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The association between treatment and systemic inflammation in acromegaly

Running title: Systemic inflammation and treatment in acromegaly

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Abstract

Objective: Acromegaly is characterized by an excess of growth hormone (GH) and insulin like growth-factor 1 (IGF1), and it is strongly associated with cardiovascular diseases (CVD). Both acute and long-lasting pro-inflammatory effects have been attributed to IGF1. Previous results suggest the presence of systemic inflammation in treated patients. Here we assessed the association between treatment of acromegaly, systemic inflammation and vascular function.

Design: Ex vivo cytokine production and circulating inflammatory markers were assessed in peripheral blood from treated and untreated acromegaly patients (*N=120*), and compared them with healthy controls. A more comprehensive prospective inflammatory and vascular assessment was conducted in a subgroup of six treatment-naive patients with follow-up during treatment.

Results: Circulating concentrations of VCAM1, E-selectin and MMP2 were higher in patients with uncontrolled disease, whereas the concentrations of IL18 were lower. In stimulated whole blood, cytokine production was skewed towards a more pro-inflammatory profile in patients, especially those with untreated disease. Prospective vascular measurements in untreated patients showed improvement of endothelial function during treatment.

Conclusions: Acromegaly patients are characterized by a pro-inflammatory phenotype, most pronounced in those with uncontrolled disease. Treatment only partially reverses this pro-inflammatory bias. These findings suggest that systemic inflammation could contribute to the increased risk of CVD in acromegaly patients.

Introduction

Acromegaly is a rare disease caused by excessive production of growth hormone (GH), mostly by a pituitary adenoma, and subsequent insulin-like growth factor 1 (IGF1) excess [1]. GH and IGF1 have numerous immunological, metabolic and cardiovascular effects [2-5]. Patients with active acromegaly suffer from cardiovascular morbidity and mortality [6, 7]. Although the mortality risk practically normalizes with adequate treatment, cardiovascular disease (CVD) risk factors often persist [8]. The mechanism underlying this phenomenon is not well understood; direct deleterious effects of GH and IGF1 on the cardiovascular and/or immune system have been suggested [9], but prospective systematic analyses are lacking. Atherosclerosis is the main pathophysiological process driving CVD, and is characterized by subclinical systemic inflammation and inflammatory arterial wall changes [10], in which immune cells play a pivotal role. Recently, the CANTOS and COLCOT trials provided proof-of-principle that targeting low-grade inflammation reduces cardiovascular events in high-risk patients [10, 11].

Given the high prevalence of cardiovascular disturbances in acromegaly patients, and the previously suggested pro-inflammatory effects of GH and IGF-1, we hypothesized that subclinical inflammation is present in acromegaly patients, which contributes to their CVD risk. In a cross-sectional study in treated acromegaly patients and healthy controls [12], we identified a pro-inflammatory phenotype and endothelial dysfunction in patients despite treatment. Building on this, we now hypothesize that treatment-naive acromegaly patients display an even more pronounced pro-inflammatory phenotype, which is only partly normalized by acromegaly treatment. We extended our cross-sectional cohort, and prospectively followed treatment-naive patients during treatment, in order to detail the effect of treatment on the inflammatory and vascular phenotype that we found previously.

Materials and Methods

This study was conducted in two academic referral centers (Radboud University Medical Center (Radboudumc), Nijmegen, the Netherlands, and the Cluj County Emergency Hospital in Cluj-Napoca, Romania).

Subjects

For the cross-sectional part, we included 44 treated (*N*=38) and treatment-naive (*N*=6) acromegaly patients that were admitted to the Cluj County Emergency Hospital (Cluj-Napoca, Romania) and nine healthy controls from this area, together with 71 treated patients from the Radboudumc and 41 healthy controls, who were described in our previous study [12].
For the prospective part, we included six additional treatment-naive patients from the Radboudumc, and six sex- and age-matched healthy controls for comparison at baseline (Table 1; Figure 1).

Subjects with inflammatory comorbidities, active malignancies or those using systemic immunosuppressive medication were excluded. In addition, patients with inadequately treated hypertension (systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥100 mmHg), poorly controlled diabetes mellitus (HbA1c >69 mmol/mol for >1 year), ischemic CVD, or an alcohol intake of >21 IU per week were excluded.
The above-mentioned exclusion criteria also applied to controls. In addition, controls with pituitary hormone disturbances were excluded.
All patients had a history of biochemically and radiologically confirmed *active acromegaly*, defined as an increased serum IGF1 level (>2 SD above the mean corrected for sex and age) and insufficient suppression of serum GH levels (≥0.4 µg/L in Nijmegen, ≥1 µg/L in Cluj-Napoca) during an oral glucose tolerance test (OGTT) [1], combined with the presence of a

pituitary adenoma on a MRI- or CT-scan.

After diagnosis, standard care was pre-treatment with a long-acting somatostatin receptor analogue (SSA) for 6 months, followed by endoscopic endonasal transsphenoidal adenomectomy (EETA), or primary medical therapy in patients who were not suitable for surgery. If biochemical control was not obtained by SSA monotherapy, the GH-receptor antagonist Pegvisomant (PEGV) or a dopamine-agonist was added. In case of recurrent or residual disease after surgery, medical therapy was (re)started. When possible, patients underwent a second surgical approach. Patients with uncontrolled disease despite surgery and/or maximal tolerable medical therapy underwent radiotherapy.

