0.32	0.31	0.31	0.382	17.1
Width	3.26	1.13	1.92	1.84	2.02	1.43	1.91	1.54	1.83	2.91	1.98	32.7
MWS33												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	14.8	13.9	13.9	13.7	12.9	12.9	12.1	11.9	11.3	13.3	9.11
Depth	0.67	0.42	0.31	0.45	0.56	0.58	0.37	0.44	0.45	0.41	0.466	23.01
Width	1.9	3.1	1.1	0.97	1.12	0.99	1.34	1.92	0.98	1.22	1.46	46.1
MWS34												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	9.72	9.49	8.94	8.92	7.82	7.81	7.73	7.28	7.24	7.19	8.21	11.7
Depth	0.39	0.53	0.41	0.44	0.38	0.47	0.63	0.35	0.45	0.57	0.462	19.4
Width	0.86	0.85	1.48	1.43	1.39	1.29	1.54	1.89	1.92	0.78	1.34	30.35
MWS35												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.4	13.9	12.5	12.4	11.9	11.9	10.1	9.9	8.9	8.7	11.7	20.37
Depth	0.42	0.47	0.37	0.45	0.31	0.41	0.31	0.21	0.35	0.27	0.357	23.3
Width	2.71	2.13	3.12	2.45	2.16	2.67	2.91	2.42	2.15	2.12	2.48	14.4
MWS36												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.6	10.29	10.11	9.98	9.43	9.29	8.91	7.22	6.81	6.67	9.23	22.3
Depth	0.18	0.49	0.31	0.41	0.64	0.72	0.45	0.36	0.38	0.28	0.422	38.6
Width	1.93	1.88	1.91	0.92	1.32	2.25	1.92	2.14	2.13	2.11	1.85	22.4
MWS37												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	9.99	9.92	9.78	9.31	9.26	8.62	7.99	7.22	7.19	9.32	20.64
Depth	0.44	0.57	0.51	0.39	0.55	0.36	0.36	0.47	0.42	0.35	0.442	18.3
Width	1.97	1.87	1.92	1.91	1.85	2.13	1.74	1.92	1.81	1.91	1.903	5.42
MWS38												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.5	10.49	10.11	9.98	9.84	8.99	8.22	7.63	7.21	7.18	9.24	18.9
Depth	0.46	0.62	0.43	0.43	0.43	0.51	0.61	0.31	0.42	0.26	0.448	25.4
Width	1.34	1.12	1.21	1.91	1.16	1.24	1.32	2.41	1.17	1.72	1.46	28.9
MWS39												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	19.1	15.3	14.9	14.3	14.1	14.1	13.8	12.3	11.6		14.4	14.7
Depth	0.31	0.32	0.42	0.36	0.36	0.48	0.48	0.42	0.41		0.396	15.8
Width	0.93	1.94	1.38	1.88	1.93	1.82	1.67	3.17	1.98		1.87	32.3
MWS40												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.62	10.49	10.11	9.98	9.72	8.99	8.83	7.87	7.45		9.34	12.1
Depth	0.63	0.72	0.56	0.51	0.55	0.57	0.58	0.38	0.55		0.561	16.2
Width	3.12	5.11	6.37	6.14	6.23	6.37	6.13	6.11	6.82		5.82	19.1
MWS41												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	9.11	8.98	7.82	7.79	7.63	7.28	6.91	6.87	6.72	6.19	7.53	12.6
Depth	0.32	0.21	0.34	0.16	0.31	0.47	0.41	0.42	0.29	0.36	0.329	28.7
Width	1.64	1.91	1.43	1.16	1.16	1.35	1.16	1.27	1.25	0.82	1.32	22.6
MWS42												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.34	10.15	9.89	9.32	8.23	8.11	7.98	7.98	7.82	7.74	8.76	11.9
Depth	0.37	0.42	0.48	0.21	0.41	0.38	0.34	0.43	0.44	0.38	0.386	19.1
Width	0.91	1.09	0.98	0.97	1.52	1.18	1.99	1.04	1.34	0.86	1.19	29.2
MWS43												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	13.9	12.9	11.3	7.91	7.88					11.5	26.3
Depth	0.51	0.41	0.41	0.43	0.37	0.45					0.43	11.01
Width	2.42	2.34	1.92	2.63	2.44	3.91					2.61	26.01
MWS44												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.3	15.3	11.3	11.2	10.72	10.67					12.4	18.1
Depth	0.61	0.52	0.46	0.36	0.34	0.33					0.437	25.9
Width	2.93	2.82	1.36	1.13	2.14	1.92					2.05	35.9
MWS45												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.6	11.9	10.82	10.39	9.91	9.78	9.23	8.99	8.73	7.87	10.021	14.5
Depth	0.41	0.25	0.26	0.27	0.23	0.34	0.23	0.24	0.27	0.19	0.269	23.3
Width	2.11	1.93	1.74	1.39	1.65	1.92	1.63	1.25	1.93	1.24	1.68	18.2
