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Wolters, TLC, van der Heijden, CDCC, van Leeuwen, N, Hijmans-Kersten, BTP, Netea, MG, Smit, JW, Thijssen, DHJ, Hermus, A, Riksen, NP and Netea-Maier, R

Persistent inflammation and endothelial dysfunction in patients with treated acromegaly.

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1 **Title: Persistent Inflammation & Endothelial Dysfunction in Patients with Treated**
2 **Acromegaly**

3

4 Running title: Persistent Inflammation in Treated Acromegaly

5

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24

25 **Keywords:** inflammation, cardiovascular disease, IGF1, endothelial dysfunction, acromegaly

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28 **Abstract**

29 **Objective:** Acromegaly is characterized by an excess of growth hormone (GH) and insulin
30 like growth-factor 1 (IGF1). Cardiovascular disease (CVD) risk factors are common in
31 acromegaly and often persist after treatment. Both acute and long-lasting pro-inflammatory
32 effects have been attributed to IGF1. Therefore, we hypothesized that inflammation persists in
33 treated acromegaly and may contribute to CVD risk.

34 **Methods:** In this cross-sectional study, we assessed cardiovascular structure and function, and
35 inflammatory parameters in treated acromegaly patients. Immune cell populations and
36 inflammatory markers were assessed in peripheral blood from 71 treated acromegaly patients
37 (with controlled or uncontrolled disease) and 41 matched controls. Whole blood (WB) was
38 stimulated with Toll-like receptor ligands. In a subgroup of 21 controls and 33 patients with
39 controlled disease, vascular ultrasound measurements were performed.

40 **Results:** Leukocyte counts were lower in patients with controlled acromegaly compared to
41 patients with uncontrolled acromegaly and controls. Circulating IL-18 concentrations were
42 lower in patients; concentrations of other inflammatory mediators were comparable with
43 controls. In stimulated WB, cytokine production was skewed towards inflammation in
44 patients, most pronounced in those with uncontrolled disease. Vascular measurements in
45 controlled patients showed endothelial dysfunction as indicated by a lower flow-mediated
46 dilatation/nitroglycerine-mediated dilatation ratio. Surprisingly, pulse wave analysis and pulse
47 wave velocity, both markers of endothelial dysfunction, were lower in patients, whereas
48 intima-media thickness did not differ.

49 **Conclusions:** Despite treatment, acromegaly patients display persistent inflammatory changes
50 and endothelial dysfunction, which may contribute to CVD risk and development of CVD.

51 **Introduction**

52 Acromegaly is caused by overproduction of growth hormone (GH), in most cases by a
53 pituitary adenoma. GH in turn induces production of insulin-like growth factor 1 (IGF1) (1).
54 Both GH and IGF1 have numerous metabolic and trophic effects (2). Apart from disease-
55 specific complications, patients with active acromegaly suffer from an increased morbidity
56 and mortality due to cardiovascular disease (CVD) (3, 4). With disease control (i.e.
57 normalized circulating GH and IGF1 concentrations), the increased prevalence of CVD
58 normalizes to a great extent (5). However, the prevalence of CVD risk factors as hypertension
59 and diabetes mellitus (DM) remains elevated (6-8), which implies persistence of the elevated
60 CVD risk in controlled acromegaly patients. The cause of this phenomenon is incompletely
61 understood, and it is debated whether it could be attributed to direct deleterious effects of GH
62 and IGF1 on the cardiovascular system or is also caused by concomitant cardiovascular and
63 metabolic disturbances that cause hypertension, insulin resistance and dyslipidemia in
64 acromegaly patients (6).

65 Interestingly, CVD is strongly associated with subclinical systemic inflammation (9, 10).
66 Vascular wall inflammation is an important driver of the initiation and progression of
67 atherosclerosis, which is the main pathophysiological process driving CVD. Circulating
68 immune cells invade the vasculature, and induce expression of adhesion molecules and
69 subsequent leukocyte adherence, which promotes a pro-inflammatory and pro-atherogenic
70 environment. Although the importance of innate immune cells in the development and
71 progression of atherosclerosis is widely accepted, the unresolving character of the low-grade
72 inflammation that drives it remains poorly understood. Recently, our group described that
73 innate immune cells can develop long-term functional reprogramming characterized by
74 hyperresponsiveness, termed 'trained immunity' (11). Short-term exposure to stimuli can
75 induce a long-term pro-inflammatory phenotype of monocyte-derived macrophages (12, 13),

76 and circulating monocytes obtained from patients with risk factors for atherosclerosis or
77 established atherosclerosis display a pro-inflammatory phenotype (14, 15).
78 Intriguingly, immune cells express GH and IGF1 receptors (16, 17). Previously, we found that
79 IGF1 can impact on monocyte inflammatory function in vitro (18). Moreover, exposure to
80 IGF1 induces trained immunity (19). However, studies on the inflammatory profile of
81 acromegaly patients rendered conflicting results: both unaltered as well as pro-inflammatory
82 phenotypes have been reported (20-22). On the other hand, previous studies on the risk of
83 CVD in (treated) acromegaly imply that the arterial structure and function of patients with
84 acromegaly is impaired, which might contribute to the development of CVD (6). We therefore
85 hypothesized that treated acromegaly patients are characterized by prolonged inflammatory
86 changes, which might contribute to the persistence of CVD risk factors and development of
87 CVD. To test this hypothesis, we comprehensively assessed vascular structure and function,
88 circulating inflammatory markers and ex vivo cytokine production capacity in acromegaly
89 patients and healthy controls. By including both patients with active disease under treatment
90 and controlled disease, we aimed to elucidate whether these properties are reversible after
91 disease control.

92 **Materials and Methods**

93 This cross-sectional case-control study was conducted in an academic referral center
94 (Radboudumc Nijmegen, the Netherlands). The study structure is displayed in Figure 1.

95

96 **Subjects**

97 Seventy-one adult patients with acromegaly and forty-one healthy controls (Table 1) were
98 included between February 2016 and April 2017. All patients that were currently treated at
99 our center or were treated within the last 5 years were selected. Subjects with inflammatory
100 comorbidities, active malignancies or those using statins or systemic immunosuppressive
101 medication were excluded. In addition, we excluded patients with inadequately treated
102 hypertension (systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 100 mmHg),
103 poorly controlled DM (HbA1c > 69 mmol/mol for > 1 year) or ischemic cardiovascular
104 diseases, or with an alcohol intake of > 21 IU per week. Non-pregnant adults with established
105 and treated acromegaly were asked to participate (N=101), 29 patients declined. Reasons for
106 decline were not being able to travel to the hospital, lack of time, and difficulties with being
107 fasted. The remaining 72 patients were enrolled in the study. One patient was excluded
108 because a previously unknown inflammatory disorder manifested during the study.

109 In order to provide a sex-, age- and body composition-matched control group, patients were
110 asked to provide a healthy volunteer from their own living environment with, preferably, a
111 similar age, sex, and physique. The above-mentioned criteria, except for the presence of
112 acromegaly, were also applied to controls. In addition, controls with hormonal disturbances,
113 except for adequately supplemented primary hypothyroidism (based on normal TSH-levels
114 after suppletion for > 3 months), were excluded. Forty-four controls were willing to
115 participate; three candidates were excluded based on above-mentioned exclusion criteria.

116 All patients had a history of biochemically and radiologically confirmed acromegaly, defined

117 as an increased serum IGF1 level (>2 SD above the mean corrected for sex and age) and
118 insufficient suppression of serum GH levels (≥ 0.4 $\mu\text{g/l}$) during an oral glucose tolerance test
119 (OGTT) (1), combined with the presence of a pituitary adenoma on a MRI- or CT-scan. All
120 patients had received treatment (i.e. surgery, radiotherapy and/or medication; Table 2).
121 Disease duration was based on patients' reports that were obtained during a thorough medical
122 history. Patients were considered *cured* if their serum IGF1 level fell within the reference
123 range for sex and age, and when patients had a sufficient suppression of serum GH levels
124 during an OGTT (GH < 0.4 $\mu\text{g/l}$), that was performed after surgery and/or radiation therapy
125 (23). *Biochemical control* was defined as IGF1 levels in the reference range for sex and age
126 with use of GH or IGF1 lowering therapy. Both cured and biochemically controlled patients
127 were considered *controlled patients*. *Uncontrolled* patients had elevated IGF1 levels despite
128 medical, surgical and/or radiation treatment.