Surgical control was defined as postoperative IGF1 levels within the sex- and age-adjusted reference range, preferably combined with a sufficient suppression of serum GH levels (GH ≤0.4 μg/L) during an oGTT, performed approximately four months after surgery, without use of GH- or IGF1-lowering drugs. *Biochemical control* was defined as IGF1 levels within the sex- and age-adjusted reference range with use of GH- or IGF1-lowering drugs [13]. Surgically and biochemically controlled patients are both considered *controlled*. Patients with active acromegaly (e.g. IGF1 levels above the reference range) despite treatment are *uncontrolled*. Both *controlled* and *uncontrolled* patients are considered *treated*.

Postmenopausal women had gonadotrophin levels were in the postmenopausal range and/or were they older than 55 years. *Hypogonadism* was defined as estrogen- or total testosterone levels below the reference range in premenopausal women and men, *adrenal insufficiency* (AI) as a serum morning cortisol <100 nmol/L, after withdrawal of glucocorticoids for 24 h, or a maximal cortisol response ≤550 nmol/L during an insulin tolerance test or a 250 μg ACTH (Synacthen) stimulation test [14], *hypothyroidism* as free thyroxin plasma levels <8 pmol/L (reference range 8–22 pmol/L), *hypopituitarism* as the presence of one or more of the aforementioned pituitary hormonal deficiencies, *hypertension* as use of antihypertensive

therapy based on a previous diagnosis of hypertension or at least three measurements of a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg on different days, and *diabetes mellitus (DM)* as use of glucose-lowering medication based on a previous diagnosis of DM or fasting glucose levels ≥7 mmol/L and/or random glucose levels ≥11.1 mmol/L at distinct timepoints.

The study was conducted in accordance with the Declaration of Helsinki and approved by our local ethical committee (CMO regio Arnhem-Nijmegen; 2015-2023). All subjects signed informed consent prior to participation.

Study protocol

Anthropometric measurements

Blood pressure and heart rate were measured in supine position on both arms after 10 minutes of rest. Height, weight, waist, and hip circumference were determined between 0830 and 1030 h. Measurements were performed by one experienced non-blinded investigator per center.

Circulating inflammatory and cardiovascular markers

Blood was drawn from the brachial vein in a fasted state, in 10 mL EDTA tubes (Vacutainer, BD; Franklin Lakes, NJ, USA). Within 3 hours, tubes were centrifuged (3800 RPM, 10 minutes, room temperature), and plasma was collected and stored at −80°C until assayed. Plasma IGF1 levels were determined by a chemiluminescent immunometric assay (Liaison, DiaSorin, Saluggia, Italy) in Nijmegen and by a Cobas e 411 immunoassay analyzer (Roche Diagnostics, Basel, Switzerland) in Cluj-Napoca. Lipid levels were measured on a Cobas 8000 analyzer (Roche) in Nijmegen and an AU 680 spectrophotometer (Beckman Coulter, Brea, California, USA) in Cluj-Napoca. LDL cholesterol levels were calculated using the Friedewald formula.

Plasma levels of E-Selectin, Matrix Metalloproteinase (MMP)2, vascular cell adhesion molecule (VCAM)1, high sensitivity C-Reactive Protein (hsCRP), and interleukin (IL)18 were measured with DuoSet enzyme-linked immunosorbent assays (ELISA; R&D Systems, Abingdon, United Kingdom), with a sensitivity of 93.8 pg/mL (E-Selectin), 625 pg/mL (MMP2), 15.6 pg/mL (VCAM1, hsCRP), and 7.8 pg/mL (IL18). IL18 binding protein (IL18BP) was measured with a high sensitivity Quantikine ELISA assays (R&D; sensitivity 2.25 pg/mL).

Ex-vivo stimulation of whole blood (WB)

E. coli lipopolysaccharide (LPS; serotype 055: B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and re-purified as previously described [15]. Phytohemagglutinin (PHA) was purchased from Sigma-Aldrich (PHA-P; L1668**)**. *Candida albicans* (C. albicans) ATCC MYA-3573 (UC 820) and Staphylococcus aureus (S. aureus) Rosenbach ATCC 25923 were grown overnight in Sabouraud and Brain Heart Infusion broth at 37°C, respectively, and harvested by centrifugation, washed twice, and resuspended in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Dutch Modification, Gibco, Thermo Scientific, Waltham, MA, USA)[16]. *C. albicans* yeasts were heat-killed for 30 minutes at 95°C. Blood was drawn from the brachial vein in a fasted state, between 0800 and 1000 h, in 4 mL lithium-heparin tubes (Vacutainer). Within three hours, 100 µL of WB was incubated at 37°C with 400 µL of stimulus (LPS 100 ng/mL, PHA 10 µg/mL, *C. albicans* $1x10^6$/mL, *S. aureus* $1x10^6$/mL) or RPMI (unstimulated condition) per well. After 48 hours, supernatants were collected and stored at −20°C until assayed.

Cytokine concentrations were measured in supernatants by commercial ELISA kits according to the manufacturer's instructions: tumor necrosis factor alpha (TNFa), IL1B, IL1 receptor antagonist (IL1Ra), IL6 (DuoSet, R&D) with a sensitivity of 3.9 pg/mL (IL1B), 4.7 pg/mL

(IL6), 7.8 pg/mL (TNFa), and 39.0 pg/mL (IL1Ra). Interferon gamma (IFNg) was measured using a PeliKine Compact kit (Sanquin; Amsterdam, sensitivity 3.9 pg/mL). For the existing cohort, IL10 was measured using a PeliKine kit (sensitivity 2.34 pg/mL), for the untreated patients, their controls and the Cluj cohort, using a kit from R&D (sensitivity 11.7 pg/mL).