MWS46												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.2	13.8	13.4	12.3	9.43	8.98	7.22				11.3	24.3
Depth	0.56	0.41	0.33	0.33	0.26	0.38	0.29				0.366	27.2
Width	2.38	2.14	1.43	0.98	1.92	0.77	0.73				1.48	45.9
MWS47												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.1	13.9	13.2	13.2	12.3	10.79	10.12	9.98	9.82	9.77	11.76	15.5
Depth	0.37	0.32	0.27	0.32	0.37	0.31	0.33	0.48	0.33	0.38	0.348	16.4
Width	0.99	0.93	0.98	0.83	0.97	0.78	0.82	0.93	0.91	0.92	0.906	7.98
MWS48												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.9	12.4	11.8	11.7	10.82	10.69	10.51	10.49	9.94	6.59	11.08	20.902
Depth	0.38	0.42	0.55	0.44	0.38	0.37	0.47	0.43	0.45	0.63	0.452	18.1
Width	1.92	0.94	2.18	1.98	1.12	2.14	1.72	1.88	1.94	1.13	1.69	26.9
MWS49												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.6	14.9	12.3	12.2	10.63	10.28	10.12	9.98	9.22	9.19	11.4	19.9
Depth	0.25	0.47	0.33	0.27	0.37	0.53	0.52	0.31	0.31	0.34	0.37	27.4
Width	2.13	1.94	1.94	1.78	1.45	2.13	1.98	2.13	1.92	1.94	1.93	10.56
MWS50												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.1	13.8	10.82	9.98	9.82	9.61	8.67	8.22	7.82	5.99	9.88	25.7
Depth	0.33	0.32	0.28	0.34	0.45	0.22	0.38	0.39	0.31	0.22	0.324	22.4
Width	1.93	1.95	1.83	1.94	1.81	1.89	1.78	1.97	1.34	1.16	1.76	15.9
MWS51												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.21	10.18	9.12	8.99	7.65	7.48					8.94	13.2
Depth	0.31	0.25	0.43	0.33	0.31	0.31					0.323	18.2
Width	3.11	2.92	2.64	2.55	2.53	2.41					2.69	9.89
MWS52												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.4	13.2	12.8	12.5	12.3	12.1	12.2	11.9	8.92	8.89	11.8	13.7
Depth	0.41	0.51	0.33	0.24	0.33	0.31	0.39	0.43	0.41	0.34	0.37	20.42
Width	1.94	1.82	1.91	1.67	1.45	1.26	1.95	2.11	1.91	1.82	1.78	14.4
MWS53												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.4	10.39	9.93	9.69	7.41	6.97	6.72	5.98	5.93	5.91	8.23	30.53
Depth	0.31	0.27	0.25	0.24	0.24	0.29	0.29	0.31	0.24	0.26	0.27	10.48
Width	1.89	1.88	1.94	1.72	0.98	1.54	1.54	0.92	0.95	0.94	1.43	30.53
MWS54												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.1	12.3	10.91	10.88	10.84	10.69	8.42	8.27	7.81	6.28	10.051	23.2
Depth	0.48	0.53	0.45	0.66	0.41	0.61	0.49	0.32	0.31	0.58	0.484	24.1
Width	1.37	1.23	1.94	1.91	1.91	1.84	1.74	1.57	1.38	1.73	1.66	15.6
MWS55												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.1	13.4	11.8	11.5	11.2	10.5	9.4	9.3	9.1	8.9	11.02	18.5
Depth	0.98	0.77	0.55	0.53	0.72	0.69	0.75	0.53	0.56	0.49	0.657	23.4
Width	3.13	4.17	3.42	3.23	2.73	3.65	3.14	3.62	2.76	2.02	3.19	18.7
MWS56												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.8	10.89	10.31	10.28	10.13	9.19	8.92	8.89	8.22	8.17	9.88	16.9
Depth	0.51	0.63	0.38	0.48	0.58	0.52	0.46	0.52	0.52	0.28	0.488	20.26
Width	2.21	1.98	2.17	1.92	2.14	1.73	1.96	2.21	1.24	2.23	1.98	15.5
MWS57												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.6	14.2	13.2	12.4	12.1	11.7	10.93	8.86	8.56	8.49	11.7	22.4
Depth	0.62	0.61	0.43	0.58	0.64	0.69	0.46	0.47	0.79	0.41	0.57	21.8
Width	2.22	1.13	1.84	2.11	1.93	2.37	1.91	1.92	1.74	1.91	1.91	17.4
MWS58												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.7	16.6	13.8	13.79	12.9	12.7	11.4	11.4	7.42	7.37	12.41	25.9
Depth	0.44	0.48	0.49	0.44	0.33	0.43	0.34	0.24	0.38	0.51	0.408	20.76
Width	1.72	1.27	0.98	1.13	0.92	1.24	1.23	1.15	1.13	1.12	1.19	18.2
MWS59												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.1	11.9	10.64	9.19	9.11	8.97	6.93	6.92	6.62	6.46	8.99	26.01
