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Immune and metabolic profile in acromegalic patients and the impact of the current medical treatment. Results from the PROMISE study

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ABBREVIATIONS

ADCC: antibody-dependent cellular cytotoxicity

ALT: appendicular lean tissue

BMI: body mass index

CI: confidence interval

DM: diabetes mellitus

DXA: dual-energy x-ray absorptiometry

FFA: free fatty acid

FPG: fasting plasma glucose

FPI: fasting plasma insulin

HOMA-IR: homeostatic model assessment of insulin resistance

HOMA-b: homeostatic model assessment of beta cell secretion

GEP-NET: gastroenteropancreatic neuroendocrine tumours

GH: growth hormone

IGF1: insulin-like growth factor I

IMAT: intermuscular adipose tissue

IQR: interquartile range

FM: fat mass

LAK: lymphokine-activated killer

LM: lean mass

OGTT: oral glucose tolerance test

PBMC: peripheral blood mononuclear cell

PEG: pegvisomant

PitNET: pituitary neuroendocrine tumour

PTEN: phosphatase and tensin homolog

QoL: quality of life

SD: standard deviation

SM: skeletal mass

SSA: somatostatin analog

SSTR: somatostatin receptor

ULN: upper level of normal

ABSTRACT

Introduction: Acromegaly is associated with several comorbidities, mainly cardiovascular, respiratory and metabolic diseases, with an increase in the last years of cancer as main cause of mortality. Conversely, little is known about the immune function in acromegaly, even if GH/IGF1 axis has long been supposed to play a role in immune modulation, mainly by affecting lymphocytes and monocytes. We aimed to evaluate the peripheral blood mononuclear cells (PBMCs) subpopulations in acromegalic patients (ACRO) in comparison with controls (CTRLs) and to investigate the impact of disease control and different medical treatments on PBMCs, metabolism and body composition.

Material and Methods: This is an observational, prospective, single site, pilot study (NCT05069324). Twenty-nine patients (16 M and 13 F, mean age 51.3 $\pm$ 15.6 years) with an active disease and 25 sex and age-matched healthy volunteers entered the study according to inclusion criteria. Twenty-five acromegalic patients underwent neurosurgery, 15 were on SSA treatment, and 10 patients on PEG (monotherapy or combined with SSA). Six patients with uncontrolled disease (IGF1 $\geq$ 1.2 x ULN) on SSA treatment changed therapy (add or switch to PEG) and were evaluated after 8 weeks from the treatment change together with another group of 8 patients with stable disease. Anthropometric, metabolic and hormonal parameters were recorded along with full quantification of PBMCs evaluated by flow cytometry. Data are expressed as means $\pm$ SD or median (IQR) and statistical analysis was performed with parametric and non-parametric tests, as appropriate.

Results: Immune cell profiling revealed in ACRO compared to CTRLs decreased monocytes with a higher proportion of non-classical and a lower proportion of intermediate subset. Moreover, ACRO had lower NK cells and $CD16^{high}$ NK with an increased proportion of the more naturally cytotoxic subset ($CD56^{dim}$) and a decreased proportion of the NK cells more responsible of cytokine production

(CD56^{bright}), without changes in T and B-lymphocytes. In ACRO group no differences were found according to disease control and medical treatment. Conversely, in treated acromegalic patients body composition parameters were similar to CTRLs, with a higher fat mass, particularly localized at trunk, in PEG treated patients compared to SSA treatment. The introduction of PEG, in comparison with the stable treatment, improved after 8 weeks glycaemia and influenced the immune cells redistribution by increasing the proportion of non-classical monocytes and CD56^{bright} NK cells, without body composition changes.

Discussion: To the best of our knowledge, this study demonstrated for the first time that acromegalic patients showed an immunological fingerprint, characterised by decreased monocytes and NK cells and by an imbalance of immune innate cells subset, supporting the role of GH/IGF1 axis in immune system modulation. These results could represent the background for further studies, particularly considering the relationship between immune function and cancer and the higher cancer risk reported in acromegaly. The treatment change (add or switch to PEG) may influence immune cells redistribution, without body composition effects, supporting a potential role of PEG in immune regulation. However, further studies are needed to confirm these data and to better clarify the underlying mechanisms and their potential clinical implications.

1 Introduction

1.1 Acromegaly

Epidemiology and clinical picture

Acromegaly is a chronic progressive disease characterized by growth hormone (GH) hypersecretion and, consequently, insulin-like growth factor I (IGF1), which in the majority of cases is caused by a GH-secreting pituitary tumour and more rarely by pituitary hyperplasia or ectopic secretion of GH or GH-releasing hormone from a neuroendocrine tumour (1). The prolonged exposure to hormone excess leads to progressive somatic changes and is associated with multi-system morbidities and increased mortality, when the disease is not adequately treated (1, 2). Acromegaly affects both genders equally and is usually diagnosed in the fourth or fifth decade of life (2). It is considered a rare disease with an estimated prevalence of 36–60 cases/1 000 000 population with 3-4 new cases per 1 000 000 population per year (3-6), however more recent studies have reported an increased incidence, reaching 7.7 cases per 1 000 000/year (7), and prevalence, from it ranges from 85-133/1 000 000 (7-11), owing to better disease awareness, improved diagnostic tools and perhaps a real increase of incidence (1). At the time of diagnosis, patients usually present with somatic changes, such as acral enlargement and facial overgrowth, including prognatism and frontal bossing; and soft-tissue hypertrophy (1, 12). Other common clinical manifestations include hyperhidrosis, osteoarthritis, visceromegalia including macroglossia, and headache. Because the average time from symptoms onset to diagnosis is usually about 7–10 years (13), at the time of diagnosis many patients

present with specific systemic complications. Cardiovascular (hypertension and heart failure) and respiratory disease (obstructive sleep apnea syndrome), bone disease (vertebral fractures), arthropathy, glucose and lipid abnormalities, hypopituitarism and impaired quality of life are common comorbidities in patients with acromegaly and should be adequately investigated and treated (2, 6). Cancer incidence seems to be increased in acromegaly, routine screening is recommended for colon cancer starting at diagnosis of acromegaly and for other cancers depending on clinical features (2). For the first time cardiovascular disease has been surpassed by cancer as main cause of death in acromegaly. According to a recent 20-year follow-up study, causes of death in patients with acromegaly progressively shifted from 44% cardiovascular and 28% neoplastic during the first decade, to 23% cardiovascular and 35% neoplastic during the second decade (14).

Metabolic complications

Acromegaly is frequently associated with glucose and lipid metabolism abnormalities, including impaired glucose tolerance, impaired fasting glucose, diabetes mellitus (DM), and dyslipidaemia, which are among the major risk factors for the increased cardiovascular morbidity and mortality (2). The insulin resistance represents the main pathogenetic mechanism underlying glucose and lipid disorders. The direct action of GH is mainly diabetogenic by increasing lipolysis and inducing insulin resistance, whereas the indirect actions of GH, via increased IGF1, may in turn facilitate insulin action (15). Excess GH diminishes glucose uptake, secondary to increased free fatty acid (FFA) levels and to reduction in the expression of glucose transporter-1 and 4. The increase in FFA production seems to be the main mechanism underlying the development of insulin resistance in patients with acromegaly. In addition, GH directly impairs the insulin-induced intracellular signalling pathway by blocking insulin-receptor

substrate-1 and phosphoinositide 3-kinase in adipose tissues. An increase in gluconeogenesis has been observed due to increased glucose synthesis in the liver and/or in the kidney (16, 17). Excess GH also alters insulin sensitivity through indirect mechanisms, including adipokine dysregulation, which causes local (adipose tissue) and systemic inflammation and leads to insulin resistance (17). GH directly promotes inflammation of human adipocytes by increasing VEGF and MCP1 levels, indicating a possible contribution of adipose tissue to the systemic insulin resistance in active acromegaly (17). Moreover, GH stimulates insulin secretion to induce β-cell proliferation, insulin synthesis and secretion. Pancreatic b-cell dysfunction has also been described, predicting glucose homeostasis after curing the acromegaly (18, 19). In acromegalic patients, the balance between the beneficial effects of GH on insulin secretion and of IGF1 on insulin action and the negative effect of GH on insulin resistance determine the patient's individual risk of developing glucose intolerance and DM. The prevalence of DM in acromegaly differs greatly among studies, ranging from 16% to 56%, and this variability is explained by heterogeneity of the study populations and differences in the criteria used for the diagnosis (2). In a recent meta-analysis, the neurosurgical treatment improves glucose metabolism with a significant decrease in fasting plasma glucose (FPG), glucose after glucose oral tolerance test (OGTT), HbA1c, fasting plasma insulin (FPI), and homeostatic model assessment of insulin resistance (HOMA-IR). The effect on FPG seems to be more related to follow-up length than to disease control, with better results in the short-term than in the long-term (20). Metabolic parameters, which improve in the first months after neurosurgery, could later worsen due to other risk factors, including genetic background, lifestyle, and β-cell function at diagnosis (20). Medical treatment of acromegaly may variably influence glucose metabolism. Somatostatin analogs (SSAs) act by binding to and activating the somatostatin receptors (SSTRs) (21). Five SSTRs have been reported, and SSAs

bind to SSTR2 with higher affinity but also activate SSTR5 (22). Somatostatin receptors are also expressed in α and in the β-cells and, thus, SSAs can impact glucagon and insulin secretion. The SSTR that is expressed at the highest levels in β-cells is SSTR5, whereas SSTR2 is expressed at the highest levels in α-cells. Finally, incretins are also modulated by SSAs (23). The effect of the different medical therapies of acromegaly on a complete panel of metabolic outcomes was recently investigated in two meta-analyses: the first evaluated the effect of first-generation SSAs (octreotide and lanreotide) and the second the effect of pegvisomant (PEG), both in monotherapy and in combination with SSAs (24, 25). First-generation SSAs, acting on insulin secretion, were found to reduce FPI levels and increase HbA1c and after-load glucose while improving disease control, without affecting FPG (24). The clinical implication of these findings is that the physician should expect some metabolic worsening when treating acromegaly with SSAs, but that this appear marginal compared with the effects of disease control, and that greater attention should be paid to avoiding postprandial hyperglycemia in these patients (24). FPG seems to increase significantly only in second-line treatments suggesting that more advanced disease, longer history of acromegaly, and, consequently, worse insulin resistance status are predictors of metabolic response to SSAs (24). This also carries clinical implications, as physicians should treat or prepare such patients more intensively prior to SSAs (24). In fact, compared with the overall group where the effects on FPG were neutral, in these patients the induced drop in insulin secretion also results in a worsening of FPG (24). SSAs affect insulin levels more likely for a drug-related rather than patient-dependent effect. This is further confirmed in the meta-regression analysis showing a mild correlation between reduced insulin and GH and IGF-I reduction (24). The link between the effects of SSAs on insulin and on disease control is further supported by in vitro studies confirming an additive effect of insulin on IGF1 generation in the liver (24). The reduction in insulin