129 Adrenal insufficiency was defined as an insufficient response (serum cortisol levels < 0.55
130 $\mu\text{mol/l}$) during an insulin tolerance test or a 250 μg ACTH (Synacthen) stimulation test (24),
131 that has been performed in each patient prior to study participation following the standard of
132 care in our hospital. Hypogonadism in premenopausal women was defined as the presence of
133 secondary amenorrhea combined with estrogen values below the reference range. The
134 physiological postmenopausal state, defined as gonadotrophin levels that fall in the
135 postmenopausal range, was not considered as hypogonadism. In men, hypogonadism was
136 defined as a testosterone level below the reference range (< 11 nmol/l). Patients with hormonal
137 deficiencies were all on stable substitution therapy, except for postmenopausal women.
138 Testosterone respectively thyroid hormone substitution therapy was monitored with serum
139 testosterone respectively fT4 levels.

140 In a subgroup of 21 controls and 33 patients, vascular measurements were performed.
141 Because our study focused on the persistent long-term risk of CVD in patients with

142 acromegaly, only controlled patients were included in the vascular analysis. The group of
143 controlled patients was divided into *cured* and *biochemically controlled* patients, given the
144 potential beneficial effects of SSA on vascular function (25). Furthermore, to avoid potential
145 interference with our results, only patients without hormonal deficiencies (except for diabetes
146 insipidus), were selected.

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

150

151 **Anthropometric measurements**

152 Blood pressure and heart rate were measured in supine position on both arms after at least 10
153 minutes of rest. Height, weight, waist and hip circumference were determined between 0830
154 and 1030 h in a fasted state. All measurements were performed by a single non-blinded
155 investigator.

156

157 **Circulating inflammatory and cardiovascular markers**

158 Venous blood was drawn from the brachial vein in a fasted state, between 0800 and 1000 h, in
159 10 ml EDTA tubes (Vacutainer, BD; Franklin Lakes, NJ, USA). Within 3 hours, tubes were
160 centrifuged (Hettich Rotina 420R, radius 183mm; 3800 RPM (RCF 2954), 10 minutes, room
161 temperature), and plasma was collected and stored at -80°C until assayed. Plasma IGF1
162 levels were determined by a chemiluminescent immunometric assay (Liaison, DiaSorin,
163 Saluggia, Italy) according to the 1st WHO International Standard for Insulin-like Growth Factor-I
164 (NIBSC code: 02/25). Levels of total cholesterol, LDL cholesterol, HDL cholesterol, non-HDL
165 cholesterol and triglycerides were determined using an in-house analyzer (Cobas 8000; Roche
166 Diagnostics, IN, USA).

167 Plasma E-Selectin, Matrix Metalloproteinase (MMP)2, vascular cell adhesion molecule
168 (VCAM)1, high sensitivity C-Reactive Protein (hsCRP), interleukin (IL)18, IL18 binding
169 protein (IL18BP) and IL6 levels were determined with enzyme-linked immunosorbent assays
170 (ELISA). For E-Selectin, MMP2, VCAM1, hsCRP, and IL18, DuoSet ELISA (R&D Systems,
171 Abingdon, United Kingdom) was used with a sensitivity of 93.8 pg/ml for E-Selectin, 625
172 pg/ml for MMP2, 15.6 pg/ml for VCAM1 and hsCRP, and 11.7 pg/ml for IL18. For
173 measurement of IL6 and IL18BP, high sensitivity Quantikine ELISA assays (R&D) were
174 used with a sensitivity of 0.11 pg/ml for IL6 and 7.52 pg/ml for IL18BP.

175

176 **Cell counts**

177 Cell counts were obtained in fresh EDTA blood with a Sysmex automated hematology
178 analyzer (XN-450; Sysmex Corporation, Kobe, Japan).

179

180 *Ex-vivo stimulation of whole blood (WB).*

181 *E. coli* lipopolysaccharide (LPS; serotype 055:B5) was purchased from Sigma-Aldrich (St.
182 Louis, MO, USA), repurified as previously described, and used as an ultrapure Toll-like
183 receptor 4 ligand (26). Phytohemagglutinin (PHA) was purchased from Sigma-Aldrich (PHA-
184 P; **L1668**). *Candida albicans* (*C.albicans*) ATCC MYA-3573 (UC 820) and *Staphylococcus*
185 *aureus* (*S.aureus*) Rosenbach ATCC 25923 were used. *C.albicans* and *S.aureus* were grown
186 overnight at 37°C in Sabouraud and Brain Heart Infusion broth, respectively. Microorganisms
187 were harvested by centrifugation, washed twice, and resuspended in Roswell Park Memorial
188 Institute (RPMI) 1640 culture medium (Dutch Modification, Gibco, Thermo Scientific,
189 Waltham, MA, USA) (27). *C.albicans* yeasts were heat-killed for 30 minutes at 95°C.

190 Venous blood was drawn from the brachial vein in a fasted state, between 0800 and 1000 h, in
191 4 ml lithium-heparin tubes (Vacutainer). Within three hours, 100 µl of WB was incubated at

192 37°C in round-bottom 48-well plates (Greiner; Kremsmünster, Austria) with 400 µl of
193 stimulus (LPS 100 ng/ml, PHA 10 µg/ml, *C.albicans* 1x10⁶/ml, *S.aureus* 1x10⁶/ml) or RPMI
194 (basal unstimulated condition) per well. After 48 hours of incubation, supernatants were
195 collected and stored at -20°C until assayed.

196 Cytokine concentrations were measured in supernatants by commercial ELISA kits according
197 to the manufacturer's instructions: tumor necrosis factor alpha (TNFa), IL1B, IL1 receptor
198 antagonist (IL1Ra), IL6 (DuoSet ELISA, R&D), IL10, interferon gamma (IFNg) (PeliKine
199 Compact, Sanquin; Amsterdam). The sensitivity of the assays was 2.34 pg/ml for IL10, 3.9
200 pg/ml for IL1B and IFNg, 4.7 pg/ml for IL6, 7.8 pg/ml for TNFa, and 39.0 pg/ml for IL1Ra.
201 The inter-assay coefficients of variability were 9.5% for IL10, 5.6% for IL1B, 12.8% for
202 IFNg, 8.9% for IL6, 6.9% for TNFa, and 8.4% for IL1Ra.

203 All samples were analyzed in the same batch without previous freeze-thaw cycles.

204

205 **Vascular measurements**

206 Subjects that underwent vascular measurements refrained from exercise and consumption of
207 caffeine, alcohol, dark chocolate, vitamin C-rich products and vitamin supplements for 24
208 hours and fasted for at least six hours. All vascular measurements were performed between
209 0900 and 1200 h in a supine position after at least 15 minutes of rest under standardized
210 conditions in a temperature-controlled room (28, 29).

211

212 *Pulse wave velocity and pulse wave analysis*

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

216

217 Heart Rate Corrected Central Augmented Pressure (C_{AP}_HR75) was calculated based on
218 PWA of the right radial artery, the median of 3 measurements was used for data analysis. For
219 calculation of PWV, 80% of the direct distance between the palpation site of the right
220 common carotid and the right femoral artery was divided by the pulse transit time (in
221 seconds) (30).