Depth	0.55	0.44	0.58	0.66	0.42	0.46	0.65	0.39	0.42	0.28	0.485	25.1
Width	2.13	1.32	1.94	2.13	1.48	1.12	1.83	1.34	1.61	0.94	1.58	26.2
MWS60												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.8	11.3	11.1	10.75	9.91	7.47	5.93	5.34	5.21	5.19	8.49	34.9
Depth	0.54	0.44	0.45	0.39	0.41	0.46	0.39	0.41	0.43	0.42	0.434	10.14
Width	1.92	1.74	1.82	2.19	2.18	2.81	1.33	3.55	2.14	3.92	2.36	34.9
MWS61												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	17.3	15.7	15.1	11.8	11.7	11.3	11.01	10.96	6.82	6.58	11.8	29.6
Depth	0.54	0.44	0.17	0.26	0.19	0.21	0.24	0.36	0.21	0.39	0.301	41.3
Width	1.27	1.82	1.34	1.13	0.79	1.18	1.23	0.82	0.68	0.73	1.099	32.1
MWS62												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	13.5	12.13	11.9	11.8	11.4	9.22	8.19	7.53	7.51	10.81	23.8
Depth	0.42	0.38	0.47	0.44	0.47	0.37	0.43	0.33	0.35	0.38	0.404	12.1
Width	1.91	2.42	2.18	1.88	2.85	2.13	1.82	1.63	1.75	1.72	2.029	18.5
MWS63												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	11.9	11.4	10.98	9.43	8.99	6.93	6.92	6.23	6.18	9.302	29.3
Depth	0.33	0.38	0.34	0.34	0.42	0.38	0.27	0.32	0.42	0.42	0.362	13.9
Width	0.79	1.88	1.83	1.64	1.17	1.72	1.19	1.18	1.82	1.13	1.44	26.8
MWS64												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.3	11.2	9.12	8.67	7.91	7.89	7.81	7.28	6.52	6.46	8.41	20.23
Depth	0.76	0.68	0.57	0.61	0.38	0.61	0.22	0.56	0.51	0.33	0.523	31.8
Width	2.13	1.91	1.28	1.37	1.43	0.92	0.87	1.82	1.24	1.12	1.41	29.9
MWS65												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.83	10.77	10.42	10.41	9.86	9.84	8.11	7.99	7.78		9.56	13.1
Depth	0.42	0.25	0.35	0.35	0.21	0.37	0.29	0.31	0.21		0.3067	23.9
Width	1.73	1.55	1.63	0.95	1.23	0.98	0.85	0.92	1.13		1.21	27.6
MWS66												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	9.81	8.98	8.92	7.99	6.52	6.37	5.53	5.18	5.12		7.16	25.1
Depth	0.33	0.26	0.25	0.24	0.28	0.22	0.21	0.17	0.19		0.239	20.34
Width	1.29	1.92	1.23	1.22	1.13	0.97	1.21	1.22	1.13		1.26	21.1
MWS67												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	9.91	9.88	9.43	9.33	9.29	9.22	7.93	7.59	6.91	6.98	8.65	13.6
Depth	0.42	0.28	0.29	0.25	0.37	0.27	0.32	0.26	0.36	0.36	0.318	17.9
Width	1.92	1.91	1.84	1.26	1.82	1.24	2.12	1.96	1.94	1.92	1.79	16.6
MWS68												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	17.3	16.9	14.4	13.9	13.8	13.6	12.9	12.8	12.2	9.28	13.7	16.7
Depth	0.48	0.45	0.41	0.42	0.45	0.37	0.31	0.35	0.56	0.37	0.417	17.3
Width	1.77	1.34	1.72	1.94	1.92	0.99	2.61	2.22	1.84	1.73	1.81	24.4
MWS69												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	8.24	7.89	7.82	7.46	6.96	6.89	6.54	6.49	6.13	6.11	7.053	10.79
Depth	0.27	0.17	0.31	0.22	0.26	0.22	0.23	0.23	0.17	0.16	0.224	21.5
Width	1.42	1.13	1.32	0.96	1.21	0.98	1.15	1.09	0.99	1.21	1.15	13.1
MWS70												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.3	12.1	10.83	10.02	9.22	9.18	8.91	8.48	7.23	6.49	9.48	20.13
Depth	0.25	0.27	0.31	0.35	0.24	0.33	0.23	0.22	0.22	0.34	0.276	18.7
Width	4.02	4.82	3.83	3.22	3.01	2.82	4.21	5.02	4.01	3.42	3.84	19.1
MWS71												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.9	15.9	12.3	11.9	11.6	11.5	11.3	10.98	10.53	10.28	12.2	16.6
Depth	0.95	1.01	0.61	0.45	0.86	0.71	0.62	0.81	0.63	0.65	0.73	23.8
Width	2.91	3.13	2.44	3.12	3.92	2.96	3.12	3.34	2.92	2.91	3.077	12.3
MWS72												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.4	14.2	13.9	10.18	9.93	9.88	8.82	7.47			11.1	24.2
Depth	0.41	0.44	0.35	0.34	0.38	0.37	0.34	0.27			0.363	14.1