Plasma and WB ELISAs were performed in three batches (existing cohort, Cluj-Napoca cohort and prospective cohort) without previous freeze-thaw cycles. Control samples from the same batch were used to evaluate the comparability between the three WB batches.

Cell counts

Cell counts were obtained in fresh EDTA blood with a Sysmex automated hematology analyzer (XN-450; Sysmex Corporation, Kobe, Japan). In Cluj-Napoca, cell counts were obtained using an automated Mindray spectrophotometer (BC-6200; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

Prospective analyses

Six treatment-naive newly-diagnosed patients were studied at their first visit (T_0), after 6 months (T_1) and after 15 months (T_2) in the Radboudumc. At each visit, venous blood was drawn in the fasted state, and anthropometric and vascular measurements were performed. Between T_0 and T_1, patients were pretreated according to the abovementioned protocol. Just after T_1, patients underwent EETA.

Flow cytometry

Monocyte subpopulations were identified with flow cytometry using the lysis-no-wash strategy (BD Pharm Lyse lysing buffer, Becton Dickinson) on fresh EDTA blood. 100µl of

blood was stained by monoclonal antibodies (CD16 FITC NKP15 Becton & Dickinson, and CD14 PE RMO52, HLA-DR Immu357 PC5.5, CD45 PC7 J33; last three Beckman Coulter). Surface expression was assessed using FC500 and CytoFLEX flow cytometer and analyzed with Kaluza software version 2.1 (Beckman Coulter). The applied gating strategy was in short; monocytes were selected in the SSC/CD45+ plot, gated to SSC/HLA-DR+ plot, identifying monocytes as CD45+ HLA-DR+ cells with monocyte scatter properties. Exclusion of lymphocytes and natural killer cells was performed by excluding CD45+ HLA-DR+ CD14-CD16- cells. In the CD14/CD16 plot, the percentages of gated monocyte subsets (classical (CD14++CD16−), intermediate (CD14++CD16+), non-classical monocytes (CD14+CD16++)) were used for analyses. Identification of monocytes subsets followed current recommendations [17].

RNA isolation

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare Biosciences). After isolation, the monocyte fraction was increased with hyperosmotic Percoll gradient isolation (Sigma). Percoll-isolated monocytes stored at baseline were isolated using a TRiZOL/RNeasy hybrid protocol. In short, per 1 mL of TRiZOL 200 µL of chloroform was added, mixed, incubated at room temperature for 5 min and spun down for 15 min (12000g) at 4°C. The upper aqueous phase was transferred to a RNA-se free Eppendorf tube, and an equal volume of 70% ethanol was added. After thorough mixing, the sample was loaded unto RNeasy mini columns (Qiagen), after which the manufacturers protocol was followed. After the last manufacturer's step, 15 µL of RNase free water was added, incubated for 5 min, and spun down.

RNA sequencing and differential gene expression analysis

The RNA concentration was determined on the Qubit; the quality using Nanodrop technology.

Library preparation was performed using the Quantseq 3‘mRNA-Seq Library Prep Kit-FWD (Cat#015.96, Lexogen) according to the manufacturer’s protocol. RNA input was normalized to 150 ng. All samples were processed in a single library preparation. After quality control of each library (using Qubit and tapestation), libraries were pooled and diluted to 4 nM. Thereafter, libraries were sequenced in one round on a NextSeq 500 (Illumina) with a 1.4 pM final loading concentration.

Low quality filtering and adapter trimming was performed using Trim Galore!, V0.4.4_dev 9, a wrapper tool around the tools Cutadapt v1.18 and FastQC v0.11.5 (Babraham Bioinformatics). Reads were mapped to a human reference genome (GRCh38.95, Ensembl) with Star v2.6.0a [18] resulting in BAM. These BAM files were counted (number of reads mapped to a feature, e.g. a gene) with HTSeq (HTSeq-count tool v0.11.0 [19]) using a complementary .gtf file, containing annotation for GRCh38.95 (Ensembl). MultiQC was used to combine results and quality checks of all samples[20]. Total reads were between 14-17 million, of which percentage uniquely assigned reads were between 52-61%, aligned reads between 79-83%**.** LogFold shrinkage was performed with apeglm for easier comparison between groups [21]. Differential gene expression analysis was carried out with DESeq2 v1.22.0 in R[22], with internal statistical and normalization method (i.e. adjustment of *P*-value for multiple testing with Benjamini–Hochberg). The average expression of patients versus controls was tested, with correction for sex.

Vascular measurements

Subjects refrained from exercise and consumption of caffeine, alcohol, dark chocolate, vitamin C-rich products and vitamin supplements for 24 hours and fasted for at least six hours. Vascular measurements were performed in a supine position after at least 15 minutes of rest under standardized conditions in a temperature-controlled room between 9 and 12 AM

[23].

Pulse wave velocity and pulse wave analysis

Pulse wave velocity (PWV) and pulse wave analysis (PWA) were performed with a SphygmoCor EM3 tonometry device (AtCor Medical, Sydney, Australia) by a single investigator according to the manufacturer's instructions.