levels is therefore not necessarily detrimental but could reflect better disease control (greater sensitivity to SSAs) or reduction in a factor stimulating IGF1 levels (24). The resulting improved disease control (whether through GH or IGF1 reduction) also improves insulin sensitivity, as confirmed by our data on HOMA-IR and HOMA-b (beta cell function). SSAs reduce the insulin response to a meal or OGTT and, conversely, GH impairs insulin signalling. The net balance between the opposite effects of SSAs may vary among patients depending on their individual family history, predisposition to DM, body mass index (BMI), and the presence of other known risk factors. In contrast, PEG in monotherapy has a positive impact on glucose metabolism, inducing a considerable decrease in FPG and, accordingly, a marked decrease in HbA1c (25). It also induces a substantial decrease FPI and, in accordance with the drop in both glucose and insulin levels, we found a noteworthy decrease in HOMA-IR (25). These effects were independent of disease control (25). Conversely, PEG plus SSA treatment has no effect on glucose metabolism, except for the decrease in FPI. Because SSAs may worsen glucose metabolism, it is reasonable to argue that the addition of PEG can mitigate this effect toward a neutral balance. The improved glucose metabolism observed after PEG treatment could be explained by the blockade of the GH receptor on peripheral tissues, especially the liver, muscles, and adipose tissue, thus removing the detrimental effect of GH on insulin signalling, lipolysis, and gluconeogenesis. However, we could not exclude an additional effect due to SSA discontinuation. Second-generation SSA, pasireotide, binds to SSTR3 and SSTR5 with higher affinity than first-generation SSAs. Based on the results from studies of healthy volunteers, pasireotide elevates both fasting and postprandial plasma glucose levels. These elevations are a consequence of a marked suppression of insulin secretion, with only a mild inhibition of glucagon secretion and are also secondary to a suppression of incretin production: glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide (2). In the PAOLA

study, in which patients whose disease was not controlled with first-generation SSA were randomized to maintain the treatment or to switch to pasireotide LAR, hyperglycemia-related adverse events were reported in 67%, 61% and 30% of the patients in the pasireotide LAR 40 mg, pasireotide LAR 60 mg and SRL groups, respectively and five patients discontinued the drug due to hyperglycemia (26). In addition, 24 (38%) patients in the pasireotide LAR 40 mg group and 24 patients (39%) in the pasireotide LAR 60 mg group patients required a new antidiabetic treatment. Interestingly, the prevalence of hyperglycemia was similar in patients who did or did not exhibit a biochemical response to pasireotide treatment, and hyperglycemia was more frequently observed in patients with baseline FPG levels greater than 100 mg/dL (27). Pasireotide-related hyperglycemia seems to be reversible after drug discontinuation, as FPG and HbA1c levels decreased to near normal levels after switching from pasireotide LAR to octreotide LAR in another study (28). The rates of hyperglycemia have been confirmed in the ACCESS trial, in which patients were treated with pasireotide LAR 40 mg in a closer to real life setting (29). Hyperglycemia-related adverse events were reported in 46% of patients, and 48% of patients initiated an antidiabetic medication (29). The addition of PEG could theoretically mitigate the deleterious effect of pasireotide on glucose levels. However, in the PAPE study patients whose disease was controlled with SSA plus PEG were switched to pasireotide plus PEG and worsening of glucose levels was noted (30).

In summary, different treatment modalities can impact differently on glucose metabolism in patients with acromegaly. Diabetes is not reversible in some patients despite biochemical control of acromegaly. Surgery and PEG could exert beneficial effects, while first-generation SSA may slightly worse glucose metabolism. Pasireotide, on the other hand, exerts a deleterious effect on glucose levels. No studies have evaluated the effect of the dopamine agonist, such as cabergoline, on glucose metabolism in patients with acromegaly (2).

The disorders of lipid metabolism associated with acromegaly mainly include hypertriglyceridemia and decrease of HDL-cholesterol (2). The prevalence of hypertriglyceridemia in acromegaly is three-times higher than that of the general population, and ranges from 33% and 40% of patients, whereas the prevalence of low HDL-cholesterol ranges from 39% to 47% (31). Moreover, acromegaly is also associated with alteration of the lipoprotein metabolism, particularly with an increase of circulating levels of Lp-a, Apo A-I, and Apo E, involved in the transport of triglycerides and cholesterol, as well as small dense LDL particles, possibly as a consequence of insulin resistance, so contributing to the development of cardiovascular risk (32). The effect of the therapy on lipids is less well described. However, control of acromegaly, induced by either pituitary surgery or medical therapy improves dyslipidaemia. Neurosurgery improves lipid metabolism with a significant decrease in triglycerides, and LDL cholesterol and increase in HDL cholesterol (20). SSAs reduce triglycerides, while PEG monotherapy induces a borderline increase of triglycerides and HDL cholesterol, without changes of total cholesterol and LDL cholesterol (24, 25).

Body composition

GH and IGF-I have important effects on body composition. Regarding skeletal muscle, GH and IGF-I together are anabolic and promote protein synthesis; about adipose tissue GH has a catabolic effect by promoting lipolysis and preventing lipogenesis (33). GH signaling is accompanied by up-regulation of phosphatase and tensin homolog (PTEN) and suppression of insulin signaling in both muscle and fat (34). Insulin resistance is generally associated with increased fat body mass (FM) and reduced lean body mass (LM) in the context of the metabolic syndrome, therefore first line approach consists in life style interventions that promote physical activity and diet changes in order to reduce FM and increase LM (34). Acromegaly represents a paradoxical condition in which insulin

resistance coexists with a lean phenotype, in which the increase of LM is likely related to the increase of soft tissues and organs, the major components other than skeletal mass (SM) of total LM as measured by dual-energy x-ray absorptiometry (DXA) (33). The disease control restores insulin sensitivity in concomitance with increased FM and reduced LM (34). Moreover, acromegaly may present a unique type of lipodystrophy characterized by reduced storage of adipose tissue in central depots, most markedly visceral adipose tissue, and a shift of excess lipid to intermuscular adipose tissue (IMAT), which could play a role of GH induced insulin resistance (35). After surgery, this pattern partially reverses, but differentially in men and women (36). Visceral adipose tissue and subcutaneous adipose tissue increased to a greater extent in men than in women, while SM decreased in men and IMAT in women after surgery (36). A recent study has demonstrated that co-treatment with PEG and a reduced SSAs dose in acromegaly patients increase intrahepatic lipid and decrease intramyocellular lipid compared with SSAs monotherapy. These changes related neither to insulin sensitivity nor inflammatory markers (37). In a recent study evaluating 21 patients with an active acromegaly, long-term PEG therapy is accompanied by increases in adiposity (visceral, subcutaneous and intrahepatic) that do not differ from predicted or escalate over time, while metabolism improves and SM remains stable (38). These findings suggest that GH antagonism does not produce a GH deficiency like pattern of body composition (38). A recent large retrospective study showed that the different treatment (surgery, SSAs, PEG) of acromegaly strongly impacts body composition until biochemical disease remission, characterized by an increase in FM, independently from the treatment modality, and a decrease in LM, less pronounced after PEG treatment (39). These changes are closely associated with the normalization of IGF1, but once the disease has been controlled, body composition changes are similar to what is observed with aging (39).

1.2 Immune system

Relationship between GH/IGF1 axis and immune system

GH/IGF-1 axis has long been supposed to play a major role in immune-modulation. GH specific receptors have been found on peripheral blood mononuclear cells (PBMCs) (40) and GH modulates lymphoproliferation in vitro (41) and is considered a minor growth factor for normal lymphocyte in vivo (42). In a small study population Kotzamann et al. showed that a long-lasting and pronounced elevation of GH in acromegaly induces significantly enhanced phagocytic activity, but only negligible changes in most patients in lymphocyte phenotype and in the lymphocyte response to phytohemagglutinin (43). A study on a large series of consecutive active acromegalic patients, evaluating the lymphocyte subset pattern, confirmed an increased T-cell activity together with a decreased B-cell activity in these patients, supporting the existence of abnormalities in immune system in patients with chronic GH/IGF-1 excess (44). Few studies have assessed the direct effects of GH and/or IGF-1 on cytokine production (45). The immune-modulating effects of IGF-1 have been described in various types of immune cells, mainly in lymphocytes (46) and monocytes (47), with both pro- and anti-inflammatory effects being reported (48). A study assessed both monocyte-derived and Th-derived cytokine production and the signaling pathways involved in these processes. The authors reported that IGF-1, present in high circulating concentrations in patients with active acromegaly, potentiates the microbial Toll-like receptor (TLR) ligand-induced inflammatory cytokine production (49). GH or IGF-1 alone did not influence cytokine production in PBMCs. GH did not affect TLR-induced cytokine production, but co-stimulation with IGF-1 dose dependently increased the TLR ligand-induced production of IL6, TNF alpha and IFNγ, as well as the production of the anti-inflammatory cytokine IL10, possibly a counteracting mechanism for some of the pro-inflammatory effects of IGF-1. This effect is suggested to be mediated via the

MAPK pathway and can possibly explain the increased cardiovascular risk in acromegaly (49). The same group recently confirmed that the interplay between IGF1 and the immune system was skewed toward inflammation in acromegaly patients who are controlled or uncontrolled under treatment (50) (51). However, also GH seems to have direct effect on immune system, independently of IGF1, as demonstrated by a study reporting that GH increased the production of IFNγ and IL1B in murine peritoneal macrophages (52). Another study showed that GH, but not IGF-I, significantly increases MBL concentrations, that is a plasma protein of the innate immune system that initiates the complement cascade and activates inflammation after binding to carbohydrate structures on microbial surfaces (53). The clinical consequences of this new link between the endocrine and the immune system remain to be elucidated.

Immune cells subset classification

Monocytes circulate in blood and eventually migrate into tissue where they further mature and serve various functions, most notably in immune defense (54). Monocytes were identified as $CD3^{-}CD14^{+}$ cells and were further divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) monocytes according to the classification, which has been approved by the Nomenclature Committee of the International Union of Immunological Societies (54). The use of popular terms such as “inflammatory monocytes,” or “proinflammatory monocytes” is not recommended because this leads to confusion as the label inflammatory has been used for different subpopulations in humans and mice (54). In the steady state, classical monocytes can differentiate into intermediate monocytes, and then differentiate into patrolling non-classical monocytes in circulation. Classical monocytes have a high antimicrobial capability due to their potent capacity of phagocytosis, and secrete ROS and IL10 upon LPS stimulus, whereas intermediate and non-classical

monocytes secrete inflammatory cytokines, TNFα and IL-1β upon inflammatory stimulation (55) (**Figure 1, panel a**). T lymphocytes were identified as $CD19^{-}CD3^{+}$ cells after lymphocyte gating and were subsequently analyzed for surface expression of CD4 and CD8. Natural killer (NK) cells were identified as $CD14^{-}$ $CD19^{-}CD3^{-}CD56^{+}$ cells. $CD3^{-}CD56^{+}$ NK cells were further analyzed for surface expression of CD16. Beyond their innate cytotoxic activity, thanks to the expression of the CD16 receptor, NK cells can activate antibody-dependent cell-mediated cytotoxicity (ADCC) and, therefore, interface with adaptive immunity (56). NK cells were further divided into two subsets based on CD56 density: $CD56^{dim}$ cells with higher cytotoxic activity and $CD56^{bright}$ cells with lower cytotoxic activity (56) (**Figure 1, panel b**). In fact, $CD56^{dim}$ NK cells contain higher amounts of perforin, granzymes and cytolytic granules. $CD56^{bright}$ NK cells are responsible for higher cytokine production than $CD56^{dim}$ NK cells. $CD56^{bright}$ NK cells are more immature forms than $CD56^{dim}$ and differ in $CD56^{dim}$ under the influence of the surrounding microenvironment.