222

223 *Ultrasound measurements*

224 All ultrasound measurements were performed by a single technician on a Terason t3000
225 ultrasound device (Aloka, UK). All ultrasound images were analyzed by a single observer
226 using computer-assisted analysis with edge-detection and wall-tracking software (DICOM
227 Encoder Analysis Combo) (28).

228

229 *Flow-mediated dilation (FMD)*

230 FMD (% diameter change: (peak diameter – baseline diameter)/baseline diameter) and shear
231 rate (Arbitrary Units; AU) were measured in the distal third of the brachial artery of the right
232 arm using high-resolution B-mode 10 MHz ultrasonography and simultaneous acquisition of
233 pulsed-wave Doppler velocity signals according to a validated protocol (28, 29).

234

235 *Nitroglycerine-mediated dilation (NMD)*

236 One minute prior and ten minutes after 0.4 mg nitroglycerine sublingually, brachial artery
237 diameter and blood flow velocity were measured and analyzed following the same protocol as
238 was used for FMD analysis.

239

240 *Intima-media thickness (IMT)*

241 IMT was measured using high-resolution B-mode 10 MHz ultrasonography in the common

242 carotid artery on the far wall, at three different angles (31). IMT was identified as the region
243 between the lumen-intima border and the media-adventitia border. Regions of interest were
244 manually marked and at least 50 frames per scan were analyzed to gain a representative mean
245 of lumen diameter and IMT. These analyses were randomly repeated in order to retain
246 accuracy. Mean IMT was calculated from at least 40 useful frames at three different angles.

247

248 **Statistical analysis**

249 Data were analyzed with SPSS 25.0. Data are presented as unadjusted means with SD or
250 medians with minimum and maximum values for continuous variables, depending on the
251 normality of the distribution, which was tested by the Shapiro-Wilk test. Differences between
252 patients and controls were tested with an independent samples T-test or a Mann-Whitney U-
253 test (depending on the normality of the distribution) for continuous parameters and with the
254 Fisher Exact test in case of categorical data. Differences between subgroups were tested using
255 ANOVA. Group matching of patients and controls was performed by testing for differences in
256 age, gender, and Body Mass Index (BMI).

257 Data on cytokines and circulating parameters was log-transformed using the natural logarithm
258 prior to analysis with ANCOVA; *BMI*, *age*, and *leukocyte count* were associated with
259 cytokine production and circulating parameters and were therefore included as covariates. For
260 leukocyte counts, *IGF1 levels*, *BMI* and *age* were used. We performed a sensitivity analysis
261 using forward selection, by alternately adding *estrogen depletion*, *use of antihypertensives*
262 and *presence of diabetes mellitus* as covariates to our ANCOVA-model, which did not
263 improve goodness of fit of the model.

264 For vascular ultrasound and PWV, *age* and *systolic blood pressure* were used as covariates
265 (32, 33) and for C_AP_HR75, *sex* and *systolic blood pressure* were used. Correlations were
266 determined on non-transformed data using Spearman rank correlation. All tests were two-

267 tailed. P-values of <0.05 were considered statistically significant. When comparing three
268 groups of subjects, the Bonferroni correction for multiple testing was applied, which rendered
269 an adjusted P-value of 0.0167; corrected P-values were displayed.

270

271 **Results**

272 *Subject characteristics – total group (inflammatory parameters; Table 1 and 2)*

273 Of the 71 patients, 60 were controlled (of whom 32 were cured and 28 biochemically
274 controlled), and 11 uncontrolled. The prevalence of hormonal deficiencies differed between
275 patients and controls, but not between the subgroups. Sex, age and anthropometrical
276 measurements were not statistically different between the total patient group and controls,
277 which indicates adequate group matching regarding these parameters. Use of antihypertensive
278 medication was more prevalent in patients, but blood pressure did not differ between patients
279 and controls nor between patient subgroups. DM was not present in controls, but was present
280 in eight patients; all but one had a $HbA_{1c} < 58$ mmol/mol (median 53 (40-69)). None of the
281 subjects had established coronary artery disease. The control group contained more current
282 smokers and the patient group more former smokers.

283 In the subgroup analysis, we observed that uncontrolled patients were younger, and had a
284 higher weight and BMI. Disease duration tended to be shorter in uncontrolled patients,
285 although this difference was not statistically significant. All other features were similar in
286 both patient subgroups (Table 2).

287

288 *Subject characteristics – subgroup selected for vascular measurements*

289 Thirty-three controlled patients without hormonal deficiencies and 21 healthy controls
290 underwent additional vascular measurements. They were comparable to the subjects of the
291 total group, except that the patients in this subgroup used slightly more antihypertensive

292 drugs. However, including use of antihypertensive medication in our model did not change
293 our results.

294

295 ***IGF1 levels***

296 There was no difference between the plasma IGF1 levels of controlled patients (17.6 ± 4.1
297 nmol/l) and controls (17.3 ± 5.4 nmol/l; $P=0.7$). Uncontrolled patients had higher IGF1 levels
298 (32.6 ± 6.9 nmol/l) than the other two groups ($P<0.001$).

299

300 ***Cell counts (Figure 2)***

301 Total leukocyte count was lower in patients ($5.38 (3.36-12.06) \times 10^9/l$) compared to controls
302 ($6.81 (3.66-11.62) \times 10^9/l$; $P<0.001$), as were monocyte, lymphocyte and neutrophil counts.
303 However, the lower leukocyte count was triggered only by the controlled patients, while the
304 leukocyte count in uncontrolled patients ($7.24 (4.68-8.63) \times 10^9/l$) was not different compared
305 to controls. Relative leukocyte counts did not differ between patients and controls. The
306 inflammatory marker neutrophil-to-lymphocyte (NtL) ratio did not differ between groups,
307 whereas its analogue, the platelet-to-lymphocyte (PtL) ratio was higher in patients compared
308 to controls ($158.2 (62.5-365.2)$ vs. $137.6 (74.4-305.4)$; $P=0.007$). In patients, we observed a
309 positive correlation between IGF1 levels and leukocyte counts ($R=0.28$; $P=0.02$), whereas in
310 controls, a negative correlation was present ($R=-0.32$; $P=0.04$). IGF1 levels were also
311 positively correlated with monocyte counts in patients ($R=0.30$; $P=0.01$), but not in controls.

312

313 ***Circulating markers of inflammation and endothelial dysfunction (Figure 3)***

314 In the total patient group, plasma IL18 concentrations were significantly lower than in
315 controls ($151.9 (58.6-387.4)$ vs. $178.5 (49.2-1528.3)$ pg/ml; $P=0.01$). IL18BP concentrations
316 were higher in patients compared to controls ($356.1 (265.6-1341.2)$ vs. $265.6 (265.6-601.1)$)

317 pg/ml; $P < 0.001$). Consequently, the IL18/IL18BP ratio was significantly lower in patients
318 than in controls (0.44 (0.11-1.29) vs. 0.65 (0.19-5.75); $P < 0.001$); these differences were
319 triggered by controlled patients, since uncontrolled patients did not differ from controls.
320 VCAM1 levels were lower in patients compared to controls (320 (177-565) vs. 326 (215-561)
321 pg/ μ l; $P = 0.008$), which was caused by lower levels in controlled patients compared to
322 controls (322 (177-565) pg/ μ l; $P = 0.003$). IL18 levels correlated with VCAM1 levels ($R = 0.514$;
323 $P < 0.001$). The other circulating factors investigated did not differ significantly between
324 patients and controls nor between patient subgroups.

325

326 ***Ex vivo cytokine production in whole blood***

327 *Monocyte-derived cytokine production (Figure 4)*

328 Uncontrolled patients had higher *S.aureus*-stimulated IL1B production compared to
329 controlled patients (381.6 (140-1387.9) vs. 194.8 (87.1-653.4) pg/ml; $P = 0.02$) and higher
330 IL1Ra production compared to controls (5847.7 (4197-11760.2) vs. 3375.9 (874.1-8797.5)
331 pg/ml; $P = 0.03$). Controlled patients showed a IL1B and IL1Ra production that was
332 comparable to controls. A similar pattern was seen for IL1B and IL1Ra production in
333 response to other WB stimuli, although these differences were not statistically significant.
334 No differences were observed in the production of monocyte-derived pro-inflammatory
335 cytokines IL6 and TNFa between patients and controls, nor between patient subgroups.