Heart Rate Corrected Central Augmented Pressure was calculated based on the median of 3 PWA measurements of the right radial artery. PWV was calculated as 80% of the direct distance between the palpation site of the right common carotid to the right femoral artery divided by the pulse transit time[24].

Ultrasound measurements

Ultrasound measurements were performed by a single technician on a Terason t3000 ultrasound device (Aloka, UK), and analyzed by a single observer using computer-assisted analysis with edge-detection and wall-tracking software (DICOM Encoder Analysis Combo)[25].

Flow-mediated dilation (FMD)

FMD (% diameter change: (peak diameter – baseline diameter)/baseline diameter) was measured in the distal third of the brachial artery of the right arm using high-resolution B-mode 10 MHz ultrasonography and simultaneous acquisition of pulsed-wave Doppler velocity signals according to a validated protocol [23].

Nitroglycerine-mediated dilation (NMD)

One minute prior, and ten minutes after 0.4 mg nitroglycerine sublingually, brachial artery

diameter and blood flow velocity were measured and analyzed following above-mentioned FMD analysis protocol.

Intima-media thickness (IMT)

IMT was measured using high-resolution B-mode 10 MHz ultrasonography in the common carotid artery on the far wall, at three different angles [26, 27]. IMT was identified as the region between the lumen-intima border and the media-adventitia border. Regions of interest were manually marked and at least 50 frames per scan were analyzed to gain a representative mean of lumen diameter and IMT. Analyses were randomly repeated in order to retain accuracy. Mean IMT was calculated from at least 40 useful frames at three different angles.

Statistical analysis

Data were analyzed with SPSS 25.0. Data are presented as unadjusted means with SD or medians with minimum and maximum values for continuous variables, depending on the normality of the distribution as determined by the Shapiro-Wilk test. Differences between patients and controls were tested with an independent samples *T*-test or a Mann-Whitney *U*-test (depending on the normality of the distribution) for continuous parameters and with the Fisher Exact test in case of categorical data. Data on cytokines and circulating parameters was log-transformed prior to analysis with ANCOVA; *BMI* and *leukocyte count* were associated with cytokine production and circulating parameters, and were included as covariates. For leukocyte counts, *BMI* and *age* were used as covariates. Since leukocyte counts were not measured in nine controls of the Cluj cohort (5.1% of total cases), these values were considered *missing at random,* and were imputed based on subject characteristics (age, sex, IGF1 concentration, group (control/patient)) and leukocyte counts obtained in other controls using multiple imputation (5x) to be able to use *leukocyte count* as a covariate.

Since cytokine concentrations were measured in three batches, we included batch number as a fixed factor in our ANCOVA model. Since concentrations of other circulating inflammatory factors were not significantly influenced by batch, batch was only included as a covariate in analyses on IL18BP concentrations. We also included DM type 2 as a covariate, which turned out to be a significant covariate only for VCAM1, but did not influence our outcomes. Correlations were determined on non-transformed data using Spearman rank correlation. All tests were two-tailed. *P*-values of <0.05 were considered statistically significant.

For the prospective part, results were plotted using Graphpad Prism. Due to the small number of subjects (*N*=6), statistical analysis was not expedient given the low power. The results of the prospective part are therefore depicted in a descriptive manner, and used to explore and validate the associations that were observed in the cross-sectional study.

Results

Subject characteristics

Of the 121 patients, 34 (28.1%) were cured, 40 (33.1%) were biochemically controlled, and 35 (28.9%) were uncontrolled. 12 (9.9%) patients were treatment-naive. DM type 2 was more prevalent in treated patients compared to untreated patients and controls (*P*=0.001), but HbA1c levels did not differ significantly between the patient groups (Table 1).

Prospective subgroup characteristics

Five patients were pretreated with medical therapy for 6 months, followed by EETA (Table 2). One patient refused pretreatment and underwent EETA three months after diagnosis and consequently did not undergo measurements at T_1.

IGF1 levels

There was no difference between the mean plasma IGF1 levels in controlled patients (17.8±4.5 nmol/L) and controls (19.3±6.15 nmol/L). Untreated patients had higher IGF1 levels (68.2±11.5 nmol/L) than uncontrolled patients (38.7±18.6 nmol/L; $P<0.001$), and those two groups had higher IGF-1 levels than controls and controlled patients ($P<0.001$). IGF1 levels decreased during treatment in all 6 patients that were prospectively followed during treatment.

Peripheral blood cell composition

In patients, platelet (242 (124-381) vs. 271 (154-419) $\times 10^9$/L; $P=0.036$) and leukocyte (5.63 (3.36-12.06) vs. 6.51 (3.39-11.62) $\times 10^9$/L; $P=0.002$) counts were lower compared to controls. The lowest leukocyte counts were found in controlled patients (5.43×10^9/L (3.36-12.06); Figure 2C). Leukocyte counts correlated negatively with IGF1 levels (R-0.334; $P=0.022$) in controls, and positively in patients (R 0.287; $P=0.001$).

Leukocyte counts tended to be lower in untreated patients compared to the total group of controls (Figure 2C), but did not evidently change during SSA treatment (Figure 2D), nor did platelet counts.

***Ex vivo* anti-inflammatory cytokine production**

The production of TNFa, IL6, IL1B, IL1Ra and IFNg did not differ between patients and controls, nor between the patient subgroups.