2 Aim

The aim of this study is

1) To evaluate the immune function through the quantification of PBMCs subpopulations in acromegalic patients in comparison with a control population;
2) To investigate the impact of disease control and different medical treatments on immune function (PBMCs) and its implication on insulin resistance, metabolic complications, and body composition changes in acromegalic patients.

3 Material and Methods

3.1 Study design and population

This is an observational, prospective, single site, pilot study. Consecutive patients with acromegaly with active disease were recruited from the outpatient endocrinology clinic of the Department of Experimental Medicine at “Sapienza” University of Rome from July 2020 to July 2022. The study was approved by the Ethical Committee of Policlinico Umberto I (ref. number 5809) and registered on clinicaltrial.gov (NCT05069324). The inclusion criteria were ages 18-75 years; both sexes; previously diagnosed acromegaly according to current criteria (57) not cured by surgery and/or radiation therapy and in whom an appropriate medical treatment was indicated. The exclusion criteria were severe infections, surgery, trauma; severe chronic kidney disease (stage 4-5); any active blood or rheumatic disorders in the last 5 years. The control group included healthy volunteers matched with patients for age and sex. All patients and controls provided written informed consent after full explanation of the purpose and nature of all procedures used. The study has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. This study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting.

3.2 Visits

During the study, each patient underwent tests and procedures according to common clinical practice. The data were prospectively collected and extracted from the patient's medical records and entered into a case-report-form (CRF). All data were securely stored in a database of the Hospital.

3.3 Procedures

Body measurements

Body weight was measured in kilograms (kg) without shoes and only wearing light clothing, in fasting state. Body weight was assessed on the same equipment throughout the study, if possible, and was recorded with one decimal. Height was measured without shoes, in centimeters, rounded to the nearest cm. BMI was calculated as follows: BMI kg/m^2= Body weight (kg)/ (Height $(m)^2$. Waist circumference was measured in centimeters between the lower end of the rib cage and top of the iliac crest in a standing position, which is usually 3 cm above the anterior superior iliac spine. Hip circumference was measured in centimeters at the level of the greatest protrusion of the buttocks when the subject is standing erect with the feet together.

Laboratory assessments

Blood samples were taken from a peripheral vein in fasting state and collected as required by subsequent analysis. Analysis were performed in a Central Laboratory as follows:

Immune function assessment

Isolation of PBMCs

1. Add Ficoll to a 15 mL tube. The quantity depends on the volume of blood; for example, use 2.5 mL of Ficoll for 5 mL of blood;
2. Slowly add the blood above the Ficoll so that the two fluids remain separate;
3. Centrifuge at 1500 rpm for 30 minutes at 25 °C with acceleration = 1 and deceleration = 0;
4. After centrifugation 4 phases should be observed (red blood cells at the bottom, followed by transparent Ficoll, followed by a thin ring of mononuclear cells, followed by yellow serum at the top);
5. Using a p1000 pipette, aspirate the cell ring with delicate circular movements around the perimeter of the Falcon tube (so as to aspirate the cell

ring uniformly), taking care not to aspirate any other substance, especially the underlying red blood cells;

6. Transfer the aspirate to a 15 mL falcon tube and add PBS1x to 15 mL, mixing gently;

7. Centrifuge at 1500 rpm for 15 minutes at 25 °C to wash;

8. Discard the supernatant and resuspend the pellet in 4 mL of PBS;

9. Transfer the resuspended cells to 4 Eppendorf pipettes (1 mL in each);

10. Centrifuge the pipettes at 1500 rpm for 10 minutes at 4 °C;

11. Discard the supernatant and resuspend the cells in 500 µL of freezing medium (FBS + 10% DMSO).

Phenotypic analysis of PBMC samples

The absolute white blood cell count in the peripheral blood was determined using the Sysmex optical hematology analyzer (Roche) and was reported as the number of cells per microliter of whole blood. PBMCs were isolated from fresh whole blood using a Ficoll-Paque density gradient for cytometry analyses. The samples were analyzed using the CytoFLEX S flow cytometer (Beckman Coulter). Biexponential analysis was performed using CytExpert (Beckman Coulter) and FlowJo V10 (TreeStar) software. Monocytes were identified as $CD3^{-}CD14^{+}$ cells and were further divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) monocytes (54). T lymphocytes were identified as $CD19^{-}CD3^{+}$ cells after lymphocyte gating and were subsequently analyzed for surface expression of CD4 and CD8. Natural killer (NK) cells were identified as $CD14^{-}CD19^{-}CD3^{-}CD56^{+}$ cells. $CD3^{-}CD56^{+}$ NK cells were further analyzed for surface expression of CD16. NK cells were further divided into two subsets based on CD56 density: $CD56^{dim}$ cells and $CD56^{bright}$ (56).

Metabolic assessments

Evaluation of glucose and lipid metabolism, liver and renal function was performed. Insulin resistance and β cell function were assessed by HOMA-IR index and HOMA-β, respectively.

Body composition

Whole-body and regional body composition were estimated by a whole-body DXA scan (Hologic® Horizon). The software provided the mass of lean tissue, fat, and bone mineral for the whole body and specific regions. Appendicular lean tissue (ALT) mass was considered equivalent to the sum of lean tissue in both the right and left arms and legs. Skeletal mass was estimated from DXA according to the prediction model published by Freda et al. (33).

Biochemical control

Blood samples were taken from a peripheral vein in fasting state for IGF-1, GH.

Quality of life

Quality of life (QoL) was measured by Acroqol questionnaire. Each of the 22 items of the AcroQoL is answered in a 1 to 5 Likert scale measuring either the frequency of occurrence (always, most of the time, sometimes, rarely, or never) or the degree of agreement with the items (completely agree, moderately agree, neither agree nor disagree, moderately disagree, completely disagree). A global score is obtained adding the results of the 22 items using the following formula: [(X)-22/(110-22)] x100 where X is the sum of the answers (between 1 and 5 for each answer) (from a minimum of 22 – worse QoL – until 110 – best QoL –). The same formula was adapted for each domain (58).

Sleep apnea

Sleep disturbances were measured by Epworth Sleepiness Scale (ESS).

3.4 Outcomes

The primary outcome was the quantification of PBMCs subpopulations in acromegalic patients in comparison with a control population.

The secondary outcomes were the evaluation of PBMCs, anthropometric measurements, glucose and lipid metabolism, body composition, biochemical disease control, quality of life and sleep apnea in acromegalic patients with active disease under different medical treatments.

3.5 Statistical Methods

Clinical and laboratory findings have been compared between patients and controls (ACRO vs CTRLs). In ACRO group comparisons were performed according to disease control, IGF1 < or ≥ 1.2 x upper level of normal (ULN), and medical treatment (SSA vs PEG –monotherapy/combination with SSA-). Moreover, comparison before and after 8 weeks of follow-up were performed in patients with uncontrolled disease who switched to PEG monotherapy or added PEG to SSA treatment (group 1) and in a second group of patients with controlled disease with stable treatment (group 2). Statistical analyses were performed using the SPSS software package (latest available version from the University software agreement). All subjects were analyzed according to the actual treatment. Continuous variables are reported as mean ± SD or median and interquartile range (IQR), as appropriate. Normally distributed variables were assessed using the Shapiro-Wilk test. Homoscedasticity and homogeneity of variances were assessed by visual inspection and with Levene's test. Treatment-induced changes are expressed as delta change (post treatment value - pre treatment value). Dichotomous variables are reported as frequencies and percentages when relevant. Differences between groups were evaluated by Student's t-test for normally distributed variables and by the non-parametric Mann–Whitney test for not normally distributed variables. Comparison of longitudinal changes within a group was performed by using paired Student's t-tests or by non-parametric Wilcoxon test. Comparisons between more than two different groups were performed by using one-way analysis of variance (ANOVA)

or the Kruskal–Wallis test, as appropriate. Comparison of variable between groups correcting data by covariates, were performed by analysis of covariance (ANCOVA). We calculated least-squares mean estimates with 95% confidence interval (CI) of treatment differences between the groups using Bonferroni correction. Differences between the binomial proportions of the 2 independent groups of a dichotomous-dependent variable were assessed for homogeneity using the chi-square test or Fisher's exact test, as appropriate. Correlations were estimated by Pearson's correlation for normally distributed variables and by Spearman's correlation for not normally distributed variables. A p value of less than 0.05 was regarded as significant.

4 Results

From July 2020 to July 2022 36 patients with acromegaly were screened and 29 with an active disease entered the study according to the inclusion criteria of the study (16 M and 13 F) with a mean age (SD) of 51.3 (15.6) years. Twenty-five sex- and age- matched healthy volunteers were enrolled (9 M and 16 F). **Figure 2** shows the study flow-chart. Among acromegalics the median disease duration was 10 years (6-15); 25 patients underwent neurosurgery treatment; 15 patients were on SSA treatment (9 on monotherapy and 6 on combined therapy with cabergoline). Particularly, SSA treatment included 12 patients on first-generation SSA – 8 lanreotide and 4 octreotide- and 3 patients on second-generation SSA – pasireotide. Ten patients were treated with PEG (6 on monotherapy and 4 on combination with first generation SSA). Six patients on SSA treatment were uncontrolled (IGF1 $\geq$ 1.2 x ULN) at first evaluation and according with the common clinical practice a treatment change was proposed: 4 patients added PEG to SSA treatment and 2 patients switched to PEG monotherapy, these patients were evaluated after 8 weeks from the treatment change. A second

group of 8 patients with controlled disease (4 SSA and 4 PEG) was also evaluated after 8 weeks.