336

337 *Th-derived cytokine production (Figure 5)*

338 We found unstimulated IFNg production in patients, but not in controls (figure 5C). In
339 addition, the *S.aureus*-stimulated IFNg production was significantly higher in patients
340 compared to controls (148.1 (78-5672.7) vs. 92.7 (78-701.7) pg/ml; $P = 0.02$); the highest IFNg
341 production was observed in uncontrolled patients (374.4 (148.1-5389.5) pg/ml; $P = 0.001$ vs.

342 controls, and $P=0.012$ vs. controlled patients (118.8 (78 - 5672.7) pg/ml). Again, a similar
343 pattern was seen for the other WB stimuli. LPS-stimulated anti-inflammatory IL10 production
344 was lower in patients compared to controls (208.1 (57.2 - 890.4) vs. 275.5 (74.9 - 1285.8) pg/ml ;
345 $P=0.04$). IGF1 levels positively correlated with IL6 ($R0.31$; $P=0.008$), IL1B ($R0.42$;
346 $P<0.001$), IL1Ra ($R0.51$; $P<0.001$), and IFN γ production ($R0.34$; $P=0.004$) in patients.

347

348 **Subgroup – vascular measurements (Figure 6)**

349 Serum lipid and IGF1 levels were not significantly different between the groups. All subjects
350 had IGF1 levels that were in the normal reference range for age and sex.

351 FMD was lower in patients than in controls ($5.22\pm 3.58\%$ vs. $8.68\pm 4.87\%$; $P=0.06$), but did
352 not differ significantly between the patient groups. The FMD/NMD ratio was lower in
353 patients compared to controls (0.27 (-0.08 ; 0.15) vs. 0.42 (0.12 - 5.95); $P=0.04$). Shear rate was
354 lower in patients compared to controls (15997 (4676 - 39954) vs. 26245 (14287 - 53297) AU ;
355 $P=0.002$).

356 Compared to controls, patients had both a lower C_{AP}HR75 (7.75 ± 4.03 vs. 6.68 ± 6.12
357 mmHg ; $P=0.04$) and PWV (9.14 (7.1 - 15.36) vs. 8.83 (6.63 - 13.46) m/s ; $P=0.002$). When
358 comparing patient subgroups to controls, the lower C_{AP}HR75 was only present in
359 biochemically controlled patients (5.57 ± 5.5 mmHg ; $P=0.02$), whereas PWV was lower in both
360 cured (9.09 (6.63 - 13.27) m/s ; $P=0.02$) and biochemically controlled patients (8.74 (7.24 - 13.46)
361 m/s ; $P=0.03$). Patients using Somatostatin analogues (SSA) had a lower C_{AP}HR75
362 (8.8 ± 6.3 vs. 3.6 ± 3.9 mmHg ; $P=0.006$). IMT did not differ between controls and patients.

363

364 **Discussion**

365 To our best knowledge, this is the first study that comprehensively examined the multifaceted
366 aspects of inflammation in patients with treated acromegaly and relates them to structural and

367 functional vascular characteristics. We hypothesized that persistent inflammation contributes
368 to the persistence of CVD risk and the development of CVD in acromegaly patients despite
369 adequate treatment. Indeed, we observed pro-inflammatory changes in the function of the
370 immune system in patients with acromegaly, most pronounced in those having active disease,
371 but partly persisting in those with controlled disease. This was paralleled by persistent
372 endothelial dysfunction in controlled patients. These findings suggest that chronic
373 inflammation could contribute to the high prevalence of CVD risk factors in acromegaly both
374 during active disease as well as in adequately treated patients.

375
376 Recent evidence suggests that IGF1 levels are related to CVD in an U-shaped fashion, with
377 both low and high circulating IGF1 levels being associated with an increased CVD risk (34,
378 35). Since the importance of inflammation in the development of CVD is well established (9,
379 10), we previously investigated the effects of IGF1 in vitro. We showed direct pro-
380 inflammatory effects of IGF1 on human white blood cells in supraphysiological
381 concentrations that reflect IGF1 levels in patients with acromegaly (18). Our group has shown
382 long-lasting pro-inflammatory effects of IGF1 on human monocytes, a phenomenon termed
383 ‘trained immunity’ (11). In this study we observed that acromegaly patients with active
384 disease under treatment are characterized by high IL1B and IL1Ra as well as IFN γ production
385 capacity ex vivo in stimulated whole blood (WB). The finding that these changes in cytokine
386 production were more pronounced in reaction to certain stimuli (i.e. all stimuli gave a similar
387 pattern of cytokine production, but not all differences between groups were significant),
388 suggests that the functional reprogramming of monocytes in acromegaly is selective. These
389 pro-inflammatory changes appeared reversible after normalization of IGF1 levels, since these
390 findings were not observed in patients with controlled disease. However, we did observe a
391 lower production capacity of the anti-inflammatory IL10 in the total patient group, suggestive

392 of a pro-inflammatory change in immune cell function that persisted after normalization of
393 IGF1 levels. In addition, WB cells produced IFN γ in the absence of a stimulus in patients but
394 not in controls, indicating a more inflammatory tendency in patients. Intriguingly, our data
395 furthermore suggest an altered interaction between IGF1 and the immune system in
396 acromegaly, as was reported earlier (36, 37): IGF1 levels were positively correlated with IL6,
397 IFN γ , IL1B and IL1Ra production in stimulated WB of patients, but not in WB of controls.
398 Moreover, whereas IGF1 levels and leukocyte counts were negatively correlated in controls,
399 we observed a positive correlation in patients. Also, the platelet-to-lymphocyte ratio was
400 significantly higher in patients compared to controls. This inflammatory biomarker was
401 recently shown to predict inflammatory and cardiovascular events (38). These findings are in
402 concordance with previous reports that CVD risk decreases, but not normalizes with treatment
403 of acromegaly (8, 39), although the prevalence of evident CVD as myocardial infarction and
404 stroke was comparable in acromegaly patients treated in specialized centres compared to the
405 general population (5).

406 An additional argument suggesting that acromegaly leaves a long-lasting immunological
407 imprint is the observation that patients have lower circulating IL18 levels, paralleling higher
408 IL18 binding protein (BP) levels. This is the first study to report on IL18 homeostasis in
409 acromegaly. The effects of IL18 on CVD are controversial: some report IL18 to be associated
410 with atherosclerosis, while others have shown that IL18 improves insulin sensitivity and
411 attenuates the metabolic syndrome (40-42). These effects of IL18 are counteracted by
412 IL18BP, which binds to IL18 and reduces the amount of free (active) IL18. The low IL18
413 biological activity in the circulation of acromegaly patients could therefore have deleterious
414 metabolic effects. Interestingly, IL18 induces VCAM1 expression (43), and IL18 levels
415 strongly correlated with VCAM1 levels in our patient cohort. We found lower VCAM1 levels
416 in controlled patients compared to controls, which raises the question whether the lower

417 VCAM1 levels in our cohort are a consequence of lower IL18 levels. In previous studies both
418 similar and higher levels of VCAM1 have been reported in active acromegaly patients
419 compared to controls (20, 44). Differences in disease activity, metabolic profiles and
420 treatment between study populations could explain these discrepant results, since previous
421 studies reported on untreated patients. In addition, we applied strict correction for potential
422 confounders (age, BMI and leukocyte counts).

423 Given the fact that the vast majority of our patients had controlled disease with normal IGF1
424 levels, it was expected that the levels of hsCRP, which is a surrogate marker of low-grade
425 inflammation, were not different between patients and controls. Previously, levels of hsCRP
426 were reported to be similar in patients with controlled disease and healthy controls (3, 45).