In line with our previous study, stimulated anti-inflammatory IL10 production was higher in controls compared to controlled patients ($P=0.01$ for PHA; $P=0.06$ for LPS; Supplementary Table A.1). IL10 production tended to be higher in controlled patients compared to untreated and uncontrolled patients, although these differences were not statistically significant.

Prospectively, LPS-induced IL10 production increased during treatment with SSA in all but one patient, thereafter they decreased again, resulting in slightly higher IL10 production at T_2 compared to T_0 (Figure 2).

IGF1 concentrations positively correlated with IL6 (R 0.3; *P*=0.001) and IL1Ra (R 0.3; *P*<0.001) production in patients, but not in controls. There was a tendency towards a negative correlation between IGF1 concentrations and LPS-induced IL10 production (R-0.169; *P*=0.06) in patients.

Circulating markers of (vascular) inflammation

The circulating levels of the endothelial dysfunction marker VCAM1 were highest in untreated patients compared to controls and controlled patients (both *P*<0.001), and compared to uncontrolled patients (*P*=0.024). Also, in uncontrolled patients, VCAM1 levels were higher compared to controls (*P*=0.011) and controlled patients (*P*<0.001). Likewise, E-selectin levels were higher in patients compared to controls (*P*=0.03); the highest levels were found in uncontrolled patients (*P*=0.02) compared to controls; the same trend was observed for untreated patients compared to controls (*P*=0.06). Levels were comparable between controls and controlled patients (Figure 3; Supplementary Table A.1).

MMP2 concentrations were comparable in controls and treated patients. However, they were higher in untreated patients compared to uncontrolled patients (*P*=0.02), and even more pronounced compared to controls and controlled patients (*P*<0.001). In uncontrolled patients, MMP2 concentrations were also higher compared to controlled patients (*P*<0.001) (Figure 3). hsCRP concentrations were lower in untreated patients compared to the other three subgroups (*P*<0.001), whereas they were comparable in treated patients and controls (Figure 4;

Supplementary Table A.1).

IL18BP concentrations differed between the groups (P<0.001), and were lowest in controls. Controlled and untreated patients had higher levels than controls (both P<0.001; Figure 4). Untreated patients had higher IL18BP levels than all other groups (P<0.001). IL18 concentrations were higher in controls compared to patients (P=0.004); the lowest concentrations were found in uncontrolled patients (P=0.02 compared to controls; Figure 4). Patients had a lower IL18/IL18BP ratio than controls (P=0.04), although differences between the patient subgroups were not statistically significant.

In controls, IGF1 and IL18BP concentrations were positively correlated (R 0.43; P=0.001), whereas IGF1 concentrations and IL18/IL18BP ratio correlated negatively (R -0.45; P=0.001). In addition, IGF1 concentrations showed a positive correlation with VCAM1 (R 0.38; P=0.003), and a trend towards a negative correlation with hsCRP concentrations (R -0.22; P=0.09). In patients, IGF1 concentrations correlated positively with IL18BP concentrations (R 0.4; P<0.001), and negatively with IL18/IL18BP ratio (R -0.4; P<0.001). In addition, IGF1 correlated strongly with MMP2 (R 0.34; P<0.001), VCAM1 (R 0.49; P<0.001) and E-selectin concentrations (R 0.31; P<0.001), and negatively with hsCRP concentrations (R -0.18; P=0.046).

During treatment, circulating IL18 concentrations increased, the highest levels were measured at T_1. This was paralleled by a mild increase in IL18BP levels at T_1, whereas levels at T_0 and T_2 were comparable. hsCRP concentrations increased in all prospectively followed patients, except for the uncontrolled female patient (no.3), although her hsCRP concentration increased during SSA treatment (Figure 4). MMP2 and VCAM1 concentrations decreased in all patients

except patient no.3. E-selectin concentrations did not change (Figure 3).

Prospective analysis of monocyte subtypes

Flow cytometry revealed a trend towards a shift of monocyte subtypes during treatment, with an increase in nonclassical monocytes from T_0 to T_2 (P=0.09). At baseline, nonclassical numbers were lower in 5 out of 6 patients than their controls, but this was not significant.

The monocyte transcriptome of treatment-naive patients

We performed RNA sequencing on Percoll-isolated monocytes from the untreated patients and their controls. Using a cut-off of False Discovery Rate (FDR)<0.05 and log(2) fold change of >1.5 or <-1.5, no genes were differentially expressed. To increase sensitivity to detect potentially relevant transcriptomic changes, we explored additional signals with an FDR<0.05 and log(2)fold change of >0.5 or <-0.5 (Appendix A-Supplementary Table A.2). The PCA (Principal component analysis) plot (Appendix A-Supplementary Figure A.1) indicates that the separation between patients and controls was more distinct for women than men; the top up- and downregulated genes for female patients and controls are depicted in Appendix A-Supplementary Table A.3.