4.1 Outcomes assessment at baseline

4.1.1 Clinical and biochemical evaluation

As expected, there were statistical significant differences between ACRO and CTRLs in comorbidities prevalence as well as in glycaemia (105.4 ± 18.7 vs 86.9 ± 7.6 mg/dl, p<0.001), HbA1c [5.9 (5.4-6.3) vs 5.3 (5.1-5.6) % p<0.001], BMI [27.2 (24.3-29.3) vs 24.4 (22.9-26.5) kg/m^2, p=0.015] and in GH levels [1.3 (0.7-3.1) vs 0.3 (0.1-0.6) ng/ml, p=0.006]. There were no significant differences in lipid profile, IGF1 levels, and blood cells. **Table 1** summarizes the main general characteristics of the study population at baseline. In ACRO group no differences were found in age, sex, BMI and comorbidities prevalence in patients with IGF1 < 1.2 x ULN (n=12) or ≥ 1.2 x ULN (n=17), but disease duration was higher in patients with IGF1 < 1.2 x ULN (10 (8-15) vs 4.5 (1-13.5) years, p=0.038). No differences were found in biochemical parameters except for higher value of total cholesterol adjusted for disease duration, [mean (95%CI): 209 (192 to 226) vs 169 (148-189) mg/dl, p= 0.006] with an increase in LDL cholesterol [mean (95%CI): 130 (108 to 152) vs 97 (74-118) mg/dl, p=0.044] in patients with IGF1 < 1.2 x ULN. Considering medical treatment at baseline SSA (n=15) or PEG (n=10) no differences were observed in clinical characteristics (age, sex, BMI, disease duration, disease control, and comorbidities) and biochemical parameters (metabolic profile and blood cells). Only a borderline difference was found in hypocortisolism prevalence (0 vs 3, p=0.052).

4.1.2 Immune cell profiling

Immune cell profiling revealed a lower total monocytes count in ACRO than in CTRLs [197 (101-355) vs 334 (279-411) cells/µL, p=0.049], with a reduced percentage of intermediate monocytes [2.9 (1.5-4.8) vs 7.3 (5.5-10.4) %,

p<0.001] and an increased percentage of non-classical monocytes [7.9 (5.3-11.37) vs 1.7 (1.0-3.3), p<0.001], without significant differences in classical monocytes percentage [86.5 (81.6-90.3) vs 87.8 (84.9-91.3) %, p=0.452]. Regarding NK cells, ACRO showed a lower total count [123 (62-261) vs 279 (198-410) cells/μL, p=0.003] with a lower percentage of $CD16^{high}$ NK [84.9 (68.4-96.5) vs 96.5 (90.8-97.6) %, p=0.013] and $CD56^{bright}$ NK [0.8 (0.1-2.9) vs 7.8 (6.9-10.2) %, p<0.001]. Conversely, ACRO showed a higher percentage of $CD56^{dim}$ NK (99.0 (97.3-99.8) vs 91.3 (88.2-92.8) %, p<0.001] than in CTRLs. No differences in lymphocytes were found between the two study groups. The immune cell profiling results are summarized in **Table 2** and **Figure 3**. In ACRO group no differences were found according to disease control. Considering medical treatment at baseline patients on PEG therapy showed a lower number of CD8 lymphocytes (p=0.048), but this result was not confirmed in ANCOVA model considering IGF1 level and age as covariates [mean estimated (95%CI): 261 (159-364) vs 354 (272-446) cells/μL, p=0.158)].

To better investigate whether immune profile was related to body composition and biochemical parameters in ACRO patients, a correlation analysis was performed. CD3 lymphocytes were directly correlated with CD8 (ρ=0.687, p<0.001), CD4 (ρ=0.784 p<0.001), $CD16^{high}$ NK (ρ=0.405, p=0.036), trunk LM (ρ=0.410, p=0.034), PCR (ρ=0.474, p=0.026), and inversely correlated with age (ρ=-0.418, p=0.030). CD8 lymphocytes were directly correlated with CD4 (ρ=0.402, p=0.037), IGF1 (ρ=0.424, p=0.028), see **Figure 4**, and inversely correlated with age (ρ=0.508, p=0.007). CD4 lymphocytes were directly correlated with $CD16^{high}$ NK (ρ=0.529, p=0.005) and PCR (ρ=0.541, p=0.009). CD19 lymphocytes were directly correlated with total monocytes (ρ=0.537, p=0.004). Regarding innate immunity, total monocytes were directly correlated with total number of NK (ρ=0.419, p=0.034). Classical monocytes percentage showed an inverse correlation with intermediate (ρ=-0.637, p<0.001) and non-

classical monocytes (ρ=-0.888, p<0.001). Non-classical monocytes percentage was also inversely correlated with $CD56^{dim}$ NK (ρ=-0.403, p=0.037). $CD56^{dim}$ NK inversely correlated with $CD56^{bright}$ NK (ρ=-0.793, p<0.001) and directly correlated with trunk LM (ρ=0.431, p=0.025).

4.1.3 Body composition

ACRO showed higher height (1.74 ± 0.1 vs 1.66 ± 0.06 m, p=0.005) compared to CTRLs. Body composition assessment trough DXA total body did not reveal differences in lean and fat mass adjusted for age, sex, height. ACRO showed higher SM than CTRLs (29.1 (21.5-35.4) vs 21.4 (20.5-24.7) kg, p=0.035], not confirmed in ANCOVA model considering age, sex, and height as covariates. In ACRO group no statistical significant differences were found according to disease control. Considering medical treatment at baseline, patients on PEG showed higher total FM and trunk FM (kg and percentage) adjusted for age, sex, diabetes, and IGF1 levels compared to SSA treatment [mean estimated (95%CI): total FM 26 (22 to 30) vs 18 (15 to 22) kg, p=0.022; trunk FM 13 (11 to 15) vs 8 (7 to 10) kg, p=0.005 and 29 (26 to 32) vs 23 (20 to 26) %, p=0.024]. However, no differences were found between PEG treated patients and CTRLs (TF p=0.334; trunk FM kg p=0.197) see **Figure 5.** In ACRO group total FM (ρ=-0.503, p=0.017) and trunk FM (in percentage: ρ= -0.474, p=0.026; in Kg ρ= -0.625, p=0.002) inversely correlated with GH, whereas total LM (ρ= 0.376, p=0.049) and trunk LM (ρ= 0.365, p=0.056) directly correlated with IGF1.

4.1.4 Questionnaires

In the study population AcroQol revealed a standardized global score of 69.9 ± 15.1; regarding the sub-dimension the standardized scores were 64.8 ± 22.4 for physical function, 62.3 ± 21.2 for appearance, and 83.4 ± 13.4 for relationship. ESS revealed a total score of 4 (3-6) with a low risk of sleep apnea. No differences

were found in questionnaires scores according to disease control and baseline treatment.

4.2 Outcomes assessment at follow-up

Two groups were evaluated after 8 weeks from the baseline: group 1 (n= 6), which switched to PEG or added PEG to SSA treatment, according to common clinical practice, and group 2 (n=8), which maintained a stable treatment (SSA or PEG). No differences were found in age, sex, BMI, disease duration, diabetes and smoking between these two groups. As expected IGF1 was higher in the group 1 compared to the group 2 (620.4 ± 318.7 vs 224.6 ± 138.7 ng/ml, p= 0.016). Results regarding change between baseline and follow-up are summarized in the **Table 3**. In group 1 after 8 weeks no statistically significant differences were found in immune and body composition outcomes. As expected glycaemia [-10.8 (-18.4 to -3.2) mg/dl, p=0.015] and IGF1 levels [-289 (-445 to 133) ng/ml, p=0.005] improved after treatment change, as well as ESS scores (-1.6 (-3.0 to -0.2), p=0.035]. In group 2 after 8 weeks no changes were found in biochemical and body composition parameters. Conversely, regarding immune cells an increase of classical monocytes [11.6 (6.8 to 18.0) %, p=0.018] and $CD56^{dim}$ NK were observed [6.1 (1.9 to 19.6) %, p=0.043], together with a decrease of non-classical monocytes [-8.5 (-12.4 to -4.5) %, p=0.002] and $CD56^{bright}$ NK [-2.5 (-4.7 to -0.3) %, p=0.032].

The comparison between these two groups (group 1 vs group 2) showed a treatment related effect, adjusted for age, sex, BMI and outcome at baseline, in glycemia [estimated treatment related difference (95%CI): -13.3 (-26.1 to -0.5) mg/dl, p=0.043], in non-classical monocytes [estimated treatment related difference (95%CI): +7.5 (0.5 to 15.0) %, p=0.049], in NK $CD56^{bright}$ [estimated treatment related difference (95%CI): +6.6 (-0.4 to12.7) %, p=0.040], and in ESS

score [estimated treatment related difference (95%CI): -2.7 (-5.1 to -0.4), p=0.028], whereas the other outcomes were unaffected by treatment change.

5 Discussion

To the best of our knowledge, this observational prospective pilot study showed for the first time that acromegalic patients with active disease present a different distribution of innate immune cells without changes in T and B lymphocytes in comparison with healthy age and sex-matched controls.

Acromegalics showed:

1) **Lower count of total monocytes** with a shift in monocyte subpopulations with **higher proportion of non-classical** and **lower proportion of intermediate subset**;
2) **Lower count of total NK cells** and **CD16^{high} NK** with an **increased proportion of the more naturally cytotoxic subset (CD56^{dim})** and a **decreased proportion of the NK cells more responsible of cytokine production (CD56^{bright})**

These changes in immune cells profile, which were not evident on a routine full blood count, seem to be an immunological fingerprint of acromegaly independently from disease control and medical treatment, supporting the role of GH/IGF1 axis in innate immune system modulation. GH/IGF-1 axis has long been supposed to play a major role in development, maintenance and regulation of immune system, modulating lymphoproliferation and stimulating cytokines production, but clinical data regarding immune functions in acromegaly are limited. In a previous study with a very small population (10 acromegalic patients vs 9 controls) Kotzamann et al. showed that a chronic exposure to GH excess induces significantly enhanced phagocytic activity, without changes in lymphocyte cells profile (43). A larger study of 100 consecutive active acromegalic patients compared with 200 controls evaluated the lymphocyte

subset pattern, confirming an increased T-cell activity together with a decreased B-cell activity in these patients (44). Moreover, the same group demonstrated an impairment of mucosal immune surveillance (changes in lymphocytes pattern in colonic *lamina propria*) in acromegaly, although a causal effect in polyp formation cannot be ruled out (59). Our data did not confirm a different pattern of T and B cells but support a relationship between T lymphocytes and IGF1 levels, in fact CD8 lymphocytes are positively correlated with IGF1. Moreover, the higher proportion of $CD56^{dim}$ NK, which are potent mediators of ADCC, lymphokine-activated killer (LAK) activity and natural cytotoxicity, support that chronic GH/IGF1 stimulation can enhance phagocytic function by promoting differentiation of $CD56^{bright}$ NK into $CD56^{dim}$ NK.

Among acromegaly complications, cancer has recently become the main cause of death, surpassing the mortality due to cardiovascular disease (35% neoplastic vs 23% cardiovascular) (14). Preclinical data supports the role of GH/IGF1 signalling in cancer development and progression (60). Though controversial, epidemiological studies demonstrated higher cancer risk in acromegalics as compared to general population, particularly for gastrointestinal, endocrine, urinary tract cancer, and prostate cancer in men and breast cancer in women (61). An Italian nationwide multicenter cohort study (1512 acromegalic patients) confirmed increased standardized incidence ratios of different types of cancer in acromegaly (colon-rectum, thyroid, and kidney) with age and family history of cancer as the main independent risk factors (62). Although this elevated neoplastic risk, immune function in acromegalic patients has been poorly investigated. We can speculate a relationship between immune system impairment and the higher cancer risk in acromegalic population. In our cohort 6 patients (21%) have a personal history of cancer (3 thyroid cancer, 1 colon rectum, 1 bronchial carcinoid, and 1 seminoma), whereas no cases were reported in controls. Chronic GH/IGF1 exposure is the main player in determining

morbidity and mortality in acromegaly but absolute levels at specific time points such as diagnosis, post-treatment or last follow up are unable to accurately estimate this (2). Furthermore, other factors such as insulin, insulin resistance, IGF binding protein levels, obesity, diabetes and body composition are likely to contribute to cancer risk (2). In this context also immune system alterations with a more pro-tumoral immune profile may favour the cancer risk.