427

428 To investigate if persistent structural and functional vascular changes could be observed after
429 successful acromegaly treatment, and to assess the possible link between inflammation and
430 CVD, we performed vascular measurements in a subgroup of controlled patients without
431 hormonal deficiencies and their matched controls, a comparison that has not been previously
432 reported. We observed impaired endothelium-dependent vascular dilatation in patients
433 compared to controls as measured by the FMD/NMD ratio, reflecting an impairment of
434 arterial vasoprotective functioning (46, 47). Endothelial dysfunction is considered the earliest
435 stage of atherosclerotic disease (48), and has been reported to be present in acromegaly
436 patients (3, 6, 33).

437

438 Findings suggesting more advanced atherosclerosis, such as structural changes (IMT) or
439 arterial stiffening (PWV, PWA) (47), were not observed in controlled patients. Surprisingly,
440 our data suggested less arterial stiffness in these patients than in matched controls. A lower
441 C_{AP}HR75 was observed in biochemically controlled patients, as well as a lower PWV in

442 both biochemically controlled and cured patients. In contrast to our results, previous studies
443 reported higher (49, 50) or similar PWV in patients compared to controls (32, 33, 51) and a
444 similar (32, 33, 52, 53) or higher IMT (3, 6). These difference may be due to differences in
445 study populations, since the aforementioned studies also included patients with active
446 acromegaly, used non-cardiovascular matched controls or included patients using hormonal
447 replacement therapy (3, 32, 33): all factors known to affect vascular measurements (54-56).
448 Also, SSA – which were used by 11 out of the 14 biochemically controlled patients that
449 underwent vascular measurements in our series – are reported to lower arterial stiffness (25).
450 Indeed, we observed lower C_{AP}_HR75 values in patients using SSA.
451 Last, we excluded patients using statins, thereby indirectly excluding those with established
452 CVD. This has likely resulted in the inclusion of patients who are less cardiovascular and
453 metabolic compromised, which could explain the discrepancy between our results and earlier
454 reports.

455

456 This study has some limitations. Our cross-sectional study showed associations between IGF1
457 and cytokine levels and suggested that certain inflammatory and vascular changes are
458 reversible after disease control. However, a causal relation between IGF1 excess,
459 inflammatory and vascular changes, and the reversibility of these changes can only be
460 assessed in a prospective manner. Second, due to the relatively small number of subjects, the
461 statistical power of the subgroup analysis is limited and the presence of confounding factors
462 (e.g. DM, effects of antihypertensive drugs, and hormonal deficiencies) that may influence
463 inflammatory status cannot be ruled out. For example, serum triglyceride levels and the
464 prevalence of DM were higher in uncontrolled patients, patients used more antihypertensive
465 drugs and no controls had DM. When using *use of antihypertensives* or *presence of DM* as
466 covariates in our model, no significant influence or pro-inflammatory effect of these

467 covariates was found, which argues against the presence of important effects of these
468 potential confounders. However, a confounding effect cannot be completely ruled out. In
469 addition, we observed multiple trends that need validation in larger and better matched
470 cohorts of patients and controls. So, despite the finding that group differences were not
471 significant, they might be clinically relevant. Third, since acromegaly has an insidious onset
472 and is often diagnosed with a significant delay, it is usually impossible to define the exact
473 duration of the disease or the time that patients are exposed to high IGF1 levels; these factors
474 and previous treatments may have impacted on our outcome. Fourth, in this series we did not
475 have information on the circulating GH levels at the time of the experiments. Although most
476 studies have focused on the effect of IGF1 on CVD and inflammation, we cannot exclude
477 independent effects of GH (34, 57). Finally, a significant proportion of patients used SSA,
478 which can exert anti-inflammatory effects (58), and therefore possibly affected arterial
479 stiffness and cytokine production in peripheral blood cells (59, 60). If this is the case, use of
480 SSA may have alleviated some of the effects of the GH and IGF1 on systemic inflammation
481 and vascular impairment in patients under pharmacological treatment.

482 Although our study did not provide definitive evidence of the presence of chronic
483 inflammation in patients with treated acromegaly, we have found several clues that point
484 towards persistent pro-inflammatory changes in treated acromegaly patients. Further research
485 is needed to validate our results, especially prospective studies in larger, homogeneous
486 cohorts of patients, and research on the underlying inflammatory mechanisms that may link
487 GH/IGF-1 excess to cardiovascular disturbances.

488

489 In conclusion, the immune profile and the interplay between IGF1 and the immune system are
490 skewed towards inflammation in acromegaly patients who are controlled or uncontrolled
491 under treatment. The most profound changes in the inflammatory state were found in patients

492 that were uncontrolled despite treatment. However, even after normalization of IGF1 levels,
493 acromegaly appears to leave an immunological footprint. These persistent inflammatory
494 changes could contribute to the sustained endothelial dysfunction that we observed in patients
495 who are successfully treated and add to the development and persistence of cardiovascular
496 risk in patients with controlled acromegaly.

497

498 **Declaration of interest**

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

501

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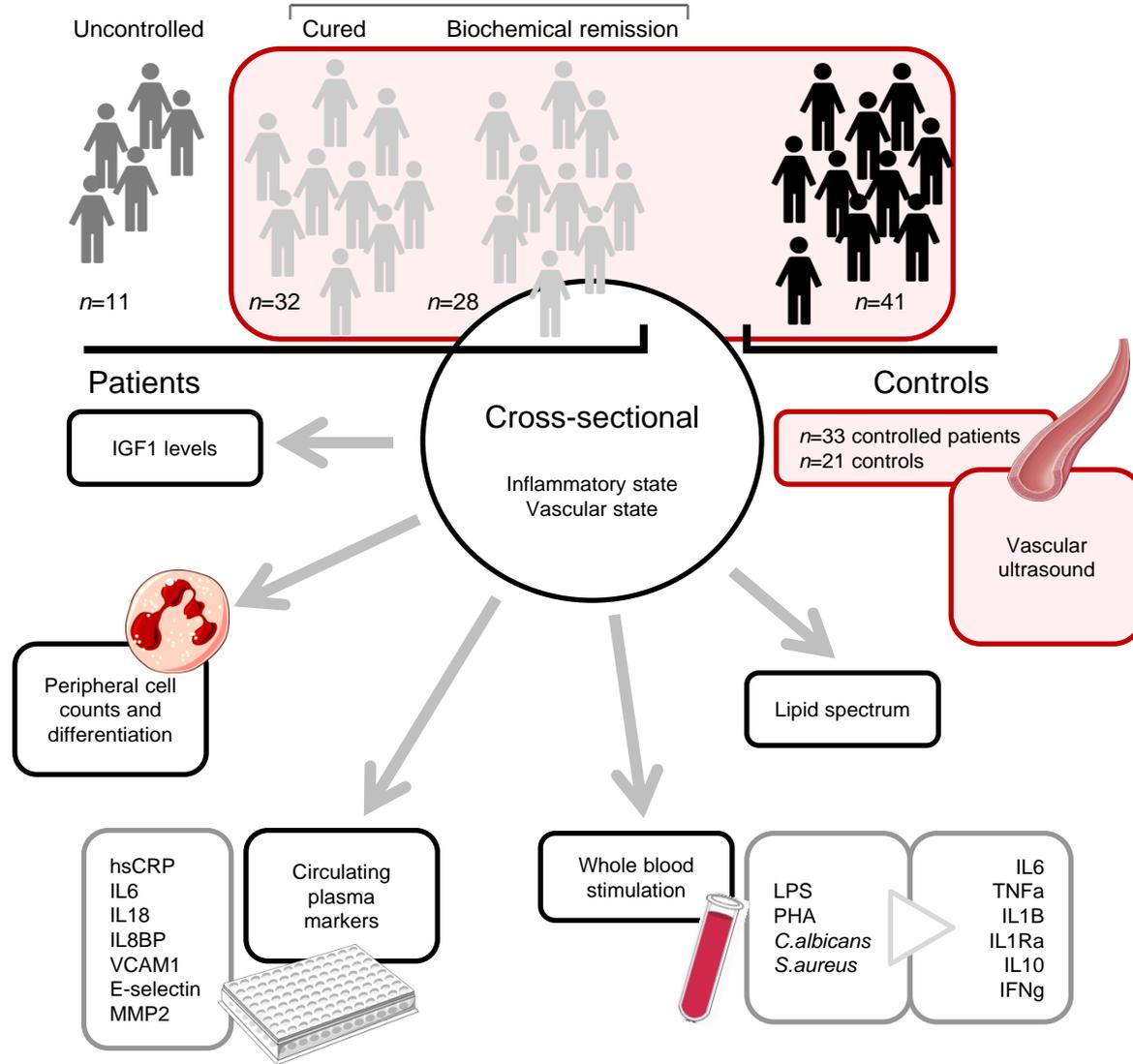
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706