Several of the upregulated genes in patients are linked to inflammation or metabolic regulation. The top upregulated gene, pyruvate dehydrogenase kinase 4 (*PDK4*), is pivotal in M1 macrophage polarization, in which *PDK2/4* deficiency prevented production of pro-inflammatory cytokines normally induced by treating macrophages with LPS + IFNg [28]. Also *ERAP2*, a central factor for peptide trimming in the generation of most HLA class I-binding peptides, was among the list, as were *LILRA5* (a selective inductor of pro-inflammatory cytokine production), *PRKAG1* (encoding a regulatory subunit of the AMP-activated protein kinase (AMPK), important in regulating cellular energy demands in states of

cellular stress), and *LGALS9*, encoding galectin-9, an important controller of AMPK. The HIF-1a target gene *DDIT4* and *ADGRG1*, an adhesion GPCR restricted to cytotoxic lymphocyte/NK cells, were among the downregulated genes.

Prospective vascular measurements

PWV and PWA did not change over time (Figure 5). IMT however, decreased in all but one patient. At T_1, FMD had increased in all 5 patients (one patient skipped T_1), compared to T_0. At T_2, FMD had improved in 4 out of 6 patients compared to T_0 (Figure 6). Interestingly, baseline diameter decreased in all but one patient, whereas FMD peak diameter increased in all patients compared to T_0.

Discussion

In this study, we show that acromegaly patients display an altered, complex immunological fingerprint and signs of endothelial damage, which is only partially normalized by disease-specific treatment. By prospectively following a subset of treatment-naive patients, we further examined the effects of treatment on inflammatory markers and vascular changes at an individual level.

In a previous study, we showed that ex vivo cytokine production (IL1B, IL1Ra, IFNg) in (uncontrolled) acromegaly patients differed from healthy controls, indicative of an altered behavior of immune cells, and that circulating markers suggest vascular inflammation in acromegaly patients [12]. Importantly, at a cellular level, anti-inflammatory and atheroprotective IL10 production was decreased in both controlled and uncontrolled patients. Since this study included few uncontrolled patients and no untreated patients, we included additional uncontrolled and untreated patients to form the present cohort, which confirmed the

defective production of IL10 in controlled patients compared to controls, and also (trending) in uncontrolled and untreated patients. In addition, IGF1 concentrations negatively correlated with *ex vivo* IL-10 production, and IL10 production modestly increased during SSA treatment. However, we did not reproduce the earlier observed increased IL1B, IL1Ra and IFNg production in uncontrolled patients compared to controls, and therefore cannot further elucidate the role of those proinflammatory cytokines in modulation of cardiovascular risk in acromegaly patients.

Importantly, we also found lower numbers of nonclassical monocytes, which are anti-inflammatory and mainly involved in tissue repair and vascular homeostasis [29], in 5 out of 6 treatment-naïve patients compared to their controls; these numbers increased during treatment. Last, by using a less stringent cut-off order to increase the sensitivity of our transcriptome analysis, we identified several genes (e.g. *PDK4, ERAP2, LILRA5;* Supplementary Table A.3) linked to inflammation or metabolic regulation to be upregulated in patients. Importantly, these latter data need further validation.

Together, these findings imply pro-inflammatory changes at the level of the immune cell in acromegaly.

The differences in circulating markers of (vascular) inflammation between patients and healthy controls are even more pronounced. In accordance with previous reports [30-33], the classical inflammatory marker hsCRP was significantly lower in untreated patients compared to controls and treated patients, and increased after initiation of treatment, which is not always accompanied by a less inflammatory phenotype. Interestingly, IL18 concentrations increased during treatment, but remained lower than the concentrations observed in controls, which corresponds with the lower IL18 concentrations and IL18/IL18BP ratio we observed earlier in controlled patients. Compared to controls, levels of the markers of endothelial damage E-

selectin and VCAM1 were higher in uncontrolled and untreated patients, and comparable in controlled patients; this was not observed previously, although others have reported higher VCAM1 concentrations in active acromegaly patients compared to controls [30, 34]. Further suggesting causality of IGF-1/GH excess in endothelial damage is the observation that VCAM1 concentrations decreased during treatment and correlated with IGF1 concentrations. Last, we observed higher concentrations of MMP2, which is associated with plaque destabilization [35], in uncontrolled and untreated patients, and MMP2 concentrations correlated with IGF1 levels and decreased during treatment, again suggesting a role for IGF1. To conclude, we found biochemical evidence for endothelial dysfunction and plaque destabilization in acromegaly patients, which respond to treatment and normalize in those with controlled disease.

The observation that levels of circulating inflammatory markers did not normalize in the patient with persistently uncontrolled disease during follow-up supports this conclusion.

The decline in concentrations of circulating inflammatory markers and pro-inflammatory cytokine production, and the increase of IL10 production at T_1, might be caused by the combined effects of (partial) disease control and SSA treatment. At T_2 we observed slightly higher levels of pro-inflammatory markers compared to T_1, which was not explained by residual disease activity as those patients had controlled disease, but might be explained by the cessation of SSAs and therefore absence of their suggested anti-inflammatory effects [36-38].

Endothelial dysfunction is considered the earliest stage of atherosclerotic disease [39], and has been reported in acromegaly patients [6, 7, 9, 40]. In the prospectively followed treatment-naive patients, we likewise found improvement of FMD during treatment, which implies

improvement in endothelial function. Interestingly, FMD was higher in most patients at T_1 compared to T_2, which might be a SSA-related effect, since SSA are reported to beneficially influence endothelial function and arterial stiffness [41]. IMT decreased during treatment in all but one patient, whereas PWV and PWA (all surrogate markers for more advanced stages of atherosclerosis) remained stable; the latter may be partially explained by the stable blood pressure that was observed during treatment, as these measures are strongly linked to blood pressure [42]. These findings correspond with earlier reports [43, 44].