The clinical implication of the changes in immune cells profile observed in our study is not clear, but we can also hypothesize that they could be related to the neoplastic nature of the disease. In our cohort acromegaly is active and sustained by a pituitary tumour, currently also called pituitary neuroendocrine tumour (PitNET) (63). Recently, studies about pituitary adenoma-infiltrated macrophages have been emerging, demonstrating a higher expression of macrophages than those in the normal pituitary (64), but little is known on the circulating cells profile and its correlation with tumour microenvironment infiltrate.

Monocytes are innate immune cells of the mononuclear phagocyte system that have emerged as important regulators of cancer development and progression (65). In our study we found in acromegalics a higher proportion of non-classical monocytes, which are involved in tumorigenesis promotion by stimulating angiogenesis and suppressing T lymphocytes function (65). However, some evidence reports also their role as anti-tumoral cells given their patrolling role as scavengers of tumour cells and debris (65). Moreover, non-classical monocytes can recruit NK in tumoral tissue and inhibit regulatory T cells (65). Conversely, in our population a lower proportion of intermediate monocytes were observed. This subset is more involved in inflammatory diseases (55) and represents the main population responsible for T lymphocyte activation (66). We recently found the same pattern of monocytes subpopulations in gastroenteropancreatic neuroendocrine tumours (GEP-NET) (67), supporting the imbalance between

non-classical monocytes and classical monocytes aside from favouring tumour growth and progression could also increase tumour angiogenesis, whereas the depletion of intermediate monocytes could indirectly promote a pro-tumoral microenvironment reducing T cells activity. Acromegaly is generally caused by a pituitary tumour (PitNET), so is not surprising find similarities in immune cells profile with NETs of other sites.

Natural killer cells are distinct lymphocytes with an important role in immune system by providing innate defence against virally infected and transformed cells (56). In acromegalic patients we found a depletion of NK cells and CD^{16} NK, likely caused by a protracted activation and subsequent down-regulation of immune system due to the natural history of acromegaly (chronic and long-standing disease). This phenomenon has been described in patients with adrenal insufficiency (68), as well as in conditions of low-grade inflammation, and also in different type of cancer, as well as in GEP-NETs (67), representing a putative mechanism of tumour immune escape leading to tumour growth and progression (69). Moreover, in our study population we observed an imbalance between NK cells subset with a higher proportion of $CD56^{dim}$ and lower proportion of $CD56^{bright}$ compared to healthy controls. The increase of the more cytotoxic subpopulation has been described in senescence, whereas there is no significant change in the numbers of $CD56^{bright}$ (70). Functionally, NK cells in the elderly are less responsive to IL2-induced proliferation (70); unfortunately data on the functional activity of NK cells in acromegaly are scarce. The depletion of $CD56^{bright}$ NK cells, which produce immunoregulatory cytokines, could play a critical role in cancer immune escape and predisposition to infection (71). Although GH specific receptors have been found on PBMCs (40) and GH may modulates lymphoproliferation in vitro and in vivo (41, 42), we did not find any differences in baseline immune cells profile between patients treated by PEG or SSA. PEG did not produce a significant change on immune cells distribution after

8 weeks of treatment, although produced a significant reduction of IGF1 and glycaemia. However, the introduction of PEG if compared to stable treatment seems to have an impact by increasing the proportion of non-classical monocytes and $CD56^{bright}$ NK cells. These results suggested that PEG could have a role as an immune regulator, both directly by GH antagonism and indirectly by reduction of IGF1 and glycaemia. However, the small population of the study limited these results, because only six patients added PEG to SSA treatment or switched to PEG monotherapy. Moreover, further studies are needed to better clarify the mechanisms underlying these cells redistribution in acromegalic patients.

Body composition is an important determinant of general health with implication in cardiovascular and neoplastic disease. Acromegaly represents a paradoxical condition in which insulin resistance coexists with a lean phenotype, related to the increase of soft tissues and organs.

The main results of our study regarding body composition showed that:

1. **Acromegalic patients** with active disease treated with different modalities present the **same lean and fat distribution** of age and sex-matched controls;
2. Patients treated with **PEG present higher FM**, particularly higher central adiposity (**trunk FM**) compared to patients treated with SSA, without differences with controls.

In our population we did not find any differences in body composition outcomes, also in estimated SM from DXA, likely because our patients (except for 3) were treated, confirming data of the literature showing that the different treatment (surgery, SSA, PEG) of acromegaly reverses body composition changes induced by the disease (39). Once the disease has been controlled, body composition changes are similar to what is observed with aging (39). We did not find any differences according to disease control, but an inverse correlation was observed

between GH and TF mass and trunk FM, whereas a direct correlation between IGF1 and total LM. Regarding medical therapy we found a significant difference in total FM and in the distribution of fat between SSA and PEG treated patients, without differences with controls, likely due to the direct effect of GH antagonism on adipose tissue, especially in the long-term treatment. In fact, when PEG has been added in the therapy did not produce significant changes in body composition parameters in the short term (after 8 weeks of treatment). A recent study evaluating 21 patients with an active acromegaly, long-term PEG therapy is accompanied by increases in adiposity (visceral, subcutaneous and intrahepatic) that do not differ from predicted or escalate over time, while metabolism improves and SM remains stable (38). These findings suggest that GH antagonism does not produce a GH deficiency like pattern of body composition change (38).

6 Conclusions

In conclusion this study demonstrated that acromegaly patients showed an immunological fingerprint, independently of disease control and medical treatment, characterised by a reduced number of monocytes and NK cells and by an imbalance of immune innate cells subset, supporting the role of GH/IGF1 axis in immune system modulation. These results could represent the background for further studies, particularly considering the higher cancer risk reported in acromegaly. Finally, in treated acromegalic patients body composition parameters are similar to healthy controls, with a higher fat mass, particularly localized at trunk, in PEG treated patients compared to SSA. The treatment change (add or switch to PEG) may influence immune cells redistribution, without body composition effects, supporting a potential role of PEG in immune regulation. However, further studies are needed to confirm these data and to better clarify the underlying mechanisms and their potential clinical implications.

7 Tables and Figures

Table 1. Baseline general characteristics. Data are expressed as mean ± SD or median (IQR) as appropriate, frequencies (%)

	ACRO (n=29)	**CTRLs (n=25)**	**P value**
Age, y	51.3 ± 15.6	53.4 ± 16.3	0.574
Sex, M/F, n	16/13	9/16	0.182
BMI, kg/m^2	27.2 (24.3-29.3)	24.4 (22.9-26.5)	*0.015*
Smoker, n (%)	14 (48.3)	8 (32)	0.274
Disease duration, y	10 (6-15)	-	-
IGF1 $\geq$ 1.2 x ULN, n	12 (41.4)	-	-
Treatment			
Neurosurgery	24	-	-
SSAs	15		
LAN autogel, n	8	-	-
OCT LAR, n	4	-	-
PAS LAR, n	3	-	-
PEG (monotherapy/combination), n	10 (6/4)	-	-
DA^, n	6	-	-
Naïve, n	3	-	-
Comorbidities			
Diabetes mellitus, n (%)	10 (34.5)	0 (0)	*0.001*
Dyslipidemia, n (%)	18 (62.1)	5 (20)	*0.002*
Hypertension, n (%)	16 (55.2)	5 (20)	*0.012*
Arthropathy, n	9 (31.0)	0 (0)	*0.001*
OSAS, n	5 (17.2)	0 (0)	*0.020*

Cardiopathy, n	20 (68.9)	0 (0)	*0.001*
Neoplasms[§],	6 (20.7)	0 (0)	0.064
Central hypocortisolism	3 (10.3)	0 (0)	0.240
Central hypothyroidism	6 (20.7)	0 (0)	*0.025*
Biochemical evaluation			
Glycaemia (mg/dl)	105.4 ± 18.7	86.9 ± 7.6	*<0.001*
HbA1c (%)	5.9 (5.4-6.3)	5.3 (5.1-5.6)	*<0.001*
Insulin (µUI/ml)	7.5 (4.6-13.9)	8.4 (6.4-10.1)	0.614
HOMA-IR	1.8 (1.1-3.5)	1.7 (1.1-2.0)	0.438
Total cholesterol (mg/dl)	192.7 ± 37.0	193.6 ± 38.3	0.924
HDL cholesterol (mg/dl)	53.3 ± 11.3	60.3 ± 16.4	0.080
LDL cholesterol (mg/dl)	113.4 ± 38.1	112.6 ± 28.8	0.934
Triglycerides (mg/dl)	103.0 (87.2-187.5)	99 (73.9-121-1)	0.306
PCR (mcg/l)	950 (600-3000)	1000 (700-2050)	0.794
GH ng/ml [#]	1.3 (0.7-3.1)	0.3 (0.1-0.6)	*0.006*
IGF-1 ng/ml	282 (183-552)	191 (155-213)	0.099
Red blood cell count, n x 10^6	4.6 (4.2-5.1)	4.7 (4.6-4.9)	0.549
Hemoglobin, g/dL	13.6 ± 1.9	13.6 ± 1.1	0923
White blood cell count, n ×10^3	5.6 (4.8-7.3)	5.3 (4.7-6.8)	0.461
Neutrophils, n × 10^3	3.2 (2.7-4.1)	2.8 (2.5-3.9)	0.310
Lymphocytes, n × 10^3	1.6 (1.2-2.2)	1.9 (1-4-2.3)	0.768
Monocytes, n × 10^3	0.3 ± 0.1	0.3 ± 0.1	0.890
Eosinophils, n × 10^3	0.1 (0.08-0.2)	0.15 (0.10-0.25)	0.142
Basophils, n × 10^3	0.04 (0.03-0.05)	0.03 (0.02-0.04)	0.260
Platelets, n × 10^3	228.0 (173.5-256.5)	206.0 (179.5-251.5	0.890

^ All in combination with SSA; [§] 3 Thyroid carcinoma, 1 colon-rectum adenocarcinoma, 1 bronchial carcinoid, 1 testicular seminoma
[#] Excluded patients on PEG treatment

Table 2. Outcomes assessment at baseline (immune cells, body composition, questionnaires). Data are expressed as mean ± SD or median (IQR) as appropriate**. ***Data are expressed as estimated mean (95% CI), covariates in the analysis of covariance model: age, sex, and height**.** AcroQoL: Acromegaly quality of life questionnaire. [§]Standardized scores.