Width	2.19	2.38	3.12	2.14	1.67	1.91	2.32	2.43			2.27	18.8
MWS73												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.4	9.87	7.42	6.89	6.81	6.37	6.13	5.47	5.42	5.28	7.201	31.3
Depth	0.42	0.51	0.31	0.42	0.46	0.48	0.45	0.55	0.95	0.5	0.505	33.5
Width	1.43	1.98	1.94	1.82	0.97	1.57	1.13	1.42	1.43	0.99	1.47	25.03
MWS74												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	18.2	17.9	15.2	14.3	13.3	12.9	12.1	11.3	10.91	10.27	13.6	20.35
Depth	0.59	0.53	0.31	0.36	0.49	0.42	0.45	0.51	0.46	0.41	0.453	18.2
Width	2.12	2.43	1.96	3.14	2.13	1.97	2.24	1.93	1.37	2.34	2.16	20.84
MWS75												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.8	14.6	13.2	11.9	10.01	9.86	9.23	9.18	6.81	6.37	10.59	28.01
Depth	0.28	0.27	0.37	0.49	0.27	0.25	0.31	0.27	0.33	0.37	0.321	22.8
Width	0.92	0.84	0.83	1.12	0.95	0.95	0.92	0.88	0.62	0.49	0.852	20.94
MWS76												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	6.88	6.86	6.22	6.17	5.93	5.88	4.91	4.97	3.33	2.98	5.41	25.1
Depth	0.26	0.24	0.41	0.42	0.31	0.46	0.34	0.35	0.44	0.31	0.354	21.5
Width	0.89	0.78	0.99	0.98	0.67	0.89	1.02	0.93	0.92	0.68	0.875	14.3
MWS77												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.9	11.8	10.63	10.61	9.93	9.89	7.98				10.39	12.8
Depth	0.51	0.52	0.47	0.42	0.43	0.44	0.41				0.457	9.59
Width	1.18	1.23	1.96	1.73	1.82	1.91	1.85				1.67	19.5
MWS78												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.03	9.68	8.46	8.24	8.22	8.21	6.48	6.35	5.98	5.96	7.76	19.2
Depth	0.48	0.41	0.61	0.34	0.43	0.36	0.48	0.27	0.29	0.48	0.415	24.9
Width	1.12	0.98	1.93	0.78	0.94	1.21	0.76	0.78	0.98	0.92	1.04	33.2
MWS79												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.2	11.9	8.91	8.68	8.33	8.27	7.31	7.28	6.24	6.22	8.55	24.6
Depth	0.42	0.36	0.38	0.31	0.49	0.32	0.33	0.41	0.47	0.47	0.396	16.8
Width	2.14	1.91	1.23	1.17	1.92	1.33	1.14	1.15	1.92	1.21	1.51	26.8
MWS80												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.6	11.5	8.43	8.38	7.41	6.97	6.81	5.49	4.97		7.95	29.4
Depth	0.29	0.32	0.39	0.26	0.38	0.25	0.23	0.21	0.36		0.299	22.4
Width	1.63	1.42	1.26	1.13	0.93	0.92	1.13	0.89	0.78		1.12	24.7
MWS81												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.96	10.94	10.72	10.69	10.62	10.37	10.21	8.99	6.93	6.28	9.67	17.8
Depth	0.29	0.39	0.43	0.47	0.33	0.26	0.31	0.39	0.39	0.39	0.365	17.9
Width	1.23	1.29	1.92	1.77	1.25	1.24	1.11	1.38	1.24	0.68	1.31	26.0003
MWS82												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.12	10.08	7.22	7.18	6.91	6.27	5.83				7.67	22.7
Depth	0.48	0.54	0.42	0.31	0.55	0.44	0.43				0.453	18.02
Width	1.23	1.15	1.32	1.14	1.02	1.22	1.03				1.16	9.39
MWS83												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	12.2	11.9	11.7	11.4	11.4	8.94	8.92	7.91	7.88	10.71	20.98
Depth	0.68	0.41	0.47	0.34	0.43	0.51	0.31	0.41	0.55	0.58	0.469	24.2
Width	1.23	1.74	1.82	1.96	1.94	1.22	1.92	1.77	1.73	1.82	1.72	15.8
MWS84												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	11.9	11.2	10.67	10.26	10.25	10.18	10.16	8.95	8.89	10.75	16.2
Depth	0.44	0.37	0.32	0.27	0.34	0.38	0.37	0.33	0.27	0.31	0.34	15.4
Width	2.94	3.98	3.93	4.37	3.16	3.05	3.24	4.12	2.93	3.14	3.49	15.7
MWS85												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.7	11.3	11.2	10.38	10.24	10.18	9.21	7.39	6.92	6.49	9.81	25.1
Depth	0.43	0.27	0.32	0.29	0.28	0.3	0.28	0.28	0.28	0.27	0.3	16.02
Width	1.44	1.23	1.28	1.15	1.14	1.61	1.23	1.18	1.91	1.24	1.34	18.4