This study has some limitations. The major limitation is our small size of the prospective subgroup. Therefore, we used a qualitative and descriptive approach in reporting the study outcomes, and these findings need further validation. While correcting for ethnicity and the presence of DM type 2 did not significantly influence our results, we cannot completely exclude their influence. Last, although most studies suggest that the effects of IGF1 on cardiovascular and inflammatory homeostasis predominate in GH/IGF1 excess [45, 46], we did not assess the independent effects of GH. In our cohort, two patients were suffering from a GH deficiency; both received adequate GH suppletion therapy. GH deficiency is known to induce a pro-inflammatory state, which is reversed by adequate GH suppletion [47, 48] so we consider it unlikely that the adequately corrected GH deficiency of those two patients did influence our results.

Importantly, since we extended our existing cohort by pooling data, the conclusions of the current study are likely related to those of the previous study.

Although controls were younger than patients, and had less comorbidities, we did not find large differences in cardiovascular and inflammatory markers between controls and controlled patients, which highlights the importance of stringent disease control.

To conclude, acromegaly induces a complex inflammatory footprint, which is mostly, but not exclusively, pro-inflammatory. Reduced cellular production of anti-inflammatory IL10, coincides with elevated levels of markers of endothelial dysfunction and MMP2, while hsCRP and IL18 levels are lower in patients. In treatment-naive patients, our findings suggest a shift in monocyte subpopulations with a smaller anti-inflammatory subset. While *ex vivo* cytokine production capacity is only partly restored after disease control, circulating inflammatory markers return to normal, and endothelial dysfunction declines. Since both inflammation and endothelial dysfunction promote atherogenesis, these findings underscore the importance of timely and aggressive treatment in order to prevent CVD.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A

Supplementary Tables and Figures.

Figure and Table Legends and Footnotes

Table 1. Clinical characteristics in patients and controls. Values are displayed as mean with SD (standard deviation) or as median with minimum and maximum, depending on the normality of the distribution. Categorical variables are displayed as numbers. BMI: body mass index in kg/m^2; BP: blood pressure; RT: radiotherapy; SSA: Somatostatin analogue;

PEGV: Pegvisomant; DA: dopamine agonist; IGF1: Insulin-like Growth Factor 1; P: P-values when comparing the three subgroups of patients and controls. *in diabetic patients

Table 2. Clinical characteristics of prospective patients (*N*=6).

PY: packyears; BMI: body mass index in kg/m^2; BP: blood pressure; IGF1: Insulin-like Growth Factor 1; SSA: Somatostatin analogue; DA: dopamine agonist; RT: radiotherapy. HG: hypogonadism; HC: hypocortisolism; HP: hyperprolactinemia; MP: menopause; PM: postmenopausal.

* SSA were discontinued due to pancreatic problems.

Figure 1. Study overview.

IGF1: Insulin-like Growth Factor 1; hsCRP: high sensitivity C-reactive protein; IL: interleukin; IL18BP: IL18 binding protein; VCAM1: vascular cell adhesion molecule 1; MMP2: matrix metalloproteinase 2; LPS: lipopolysaccharide; PHA: Phytohemagglutinin; IL: interleukin; TNFa: tumor necrosis factor alpha; Ra: Receptor antagonist; IFNg: interferon gamma.

Figure 2: LPS-induced IL10 production (A) and leukocyte counts (C) in controls and subgroups of patients (left panel) and prospective LPS-induced IL10 production (B) and leukocyte counts (D) in 6 prospectively followed patients (right panel).

LPS: lipopolysaccharide; IL10: interleukin 10.

Figure 3: Circulating VCAM1 (A), E-selectin (C) and MMP2 (E) levels in controls and subgroups of patients (left panels) and prospective VCAM1 (B), E-selectin (D) and MMP2 (F) levels in 6 prospectively followed patients (right panel).

VCAM: vascular cell adhesion protein; MMP: metalloproteinase.

Figure 4: Circulating hsCRP (A), IL18 (C) and IL18BP (E) levels in controls and subgroups of patients (left panels) and prospective hsCRP (B), IL18 (D) and IL18BP (F) levels in 6 prospectively followed patients (right panel).

hsCRP: high sensitivity cell-reactive protein; IL18: interleukin 18; BP: binding protein.

Figure 5: prospective vascular measurements. IMT (A), IMT/lumen (B), PWA (C) and PWV (D).

PWA: pulse wave analysis; PWV: pulse wave velocity; IMT: intima-media thickness.

Figure 6: prospective FMD analysis. For each patients (A-F; 1-6) FMD baseline diameter, FMD peak diameter, % FMD change and FMD/NTG ratio are depicted.