	ACRO	**CTRLs**	**P value**
Immune cells			
N°	27	16	
Total monocytes	197(101-355)	334 (279-411)	*0.049*
CD14++ CD16−, classical (%)	85.5 (81.6-90.3)	87.8 (84.9-91.3)	0.452
CD14+ CD16+, intermediate (%)	2.9 (1.5-4.8)	7.3 (5.5-10.4)	*<0.001*
CD14+CD16++, non-classical (%)	7.9 (5.3-11.7)	1.7 (1.0-3.3)	*<0.001*
T lymphocytes (cells/µL)	1109 ± 436	1069 ± 378	0.758
CD8+ T lymphocytes (cells/µL)	317 ± 166	317 ± 143	0.992
CD4+ T lymphocytes (cells/µL)	549 ± 386	690 ± 246	0.151
NK (CD3−CD56+)(cells/µL)	123 (62.0-261)	279 (198-410)	*0.003*
NK CD16 ^{high} (%)	84.9 (68.4-96.5)	96.5 (90.8-97.6)	*0.013*
NK CD56 ^{dim} (%)	99.0 (97.3-99.8)	91.3 (88.2-92.8)	*<0.001*
NK CD56 ^{bright} (%)	0.8 (0.1-2.9)	7.8 (6.9-10.2)	*<0.001*
B Lymphocytes (cells/µL)	157 (75.9-316)	147 (73.1-188)	0.436
Body composition			
N°	28	11	
Height, m	1.74 ± 0.1	1.66 ± 0.06	*0.005*
Total mass, kg*	80.4 (75.9-85.0)	75.1 (67.6-82.5)	0.235
Total fat mass, kg	21.4 (18.7-24.1)	21.0 (16.5-25.4)	0.866
Total lean mass, kg*	58.3 (55.3-61.2)	54.0 (49.2-58.8)	0.147
Fat mass trunk, %*	25.5 (22.-28.2)	28.6 (24.-33.1)	0.247
Fat mass trunk, kg*	10.2 (8.7-11.7)	9.6 (7.1-12.1)	0.668

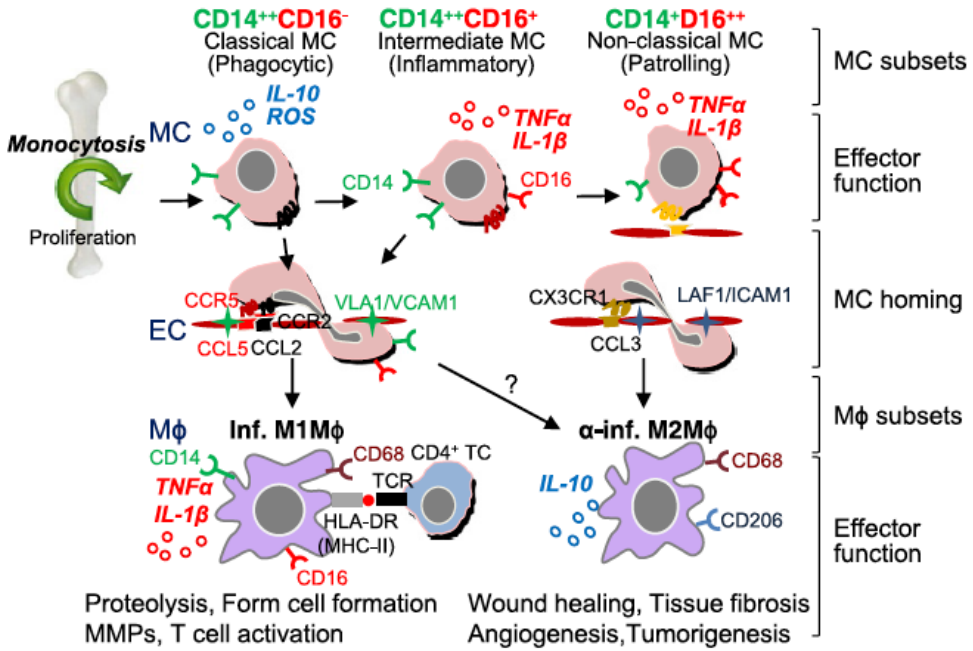
VAT, g*	557 (455-659)	403 (254-551)	0.102
Lean mass trunk, kg	32.0 (23.8-34.0)	25.8 (22.7-28.2)	0.259
Appendicular lean tissue, kg*	24.7 (23.3-26.1)	23.0 (20.8-25.2)	0.221
Skeletal mass, kg	29.1 (21.5-35.4)	21.4 (20.5-24.7)	*0.025*
Log10 Skeletal mass, kg*	1.43 (1.41-1.45)	1.4 (1.36-1.44)	0.201
AcroQol§			
Physical	64.8 ± 22.4	-	-
Appearance	62.3 ± 21.2	-	-
Relationship	83.4 ± 13.4	-	-
Global	69.9 ± 15.1	-	-
Epworth sleepiness scale	4 (3-6)	-	-

Table 3. Change between baseline and follow-up (8 weeks). Data are expressed as Mean (95% CI) or median (IQR) *, p within group was calculated by paired Student's t-tests or by non-parametric Wilcoxon test, as appropriate. AcroQoL: Acromegaly quality of life questionnaire. §Standardized scores.

	Delta change in group 1 (Add or switch to PEG)	**P within group**	**Delta change in group 2 (Stable treatment)**	**P within group**
Biochemestry				
Glycaemia mg/dl	-10.8 (-18.4 to -3.2)	*0.015*	-1.6 (-13.9 to 10.8)	0.767
HbA1c (%)	0.03 (-0.25 to 0.32)	0.777	0.04 (-0.1 to 0.20)	0.534
Insulin (µUI/ml)	0 (-18 to 2.1) *	1.000	-1.6 (-6.3 to 3.1)	0.429
HOMA-IR	-0.4 (-5.5 to 0.2) *	0.116	-0.5 (-1.8 to 0.9)	0.437
HOMA-beta	6.1 (-100 to 10.9) *	0.600	-16.6 (-70.3 to 11.9)*	0.091
Total cholesterol (mg/dl)	0.7 (-22 to 24)	0.944	0 (-31.2 to 31.4)	1.000
HDL cholesterol (mg/dl)	0.9 (-10 to 12)	0.836	5.3 (-6.2 to 16.8)	0.306
LDL cholesterol (mg/dl)	6.4 (-16 to 20.7) *	0.400	-2.3 (-22.6 to 18.0)	0.793
Triglycerides (mg/dl)	-0.95 (-41.4 to 9.1) *	0.600	-8.7 (-60 to 42.6)	0.692
PCR (mcg/l)*	0 (-250 to 775)	1.000	-200 (-3200 to 0)*	0.225
IGF-1 ng/ml	-289 (-445 to 133)	*0.005*	97.7 (-60 to 256)	0.181
Immune cells				
Total monocytes	-87 (-289 to 114)	0.315	40 (-107 to 188)	0.514
CD14++ CD16−, classical (%)	-3.4 (-8.5 to 1.7)	0.145	10.6 (5.2 to 16)	*0.004*
CD14+ CD16+, intermediate (%)	-0.2 (-0.5 to 1.9)*	0.753	-1.0 (-4.7 to 2.8)	0.525
CD14+CD16++, nonclassical (%)	2.5 (-1.6 to 6.7)	0.180	-8.5 (-13.3 to -3.5)	*0.007*

T lymphocytes (cells/µL)	150 (-392 to 693)	0.508	306 (-208 to 821)	0.187
CD8+ T lymphocytes (cells/µL)	-29 (-349 to 289)	0.819	78 (-88 to 244)	0.281
CD4+ T lymphocytes (cells/µL)	114 (-177 to 405)	0.361	203 (-276 to 683)	0.325
NK (CD3−CD56+) (cells/µL)	69 (-71 to 209)	0.262	4.7 (-69 to 60)	0.861
NK CD16 ^{high} (%)	2.7 (-6.4 to 11.9)	0.475	17.2 (-11.8 to 46.2)	0.188
NK CD56 ^{dim} (%)	-0.8 (-2.9 to 0.01)*	0.116	5.3 (1.0 to 22.1)*	*0.046*
NK CD56 ^{bright} (%)	0.8 (-0.00 to 4.2)*	0.116	-2.7 (-5.3 to -0.4)	*0.041*
B Lymphocytes (cells/µL)	127 (-51 to 306)	0.126	8.8 (-253 to 271)	0.935
Body composition				
Total mass, kg	-0.7 (-2.5 to 1.0)	0.307	0.2 (-1.9 to 1.5)	0.812
Total fat mass, kg	-0.7 (-2.8 to 1.3)	0.373	0.4 (-0.3 to 0.9)*	0.499
Total lean mass, kg	-0.05 (-2.4 to 2.3)	0.955	-0.04 (-0.2 to 2.1)	0.964
Fat mass trunk, %	-0.4 (-3.5 to 2.8)	0.765	1.6 (0.8 to 2.0)*	0.176
Fat mass trunk, kg	-0.5 (-1.9 to 0.9)	0.394	0.6 (0.4 to 0.8)*	0.237
VAT, g	13.4 (132 to -159)	0.811	-52.3 (-149 to 45)	0.236
Lean mass trunk, kg	-0.4 (-2.8 to 2.9)	0.693	-0.1 (-0.6 to 0.1)*	0.398
Appendicular lean tissue, kg	0.2 (-1.1 to 1.5)	0.688	0.9 (-1.1 to 2.9)	0.318
Skeletal mass, kg	0.2 (-1.2 to 1.7)	0.688	1.0 (-1.3 to 3.3)	0.318
AcroQoL				
Physical	4.4 (-11 to 19.8)	0.475	3.1 (-3.9 to 10.2)	0.322
Appearance	10.7 (-6.7 to 28.2)	0.164	0.5 (-8.9 to 9.9)	0.899
Relationship	5.7 (-7.4 to 18.9)	0.294	-4.6 (-13 to 3.9)	0.233
Global	6.8 (-6.7 to 20.4)	0.235	-0.2 (-5 to 4.7)	0.938
Epworth sleepiness scale	-1.6 (-3.0 -0.2)	*0.035*	0.4 (-0.7 to 1.6)	0.407