TABLE 1 Clinical characteristics	Controls (n=41)	Patients (n=71)	P
Sex: male	20	36	1.0
Age (years)	51.9 (14.3)	54.5 (12.1)	0.33
Height (m)	1.75 (0.09)	1.76 (0.11)	0.73
Weight (kg)	81.2 (54.8-129.8)	83.7 (51.4-150.8)	0.06
BMI (kg/m ²)	27.07 (18.3-46)	27.7 (20-49.1)	0.13
Waist-to-hip ratio	0.93 (0.08)	0.94 (0.08)	0.82
Systolic BP (mmHg)	124.5 (15.1)	129.5 (16.1)	0.9
Diastolic BP (mmHg)	75.3 (9.4)	79.680 (10.4)	0.52
Heart rate (/min)	64 (44-80)	60 (44-78)	0.87
Anti-hypertensives	3	18	0.02
Diabetes mellitus	0	8	0.03
Smoker; current/past	10/10	8/32	0.05
Alcohol use (units/week)	3 (0-20)	2 (0-21)	0.54
IGF1 (nmol/l)	17.5 (7.9-35.8)	18.2 (8.3-46.7)	0.08
Hormonal deficiency	2	30	<0.001
<i>Estrogen depletion</i>	13	25	0.56
<i>Hypothyroidism</i>	2	18	0.01
<i>Hypogonadism</i>	0	20	<0.001
<i>Hypocortisolism</i>	0	15	<0.001
<i>GH deficiency</i>	0	2	0.53
<i>Diabetes insipidus</i>	0	6	0.08
<i>Hyperprolactinemia</i>	0	1	0.63
Medical treatment	0	35	<0.001
<i>SSA)</i>	0	30	<0.001
<i>Dopamin agonist</i>	0	6	0.08
<i>Pegvisomant</i>	0	8	0.03
Surgery	0	65	<0.001
Radiotherapy	0	10	0.01
Total cholesterol (mmol/L)	5.51 (1.24)	5.23 (1.1)	0.13
HDL cholesterol (mmol/L)	1.47 (0.51-2.84)	1.43 (0.57-2.88)	0.55
LDL cholesterol (mmol/L)	3.27 (1.17)	3.1 (0.93)	0.23
Triglycerides (mmol/L)	1.35 (0.58-3.55)	1.11 (0.53-5.46)	0.2
Non-HDL cholesterol (mmol/L)	3.96 (1.26)	3.72 (1.02)	0.22

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/m²; BP: blood pressure; IGF1: Insulin-like Growth Factor 1; GH: Growth Hormone; Estrogen depletion (in women): postmenopausal women not using estrogen substitution; SSA: Somatostatin analogue; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

TABLE 2 Clinical characteristics	Controlled (n=60)	Uncontrolled (n=11)	P	P*
Sex: male	31	5	0.75	0.93
Age (years)	56.3 (11.1)	44.6 (13.1)	0.01	0.012
Height (m)	1.76 (0.11)	1.79 (0.11)	0.61	0.66
Weight (kg)	83.2 (51.4-150.8)	111 (64.9-147.2)	0.052	0.032
BMI (kg/m ²)	27 (20-49.1)	32.3 (24.1-41.4)	0.029	0.029
Waist-to-hip ratio	0.93 (0.77-1.16)	0.91 (0.82-1.04)	0.86	0.98
Systolic BP (mmHg)	129.2 (16.7)	125.9 (13.0)	0.25	0.31
Diastolic BP (mmHg)	80.2 (10.3)	76.2 (10.4)	0.72	0.05
Heart rate (/min)	61 (44-78)	60 (56-72)	0.81	0.95
Anti-hypertensives	17	1	0.27	0.018
Diabetes mellitus	5	3	0.1	0.006
Smoker; current/past	7/27	1/5	0.67	0.97
Alcohol use (units/week)	2 (0-21)	2 (0-21)	0.56	0.69
IGF1 (nmol/l)	17.6 (4.1)	32.6 (6.9)	0.014	<0.001
Disease duration (years)	9 (1-40)	3 (1-22)	0.05	NA
Hormonal deficiency	24	6	0.51	<0.001
<i>Estrogen depletion</i>	22	3	0.39	0.66
<i>Hypothyroidism</i>	16	2	0.72	0.012
<i>Hypogonadism</i>	15	5	0.27	<0.001
<i>Hypocortisolism</i>	11	4	0.23	<0.001
<i>GH deficiency</i>	2	0	1	0.60
<i>Diabetes insipidus</i>	5	1	1	0.11
<i>Hyperprolactinemia</i>	0	1	0.16	0.1
Medical treatment	28	7	0.34	NA
SSA	24	6	0.51	NA
Dopamin agonist	4	2	0.23	NA
Pegvisomant	5	3	0.1	NA
Surgery	55	10	1	NA
Radiotherapy	7	3	0.18	NA
Total cholesterol (mmol/L)	5.27 (1.13)	5.05 (0.94)	0.5	0.39
HDL cholesterol (mmol/L)	1.47 (0.77-2.88)	1.16 (0.57-2.26)	0.003	0.067
LDL cholesterol (mmol/L)	3.14 (0.95)	2.88 (0.73)	0.33	0.54
Triglycerides (mmol/L)	1 (0.53-3.34)	1.71 (1.06-5.46)	0.036	0.003
Non-HDL cholesterol (mmol/L)	3.70 (1.05)	3.83 (0.84)	0.33	0.5

Table 2. Clinical characteristics of patient subgroups. 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/m²; BP: blood pressure; IGF1: Insulin-like Growth Factor 1; GH: Growth Hormone; Estrogen depletion: postmenopausal women not using estrogen substitution; SSA: Somatostatin analogue; LDL: low-density lipoprotein; HDL: high-density lipoprotein. P: P-values when comparing subgroups of controlled and uncontrolled patients. P*: P-values when controls are included as third subgroup in the analysis; NA: not applicable.