MWS86												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.7	13.6	9.91	9.78	9.76	8.94	8.93	6.81	6.39		9.76	26.1
Depth	0.42	0.45	0.25	0.33	0.35	0.26	0.27	0.34	0.25		0.324	22.9
Width	1.79	1.82	1.84	1.13	1.32	1.95	2.14	1.44	2.12		1.73	20.51
MWS87												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.42	10.28	9.91	9.47	8.73	7.87	6.24	4.88			8.48	23.8
Depth	0.24	0.28	0.18	0.24	0.28	0.18	0.21	0.25			0.233	17.01
Width	1.01	1.14	1.22	1.63	1.28	1.32	1.43	0.84			1.23	19.8
MWS88												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.2	16.2	13.6	13.5	12.9	12.9	7.83				13.3	21.1
Depth	0.43	0.41	0.37	0.24	0.38	0.44	0.34				0.373	18.3
Width	1.89	1.99	1.96	1.95	1.12	1.03	1.14				1.58	28.9
MWS89												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.3	11.3	10.46	10.27	10.24	9.36	9.27	9.02	8.92	8.91	9.91	9.45
Depth	0.44	0.31	0.31	0.25	0.22	0.31	0.24	0.29	0.21	0.14	0.272	29.6
Width	1.11	1.09	1.97	1.92	1.89	1.92	2.23	1.12	1.28	1.85	1.64	26.6
MWS90												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	13.5	10.93	10.29	9.87	9.86	9.22	8.98	7.21	6.39	10.11	25.4
Depth	0.26	0.34	0.31	0.24	0.35	0.23	0.31	0.35	0.33	0.25	0.297	15.9
Width	3.28	3.99	3.81	3.82	3.27	3.02	3.27	3.01	2.13	3.23	3.28	16.2
MWS91												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.1	12.9	11.6	11.6	10.61	10.55	8.42				11.3	14.3
Depth	0.35	0.34	0.21	0.23	0.16	0.26	0.23				0.254	27.1
Width	1.99	1.98	2.43	2.17	2.82	0.98	3.24				2.23	32.2
MWS92												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	12.6	12.5	11.4	10.89	9.98	9.97	9.91	7.87	7.41	7.29	9.98	19.6
Depth	0.31	0.32	0.24	0.24	0.31	0.25	0.29	0.25	0.27	0.31	0.279	11.7
Width	4.14	4.13	1.47	4.23	3.94	2.92	0.99	4.18	3.91	3.23	3.31	35.8
MWS93												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.6	15.9	12.8	11.9	11.8	10.97	9.94	9.89	7.92	7.87	6.018	49.4
Depth	0.56	0.48	0.59	0.48	0.48	0.41	0.44	0.35	0.41	0.34	0.849	9.59
Width	1.91	1.18	1.16	1.91	0.99	0.98	1.12	1.14	1.13	0.92	1.24	29.1
MWS94												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.06	10.06	9.08	9.07	8.72	8.59	8.21	7.48	6.21	6.19	8.37	16.5
Depth	0.34	0.24	0.22	0.24	0.41	0.19	0.26	0.14	0.14	0.21	0.239	35.03
Width	2.01	2.02	1.01	0.96	0.94	0.88	2.03	1.02	0.89	0.96	1.27	40.73
MWS95												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.91	10.48	10.22	9.87	9.22	9.29	8.31	7.68	7.32	7.29	9.059	14.8
Depth	0.41	0.29	0.28	0.22	0.22	0.22	0.26	0.25	0.23	0.17	0.255	25.3
Width	2.12	1.59	2.22	1.89	1.97	1.89	1.88	1.72	1.44	2.12	1.88	13.1
MWS96												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.8	16.7	14.6	13.9	13.5	12.7	12.2	11.3	10.91	10.78	13.4	16.5
Depth	0.44	1.04	0.89	0.56	0.8	0.53	0.65	1.02	0.98	0.63	0.754	29.2
Width	2.91	1.83	2.15	2.13	3.23	2.19	1.95	1.92	3.13	3.12	2.46	23.1
MWS97												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	11.4	11.3	10.5	9.8	9.7	9.2	8.2	7.6	6.8	9.94	23.2
Depth	1.16	0.55	0.44	0.46	0.37	0.51	0.56	0.7	0.48	0.52	0.575	38.8
Width	4.5	3.4	2.9	2.2	1.9	3.3	2.9	3.5	1.9	3.7	3.02	27.7
MWS98												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.12	9.99	8.21	7.88	6.83	6.79	6.21	4.39	4.16		7.18	29.7
Depth	0.51	0.58	0.42	0.25	0.45	0.29	0.35	0.52	0.4		0.419	26.1
Width	1.95	1.78	1.17	1.24	1.83	2.11	3.17	1.93	1.72		1.88	30.801
MWS99												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.3	13.2	9.97	9.95	9.21	7.99	7.54	7.29	7.21	6.67	9.23	26.1
Depth	0.28	0.17	0.2	0.39	0.31	0.25	0.28	0.38	0.33	0.23	0.282	25.7