Figure 1. Monocytes and NK cells subset



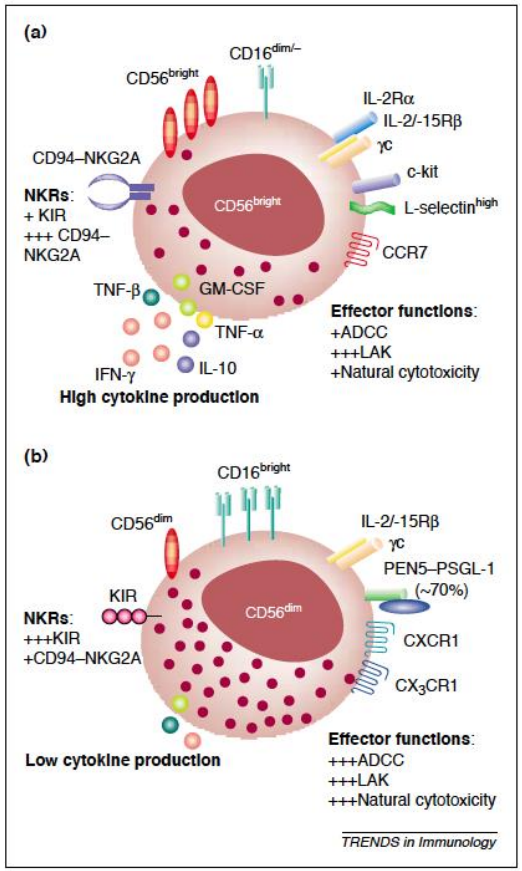


Figure 2. Study flow-chart

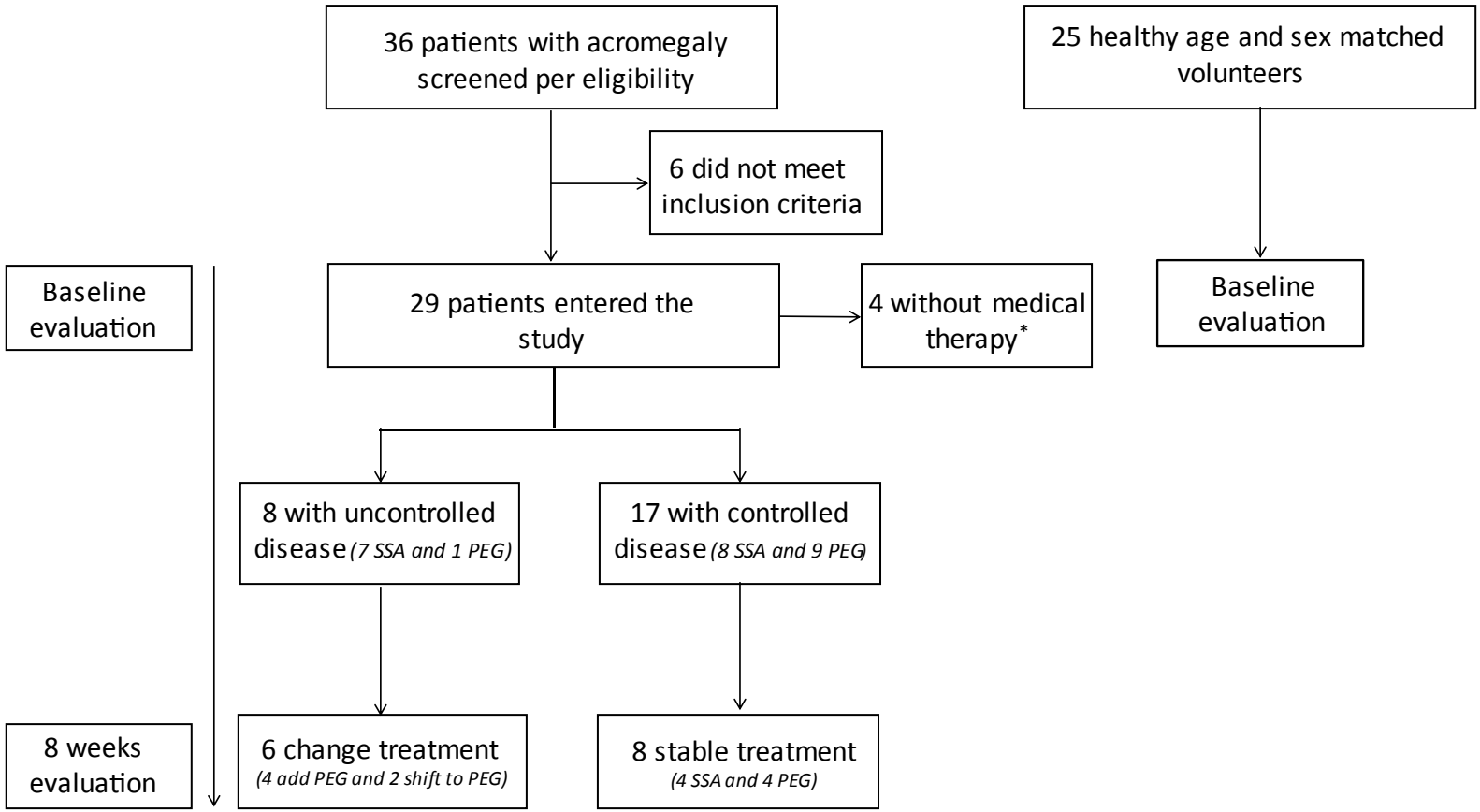
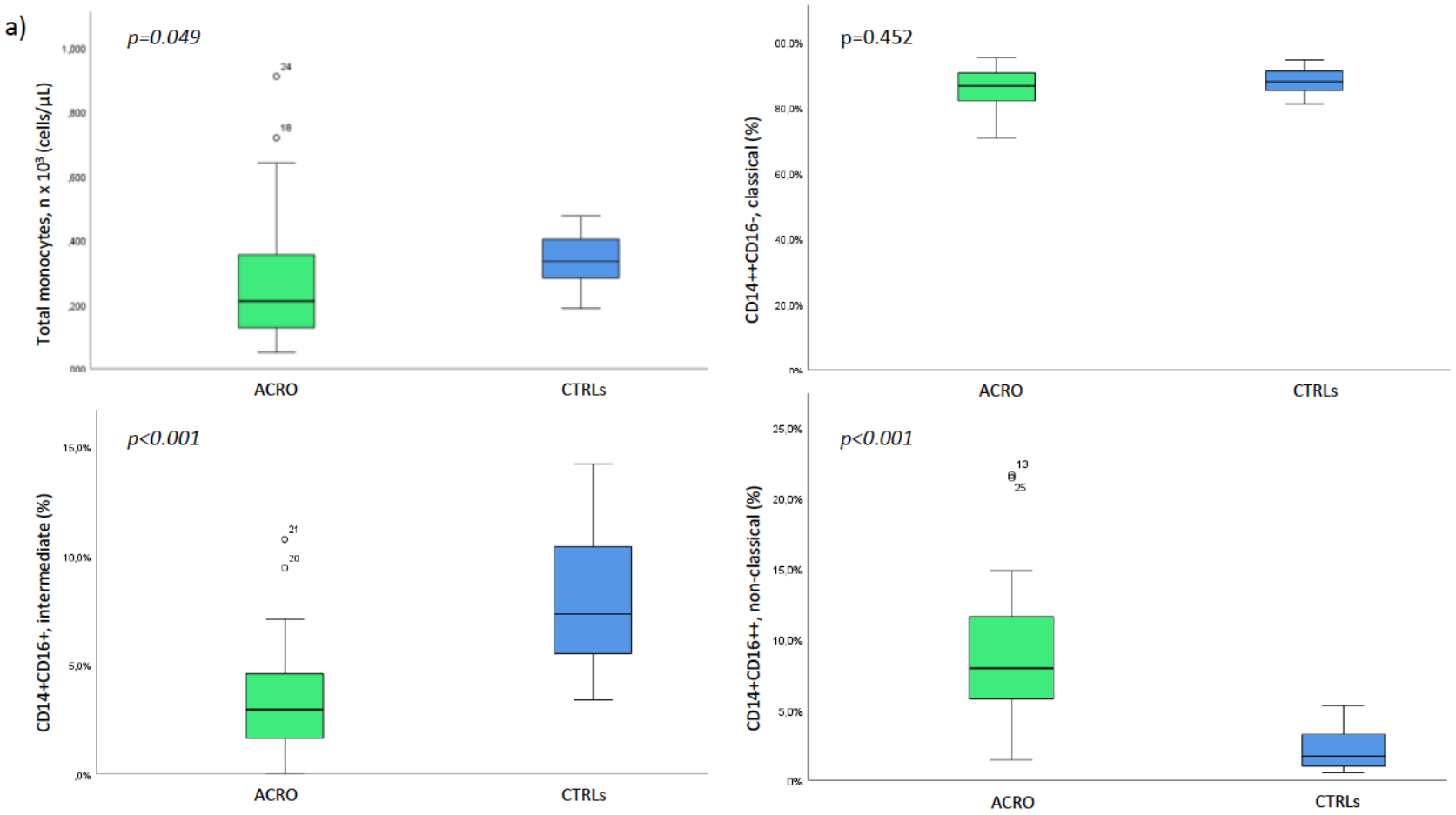


Figure 3. Boxplot of monocytes (a) and NK cells (b) in acromegalic patients (ACRO) and in healthy controls (CTRLs)