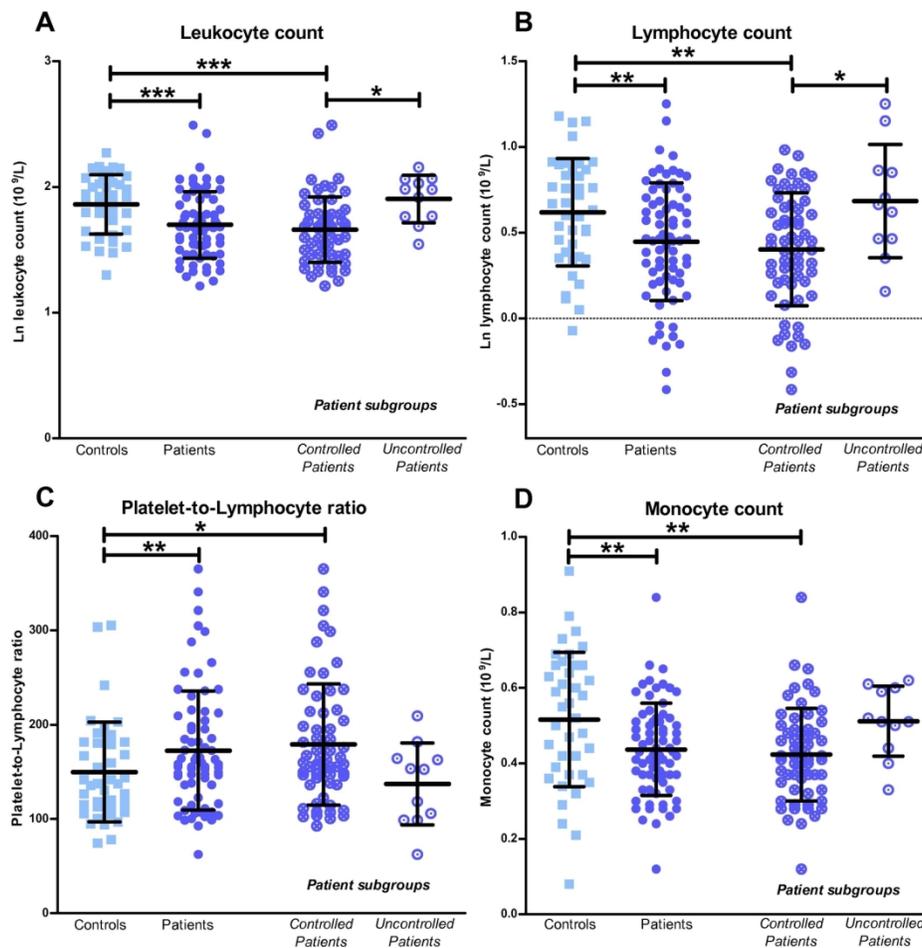


Figure 2. Leukocyte (A), lymphocyte counts (B), platelet-to-lymphocyte ratio (PtL) (C) and monocyte counts (D). Leukocyte and lymphocyte counts were log-transformed using the natural logarithm; mean with SD is displayed for all parameters.

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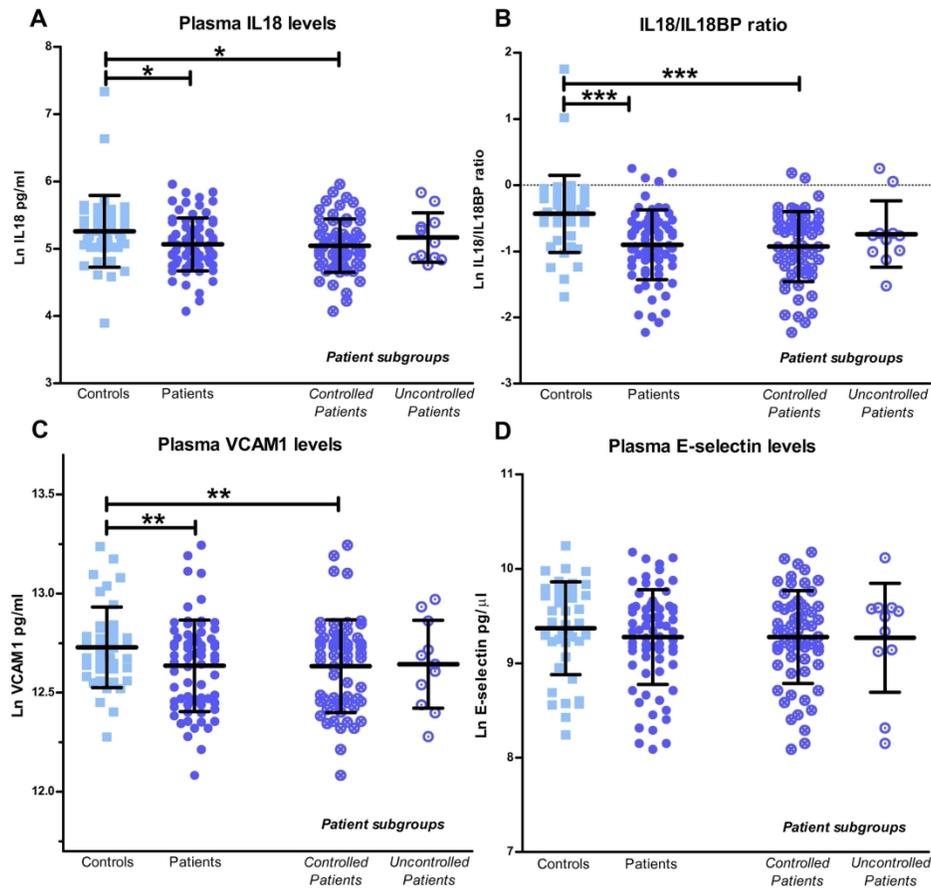


Figure 3. Circulating inflammatory markers. Anti-inflammatory IL18 (A), IL18/IL18BP ratio (B), pro-inflammatory VCAM1 (C) and pro-inflammatory E-selectin levels (D). Cytokine concentrations were log-transformed using the natural logarithm, and mean with SD is displayed. IL18: interleukin 18; IL18BP: IL18 binding protein; VCAM1: vascular cell adhesion molecule 1.

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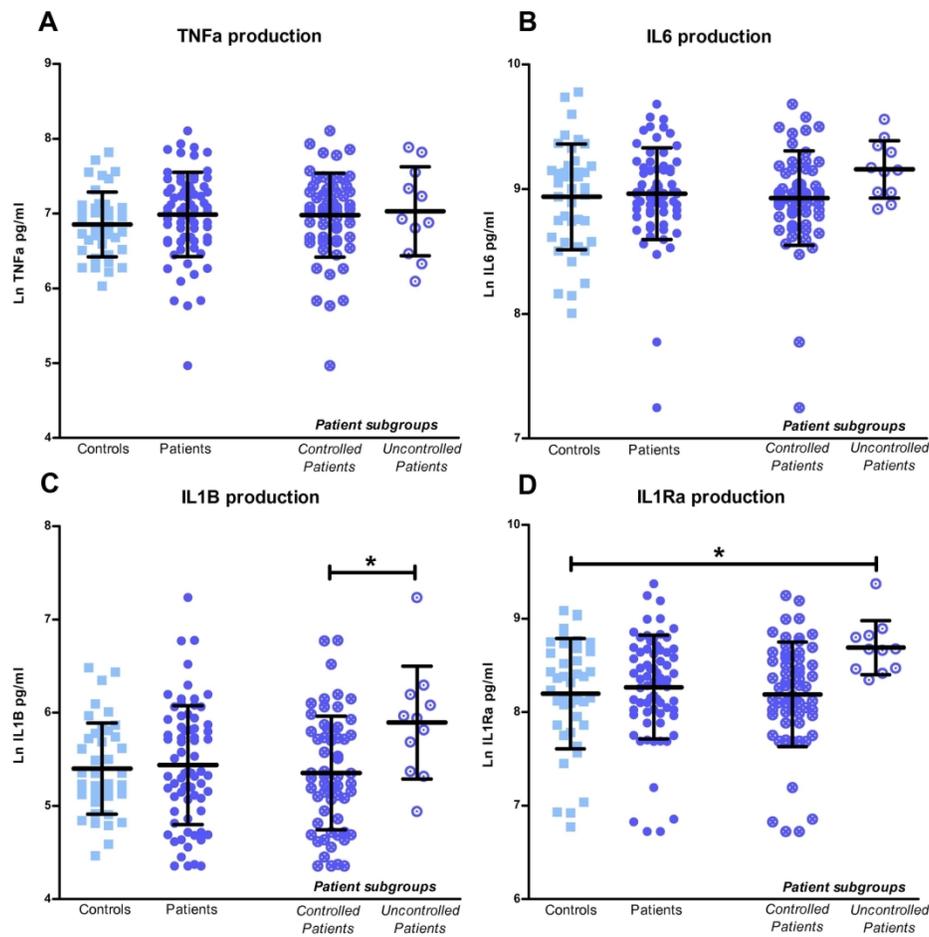