FMD: flow-mediated dilatation; NTG: nitroglycerin-mediated dilatation

TABLE 1	Controls	Controlled patients	Uncontrolled patients	Untreated patients	P
Number	56	74	35	12	
Sex (male, *N*)	24 (43%)	35 (47%)	16 (46)	7 (58)	0.8
Age (years)	47.5 (15.3)	55.8 (11.2)	47 (11.5)	52 (11.7)	0.001
Height (m)	1.74 (0.1)	1.74 (0.1)	1.74 (0.1)	1.74 (0.1)	1
Smoker (y/n, %)	13 (23.2)	10 (13.5)	5 (14.3)	2 (16.7)	0.1
Weight (kg)	77.7 (16.1)	85.8 (20.7)	95.2 (20.5)	84.4 (10.2)	0.001
BMI (kg/m^2)	26.3 (18.3-46)	27.5 (20-49.1)	31.7 (23-41.4)	27.8 (22.5-36.4)	0.001
Waist-to-hip ratio	0.94 (0.7-1.06)	0.92 (0.76-1.16)	0.9 (0.82-1.04)	0.94 (0.83-0.96)	0.487
Systolic BP (mmHg)	123.1 (14.42)	129 (16)	122.5 (16.6)	130.3 (16.2)	0.074
Diastolic BP (mmHg)	74.9 (9)	80.9 (10.3)	77.7 (11.9)	84 (15.2)	0.006
Heart rate (/min)	64 (44-80)	61 (44-78)	60 (56-72)	60 (62-80)	0.09
Hypertension (y/n)	6 (10.7)	30 (40.5)	13 (37.1)	6 (50)	<0.001
Diabetes mellitus	0	7	9	2	0.001
HbA1c (mmol/mol)*	-	52 (42-58)	55 (40-86)	49.5 (49-50)	0.32
Hormonal deficiency	2 (3.6)	28 (37.8)	20 (57.1)	4 (33.3)	<0.001
Hypothyroidism	2 (3.6)	20 (27)	15 (42.9)	1 (8.3)	<0.001
Hypogonadism	0 (0)	18 (24.3)	11 (31.4)	3 (25)	<0.001
Hypocortisolism	0 (0)	12 (16.2)	7 (20)	1 (8.3)	0.001
Alcohol use (IU/week)	3 (0-20)	2 (0-21)	2.5 (0-21)	7 (1-20)	0.16
Packyears	0 (0-37.5)	0.5 (0-48)	0 (0-76)	0 (0-40)	0.54
Treatment					
RT	0	12 (16.2)	12 (34.3)	0 (0)	<0.001
Surgery	0 (0)	66 (89.2)	29 (82.9)	0 (0)	<0.001
Medication	0 (0)	40 (54.1)	27 (77.1)	0 (0)	<0.001
SSA	0 (0)	34 (45.9)	22 (64.7)	0 (0)	
PEGV	0 (0)	10 (13.5)	5 (14.3)	0 (0)	
DA	0 (0)	7 (9.5)	14 (40)	0 (0)	
IGF1 (nmol/l)	19.3 (6.2)	17.8 (4.5)	38.7 (18.6)	68.2 (11.5)	<0.001

Table 1. Clinical characteristics in patients and controls. Values are displayed as mean with SD (standard deviation) or as median with minimum and maximum, depending on the normality of the distribution. Categorical variables are displayed as numbers. BMI: body mass index in kg/m^2; BP: blood pressure; RT: radiotherapy; SSA: Somatostatin analogue; PEGV: Pegvisomant; DA: dopamine agonist; IGF1: Insulin-like Growth Factor 1; P: P-values when comparing the three subgroups of patients and controls. *in diabetic patients

TABLE 2		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex		Male	Female	Female	Female	Male	Male
Age (years) at T_0		60	47	56	64	63	28
Height (m)		1.77	1.66	1.59	1.69	1.88	1.93
Smoker		Former (9 PY)	Never	Current (30 PY)	Former (25 PY)	Never	Never
Weight (kg)	T_0	120/75	110/70	156/87	146/92	128/84	109/50
	T_1	129/80	110/65	153/76	NA	115/79	110/58
	T_2	130/77	110/65	144/77	130/94	116/77	108/58
BMI (kg/m^2)	T_0	27.3	28.3	27.2	25.2	22.6	22.5
	T_1	27.9	29.8	27.9	NA	23.3	22.9
	T_2	28.1	30.9	26.9	25.2	22.8	22.2
Waist-to-hip ratio	T_0	0.93	0.84	0.96	0.92	0.87	0.83
	T_1	0.97	0.9	1	NA	0.86	0.83
	T_2	1	0.9	0.9	0.88	0.86	0.87
BP (mmHg; systolic/diastolic)	T_0	120/75	110/70	156/87	146/92	128/84	109/50
	T_1	129/80	110/65	153/76	NA	115/79	110/58
	T_2	130/77	110/65	144/77	130/94	116/77	108/58
Diabetes mellitus	T_0	0	0	1	0	0	0
	T_1	0	0	1	NA	0	0
	T_2	0	0	1	0	0	0
Treatment status	T_0	Naive	Naive	Naive	Naive	Naive	Naive
	T_1	SSA	SSA	None*	-	SSA	SSA
	T_2	Cured	Cured	DA + RT	Cured	Cured	Cured
Hormonal deficiency	T_0	HG	MP	PM	PM	HG	HG, HP, HC
	T_1	HG	MP	PM	PM	HG	HG, HP, HC
	T_2	HG	PM	PM	PM	HG	None

Table 2. Clinical characteristics of prospective patients (*N*=6).

PY: packyears; BMI: body mass index in kg/m^2; BP: blood pressure; IGF1: Insulin-like Growth Factor 1; SSA: Somatostatin analogue; DA: dopamine agonist; RT: radiotherapy. HG: hypogonadism; HC: hypocortisolism; HP: hyperprolactinemia; MP: menopause; PM: postmenopausal.

* SSA were discontinued due to pancreatic problems.