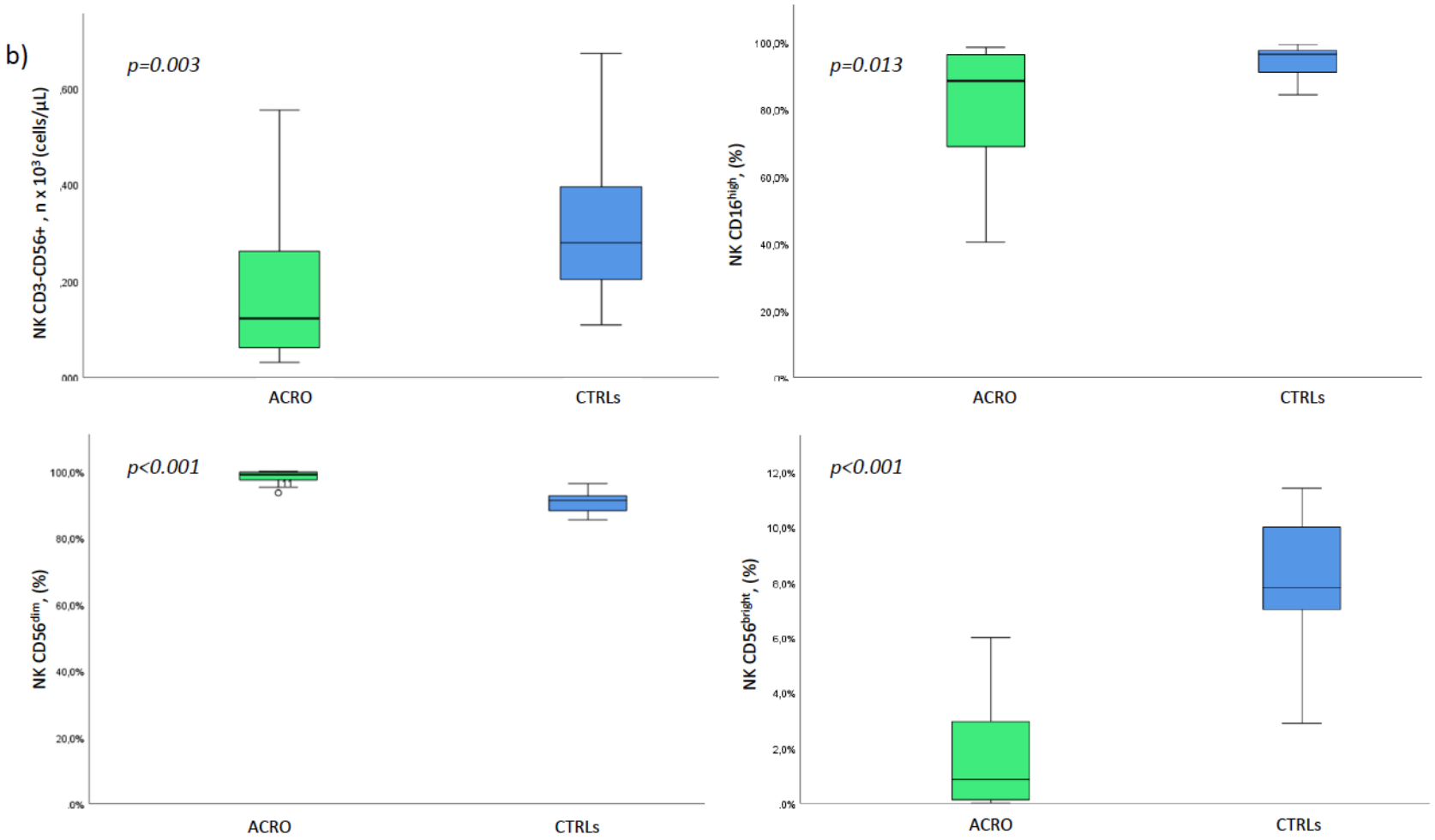

b)
p=0.003
NK CD3-CD56+ , n x 10³ (cells/µL)
,600
,400
,200
,000
ACRO
CTRLs
p=0.013
NK CD16high, (%)
100,0%
80,0%
60,0%
40,0%
20,0%
0%
ACRO
CTRLs
p<0.001
NK CD56dim, (%)
100,0%
80,0%
60,0%
40,0%
20,0%
,0%
ACRO
CTRLs
p<0.001
NK CD56bright, (%)
12,0%
10,0%
8,0%
6,0%
4,0%
2,0%
,0%
ACRO
CTRLs

Figure 4. Correlation analysis between CD8 lymphocytes (cells/µL), n x 10^3 and IGF1 levels (ng/ml)

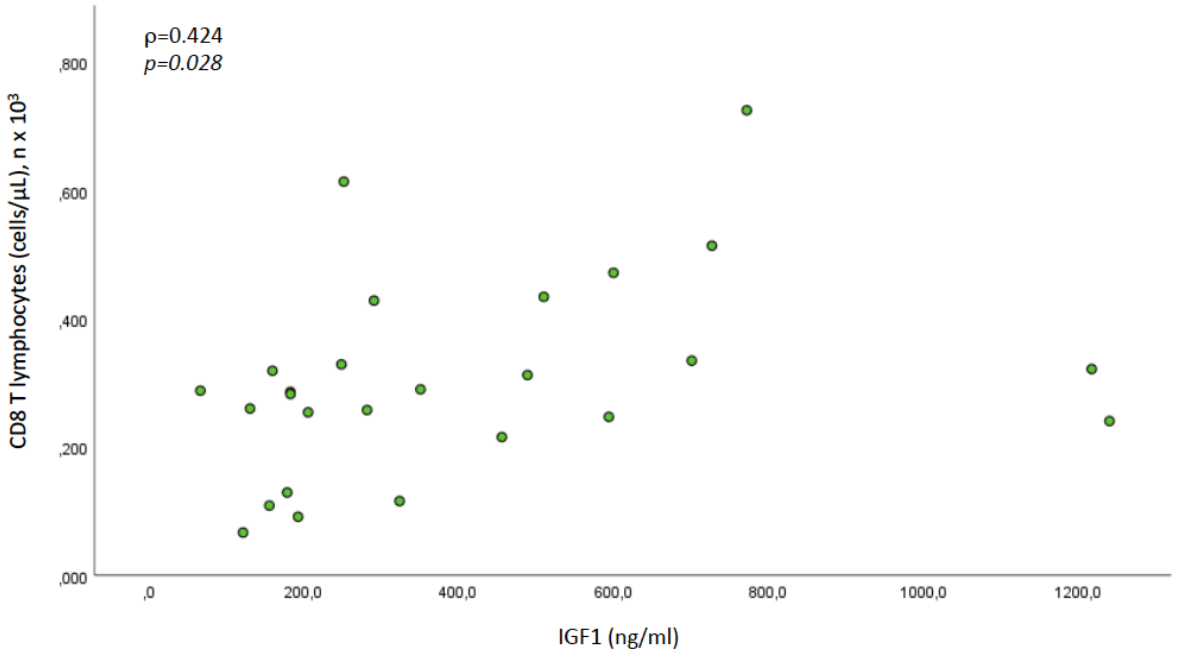
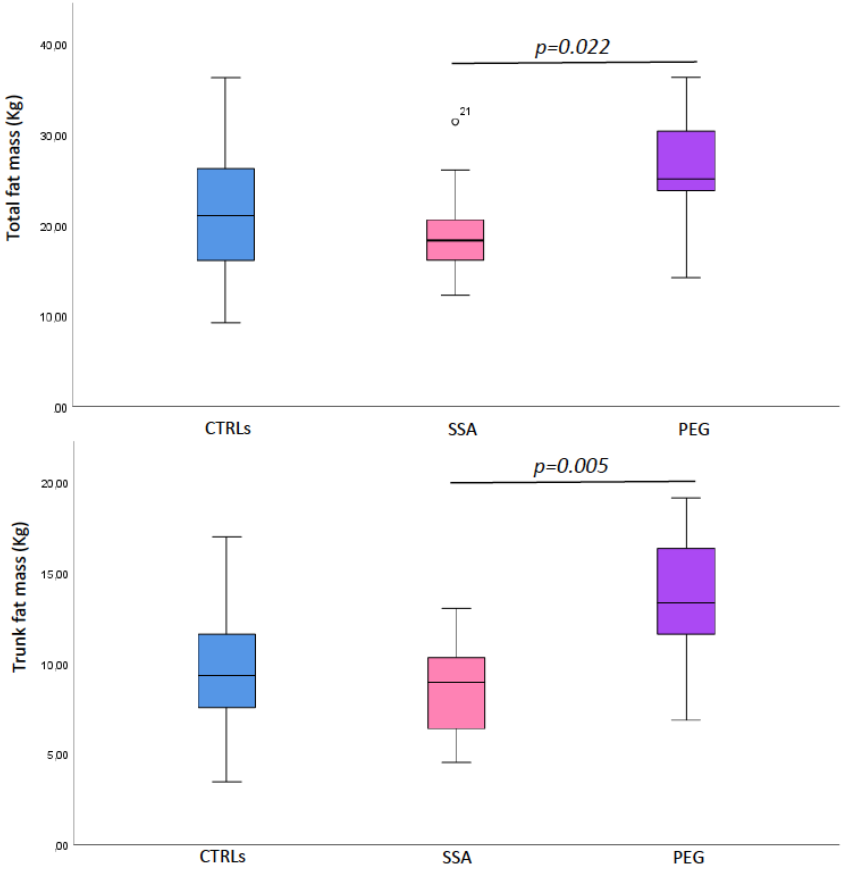


Figure 5. Boxplot of total fat mass (kg) and trunk fat mass (kg) assessed by DEXA total body in healthy controls (CTRLs) and SSA and PEG treated patients.



Legend to the figures

Figure 1. Monocytes and NK cells subset.

On the left figure from Yang et al. Biomarker Research 2014, 2:1 Page 5 of 9 http://www.biomarkerres.org/content/2/1/1. Human MC and Mφ differentiation, and distinct subset functions. Human CD14++CD16- classical MCs leave the bone marrow in a CC-chemokine receptor 2 (CCR2)-dependent manner. In the steady state, classical MCs can differentiate into intermediate MCs, and then differentiate into patrolling non-classical MCs in circulation. Classical MCs have a high antimicrobial capability due to their potent capacity of phagocytosis, and secrete ROS and IL-10 upon LPS stimulus, whereas intermediate and non-classical MCs secrete inflammatory cytokines, TNFα and IL-1β upon inflammatory stimulation. During inflammation, classical and intermediate MCs are tethered and invade tissue by interaction of complementary pair CCR2/CCL2(MCP1) or/and CCR5/CCL5(RANTES) in a VLA1/VCAM1 dependent manner. MCs then mature to M1Mφ in tissue and present self-antigen via MHC-I/II to TCR leading to TC activation. Non-classical MCs patrol the vessel wall and invade by interaction of complementary pair of CX3CR1/CCL3 via LAF/ICAM1-dependent manner. TC, T cell; MC, monocyte; Mφ macrophage; EC, endothelial cells; inf., inflammatory; α-inf. Anti-inflammatory; TCR, T cell receptor; HLA-DR, human leukocyte antigen DR (a major histocompatibility complex class II (MHC-II)).

On the right figure from Cooper et al. TRENDS in Immunology Vol.22 No.11 November 2001, Schema of human natural killer (NK)-cell subsets. (a) CD56bright NK cells produce high levels of cytokines following stimulation with monokines. This subset has low-density expression of CD16 and exhibits low natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), but potent lymphokine-activated killer (LAK) activity. CD56bright NK cells have high-level expression of the inhibitory CD94–NKG2A C-type lectin NK receptor (NKR)

but have low level expression of killer Ig-like receptors (KIRs)6,7. This NK-cell subset expresses a number of cytokine and chemokine receptors constitutively, including the high-affinity interleukin-2 receptor (IL-2Rαβγ), c-kit and CC-chemokine receptor 7 (CCR7)9,10,12,15. The adhesion molecule L-selectin, which, in combination with CCR7, is involved in trafficking to secondary lymph nodes, is also found on CD56bright NK cells. (b) By contrast, CD56dim NK cells produce low levels of NK-derived cytokines but are potent mediators of ADCC, LAK activity and natural cytotoxicity, and have a more granular morphology than CD56bright NK cells2,40. The CD56dim NK-cell subset has high-level expression of KIRs. These cells have distinct expression of cytokine (e.g. IL-2Rβγ) and chemokine (e.g. CXCR1 and CX3CR1)9,10,15 receptors. CD56dim NK cells lack L-selectin but highly express PEN5–P-selectin glycoprotein ligand-1 (PSGL-1), another adhesion molecule. Abbreviations: γc, common γ chain; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon γ; TNF, tumor necrosis factor. Adapted from Ref. 44, with permission from the American Society of Hematology.

Figure 2. Study flow-chart. *1 patient after second neurosurgery and 3 naïve patients.

Figure 3. Boxplot of monocytes (a) and NK cells (b) in acromegalic patients (ACRO) and in healthy controls (CTRLs). Panel a) boxplot of monocytes count and subset (classical, intermediate, and non-classical, %), panel b) boxplot of NK count and subset ($CD16^{high}$, $CD56^{dim}$, $CD56^{bright}$, %).

Figure 4. **Correlation analysis between CD8 lymphocytes (cells/μL), n x 10^3 and IGF1 levels (ng/ml).**

Figure 5. Boxplot of total fat mass (kg) and trunk fat mass (kg) assessed by DEXA total body in healthy controls (CTRLs) and SSA and PEG treated patients.

8 References

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