Figure 4. Monocyte-derived pro-inflammatory cytokine production. LPS-stimulated TNF α (A) and IL6 production (B), and *S.aureus*-stimulated IL1B (C) and IL1Ra production (D). Cytokine concentrations were log-transformed using the natural logarithm, and mean with SD is displayed. LPS: lipopolysaccharide; TNF α : tumor necrosis factor alpha; IL: interleukin; Ra: receptor antagonist

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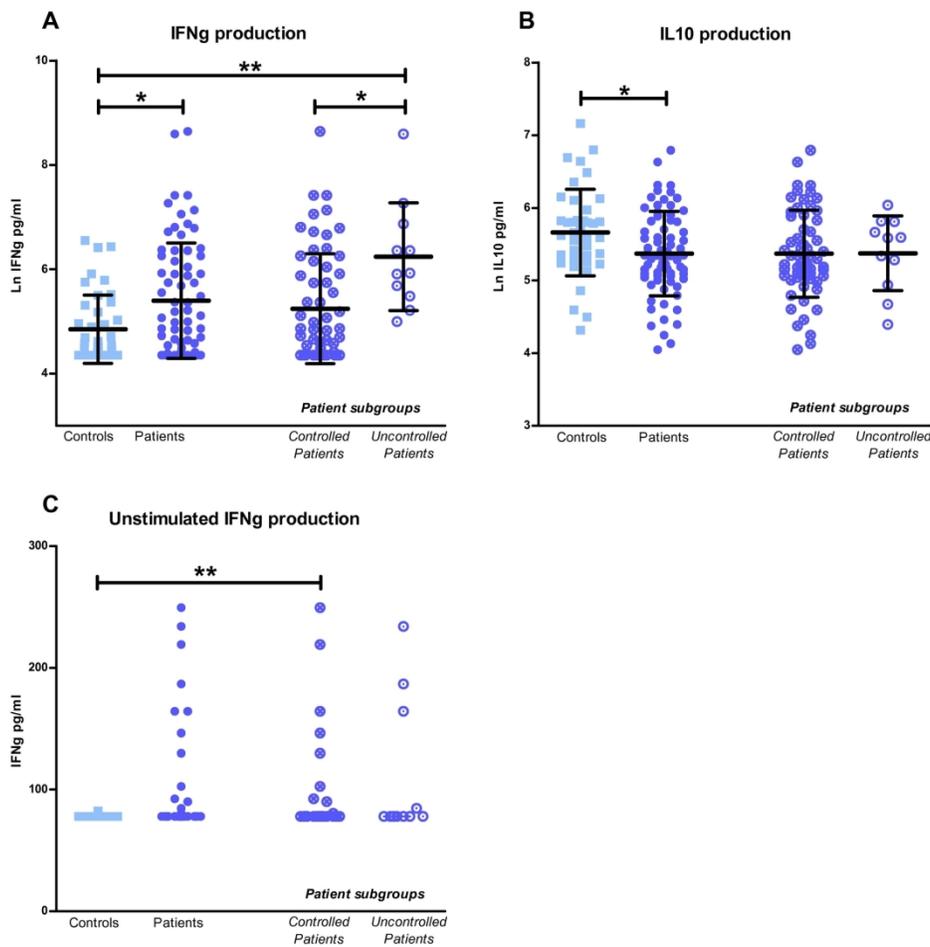


Figure 5. Lymphocyte-derived cytokine production. *S.aureus*-stimulated pro-inflammatory IFNg production (A), LPS-stimulated anti-inflammatory IL10 production (B) and unstimulated IFNg production (C). *S.aureus*-stimulated IFNg production and LPS-stimulated IL10 production were log-transformed using the natural logarithm. For *S.aureus*-stimulated IFNg production and LPS-stimulated IL10 production mean with SD is displayed. IFNg: interferon gamma; LPS: lipopolysaccharide; IL: interleukin.

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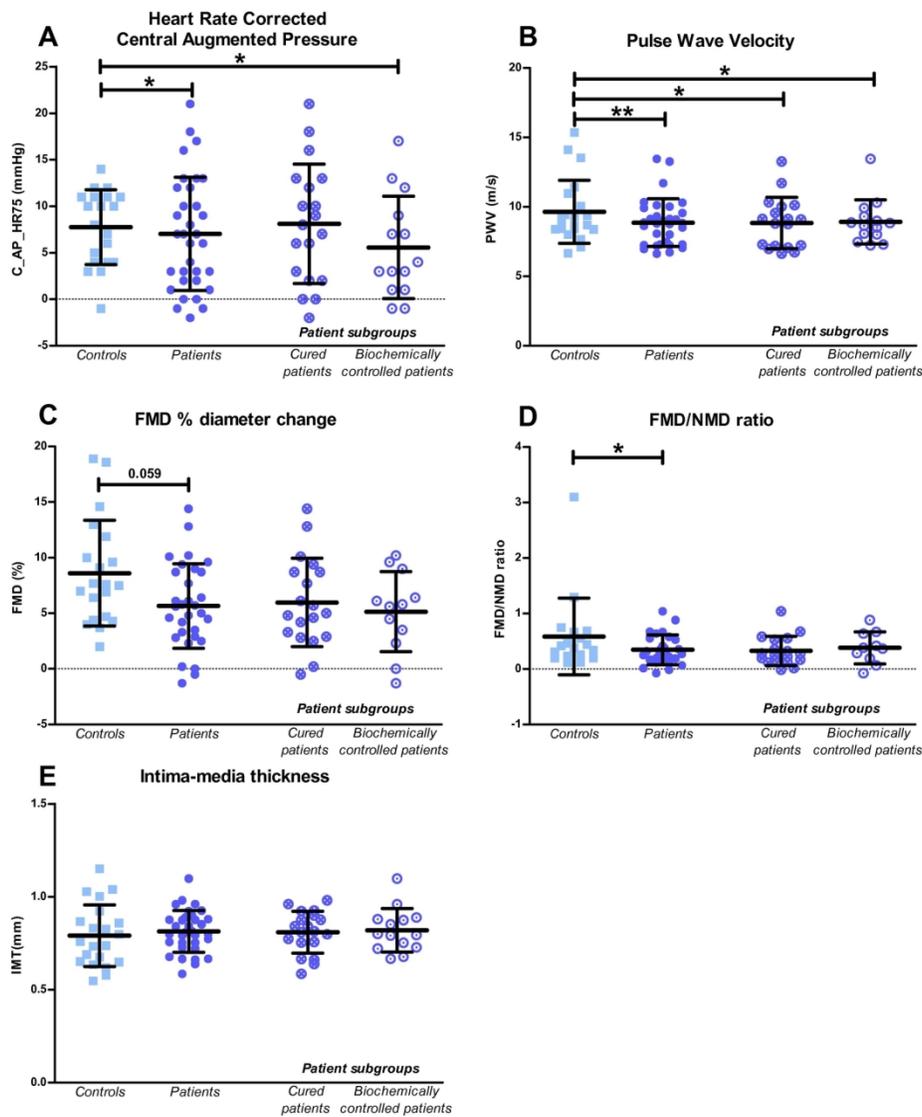


Figure 6. Vascular measurements. Heart rate-corrected Central Augmented Pressure (C_AP_HR75; A), PWV (B), FMD (C), FMD/NMD ratio (D) and IMT (E). Values were log-transformed using the natural logarithm prior to analysis and mean with SD is displayed. PWV: pulse wave velocity; IMT: intima-media thickness; FMD: flow-mediated dilatation; NMD: nitroglycerine-mediated dilatation.

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