Width	1.97	1.74	1.99	1.97	1.47	1.96	1.93	4.14	2.21	1.92	2.13	34.5
MWS100												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.2	16.2	12.4	12.3	11.5	10.38	10.21	10.19	8.93	8.92	11.7	22.6
Depth	0.79	0.93	0.72	0.97	0.41	0.64	0.62	0.67	0.8	0.32	0.687	29.9
Width	1.98	1.78	1.99	1.64	1.26	1.88	1.92	1.74	1.68	1.91	1.78	12.3
MWS101												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.9	13.4	12.8	12.7	12.3	12.3	11.1	10.68	10.22	10.19	12.1	12.6
Depth	0.51	0.36	0.45	0.36	0.52	0.3	0.37	0.46	0.28	0.32	0.393	21.9
Width	2.35	2.33	3.01	2.64	2.92	2.78	2.38	2.91	2.14	2.32	2.58	12.1
MWS102												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.8	15.3	13.9	11.9	10.93	9.99	9.23	8.97	8.91	8.49	11.4	24.3
Depth	0.57	0.55	0.31	0.29	0.25	0.24	0.25	0.43	0.27	0.28	0.344	36.7
Width	2.12	2.84	2.72	5.99	5.98	5.95	6.15	5.92	5.67	5.91	4.93	33.4
MWS103												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	10.77	10.12	10.06	9.88	9.87	9.81	9.27	7.43	6.88	9.8	19.4
Depth	0.46	0.4	0.31	0.37	0.27	0.28	0.3	0.3	0.3	0.29	0.328	18.8
Width	2.18	2.41	2.32	1.96	1.94	1.91	1.73	1.92	1.42	2.02	1.98	14.4
MWS104												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	13.9	11.3	10.89	10.11	10.28	9.87	9.86	7.21	6.89	10.42	22.3
Depth	0.49	0.47	0.47	0.29	0.34	0.28	0.48	0.35	0.25	0.29	0.371	25.9
Width	2.26	2.14	2.29	2.28	2.93	3.85	2.18	2.82	1.67	2.93	2.54	24.1
MWS105												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.4	13.3	10.45	10.44	9.92	9.87	8.24	8.19	7.83	7.78	9.94	20.91
Depth	0.47	0.29	0.32	0.34	0.34	0.27	0.26	0.3	0.51	0.43	0.353	24.6
Width	2.34	2.18	1.93	1.17	1.94	1.83	1.83	1.79	1.78	1.83	1.86	16.4
MWS106												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.9	16.9	13.9	13.6	12.9	12.9	10.52	10.37	8.91	8.89	12.6	23.2
Depth	0.43	0.48	0.39	0.31	0.29	0.28	0.39	0.39	0.33	0.41	0.37	17.6
Width	2.96	1.94	2.41	1.98	1.96	1.84	2.93	2.73	2.41	2.33	2.35	17.8
MWS107												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	18.9	14.9	14.8	14.8	13.4	12.1	11.9	11.2	9.98	6.83	12.9	25.6
Depth	0.51	0.34	0.26	0.41	0.33	0.42	0.25	0.25	0.41	0.34	0.352	24.4
Width	1.93	2.09	1.28	2.11	1.83	1.48	1.13	1.87	1.21	0.79	1.57	29.02
MWS108												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.9	15.3	14.2	13.9	13.8	12.9	12.5	10.91	10.28		13.4	15.3
Depth	0.57	0.42	0.33	0.52	0.46	0.34	0.47	0.33	0.48		0.436	19.9
Width	3.74	2.32	2.91	2.19	2.82	1.87	1.74	1.93	1.72		2.36	28.7
MWS109												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.8	13.8	11.2	10.58	9.91	9.86	9.72	7.28	6.91		10.35	23.4
Depth	0.41	0.51	0.32	0.26	0.31	0.31	0.34	0.31	0.21		0.331	26.04
Width	1.91	1.88	1.82	1.13	1.14	1.32	1.54	1.94	2.13		1.65	22.7
MWS110												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	16.1	15.9	14.5	13.7	13.2	12.9	12.8	12.2	8.82	8.49	12.9	19.9
Depth	0.42	0.39	0.24	0.24	0.28	0.29	0.19	0.27	0.2	0.19	0.271	29.3
Width	3.12	3.11	2.98	2.96	2.44	3.11	2.12	3.63	2.24	1.92	2.76	19.9
MWS111												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.1	9.98	9.84	9.83	9.81	9.19	8.92	8.78	7.71	6.49	9.17	14.2
Depth	0.56	0.54	0.52	0.46	0.42	0.49	0.34	0.38	0.48	0.35	0.454	17.3
Width	1.99	1.78	1.68	1.43	1.64	0.98	1.92	1.18	1.83	1.32	1.58	21.3
MWS112												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.9	11.7	10.62	10.57	9.93	9.92	9.85	9.83	9.71	9.67	10.37	7.92
Depth	0.21	0.21	0.23	0.22	0.21	0.25	0.24	0.24	0.2	0.22	0.223	7.34
Width	3.41	3.38	3.32	1.45	6.12	3.44	4.94	2.0	3.92	3.91	3.59	36.8
MWS113												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	10.92	10.91	10.75	10.68	10.51	10.48	9.24	7.27	7.11	6.98	9.49	17.9
Depth	0.23	0.23	0.25	0.34	0.18	0.23	0.37	0.23	0.22	0.25	0.253	23.7
Width	4.82	1.98	4.81	4.23	4.92	3.91	3.92	4.21	5.12	3.32	4.12	22.8
MWS114												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.8	11.4	11.2	10.98	10.71	10.48	9.86	9.85	8.22	8.17	10.27	12.2
Depth	0.35	0.29	0.25	0.25	0.29	0.37	0.23	0.23	0.22	0.26	0.274	18.7
Width	5.66	3.34	3.92	3.14	3.52	3.13	4.94	3.97	4.31	3.24	3.92	21.5
MWS115												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	13.7	11.7	9.99	9.82	9.77	8.93	7.59	7.41	7.19	10.002	24.4
Depth	0.44	0.45	0.59	0.4	0.34	0.39	1.41	0.28	0.36	0.25	0.491	68.6
Width	1.68	2.13	2.14	1.94	2.51	1.96	1.85	1.35	1.73	1.52	1.88	17.8
MWS116												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	17.9	16.2	10.62	10.57	9.41	8.48	8.22	7.38			11.1	34.8
Depth	0.55	0.37	0.31	0.32	0.39	0.53	0.41	0.33			0.4013	23.1
Width	2.18	1.19	1.46	1.82	1.98	2.23	1.97	2.14			1.87	19.7
MWS117												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.6	12.9	12.8	11.2	11.1	10.99	10.82	9.97	8.92	7.89	11.03	16.3
Depth	0.58	0.64	0.59	0.52	0.56	0.52	0.64	0.48	0.44	0.49	0.546	12.4
Width	3.13	2.32	2.91	2.13	1.98	2.12	2.43	1.98	2.24	2.33	2.36	16.2
MWS118												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.6	14.3	13.8	12.4	12.3	12.3	11.9	10.77	8.91		12.4	14.5
Depth	0.63	0.63	0.68	0.55	0.36	0.48	0.43	0.37	0.42		0.5056	23.9
Width	2.91	2.33	2.12	1.94	2.01	2.31	2.01	1.94	1.93		2.17	14.7
MWS119												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	13.3	11.6	11.2	10.92	9.18	9.11	8.99	6.21	4.28	9.87	30.27
Depth	0.36	0.32	0.42	0.42	0.45	0.36	0.27	0.34	0.42	0.48	0.384	16.8
Width	1.32	0.94	1.28	1.92	1.36	1.23	0.85	1.33	1.02	1.12	1.24	24.1
MWS120												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	18.9	14.7	13.5	11.9	9.23	8.28	5.67				11.8	37.8
Depth	0.32	0.48	0.43	0.33	0.41	0.36	0.27				0.371	19.5
Width	2.47	3.98	2.34	2.12	1.16	2.91	1.42				2.34	40.19
MWS121												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	15.1	14.1	13.2	12.9	8.86	8.68	7.99	7.21	6.62	6.56	10.14	32.9
Depth	0.56	0.62	0.48	0.43	0.42	0.45	0.48	0.36	0.32	0.31	0.443	22.4
Width	1.96	1.92	1.42	1.96	1.98	1.23	1.82	1.23	1.38	1.32	1.62	20.37
MWS122												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	11.3	10.89	8.97	8.97	8.68	8.67	7.92	7.91	7.73	7.29	8.84	15.03
Depth	0.49	0.46	0.33	0.32	0.29	0.34	0.33	0.34	0.32	0.43	0.365	18.8
Width	1.29	1.39	1.18	1.77	1.04	1.81	0.78	1.64	1.82	1.13	1.39	26.2
MWS123												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.9	11.9	11.4	11.3	11.1	10.38	10.31	8.99	8.81	7.86	10.61	16.5
Depth	0.48	0.39	0.53	0.52	0.37	0.36	0.48	0.32	0.33	0.35	0.413	19.6
Width	2.19	2.34	3.32	2.19	2.19	2.45	2.34	1.96	1.92	2.81	2.37	17.6
MWS124												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.3	14.3	12.31	12.01	11.9	11.8	11.6	10.98	9.99	9.92	11.9	12.5
Depth	0.56	0.54	0.52	0.49	0.45	0.51	0.49	0.53	0.55	0.53	0.517	6.39
Width	1.89	1.78	1.91	1.92	1.89	2.24	2.93	2.12	2.11	2.13	2.092	15.7
MWS125												
	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	13.4	13.3	11.2	10.88	10.06	10.06	9.09	9.07	8.82	8.19	10.41	17.4
Depth	0.52	0.51	0.47	0.44	0.48	0.42	0.36	0.33	0.33	0.29	0.415	19.9
Width	1.39	1.38	1.37	1.24	1.05	1.14	1.51	1.14	1.01	1.11	1.23	13.7
MWS126												

	A	B	C	D	E	F	G	H	I	J	Mean (mm)	RSD (%)
Length	14.01	13.03	12.5	12.09	11.08	11.07	11.01	9.02	8.62	4.01	10.65	26.9
Depth	0.42	0.41	0.36	0.33	0.42	0.34	0.41	0.25	0.27	0.23	0.344	21.2
Width	3.2	2.03	2.13	2.07	2.51	2.05	2.24	1.55	2.7	1.32	2.18	24.7

Appendix C

Appendix C1 List of Publications

Wilson, M., Al-Jumeily OBE, D., Tang, L., Birkett, J., Iftikhar, K., Abbas, I. and Assi, I (2025) Detecting Cardiovascular Diseases and Diabetes Mellitus Through Fingernails Using Attenuated Total Reflectance-Fourier Infrared Spectroscopy and Machine Learning. *Analytical Letters* [online], 1-20.

Available at: <https://doi.org/10.1080/00032719.2025.2534604>

[Accessed: 27th August 2025]

Wilson, M., Al-Jumeily OBE, D., Abbas, I., Khan, I., Birkett, J., Harper, M. and Assi, S. (2024) Using Near-Infrared Spectroscopy and Machine Learning Algorithms for the Detection of Cardiovascular Diseases and Diabetes Mellitus in Fingernails. *17th International Conference on Development in eSystem Engineering (DeSE)* [online], United Arab Emirates, 6-8th November.

Available at: <http://dx.doi.org/10.1109/DeSE63988.2024.10911971>

[Accessed: 24th June 2025]

Wilson, M., Al Hamid, A., Abbas, I., Birkett, J., Khan, I., Harper, M., Al-Jumeily OBE, D. and Assi, S. (2024) Identification of Diagnostic Biomarkers Used in the Diagnosis of Cardiovascular Diseases and Diabetes Mellitus: A Systematic Review of Quantitative Studies. *Diabetes, Obesity and Metabolism* [online], 26, 3009-3019.

Available at: <http://dx.doi.org/10.1111/dom.15593>

[Accessed: 24th June 2025]

Al Hamid, A., Beckett, R., Wilson, M., Jalal, Z., Cheema, E., Al-Jumeily OBE, D., Coombs, T., Ralebitso-Senior, K. and Assi, S. (2024) Gender Bias in Diagnosis, Prevention and Treatment of Cardiovascular Disease: A Systematic Review. *Cureus* [online], 16, e54264.

Available at: <https://doi.org/10.7759/cureus.54264>

[Accessed: 24th June 2025]

Assi, S., Abbas, I., Tang, L., Rowlands, S., Wilson, M., Coombs, T., Arafat, B., Al Hamid, M. and Al-Jumeily OBE, D. (2024) Evaluating the Detection of Cocaine and its Impurities Concealed Inside Fruit- and Vegetable- Food Products Using Handheld Spatially Offset Raman Spectroscopy. *Vibrational Spectroscopy* [online], 131, 103662.

Available at: <http://dx.doi.org/10.1016/j.vibspec.2024.103662>

[Accessed: 24th June 2025]

Wilson, M., Al-Jumeily OBE, D., Abbas, I., Khan, I., Birkett, J., Tang, L. and Assi, S. (2023) Identification of Diagnostic Biomarkers for Cardiovascular Diseases and Diabetes Mellitus Through Raman Spectroscopy and Machine Learning Algorithms. *16th International Conference on Developments in eSystems Engineering (DeSE)* [online], Istanbul, Turkey, 18-20th December.

Available at: <http://dx.doi.org/10.1109/DeSE60595.2023.10468923>

[Accessed: 24th June 2025]

Thompson, M., Assi, S., Al-Jumeily OBE, D., McCloskey, A., Sarker, S., Tang, L., Mohamed, F.A., Wilson, M. and Ehtezazi, T. (2023) Use of Raman and Fourier Transform Infrared Spectroscopy as a Quality Control of 3D Printed Linalool Fast Dissolving Oral Films. *16th International Conference on Developments in eSystems Engineering (DeSE)* [online], Istanbul, Turkey, 18-20th December.

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