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ORIGINAL ARTICLE

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Cellular composition and transcriptomics of subcutaneous adipose tissue linked to blood glycated haemoglobin

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Abstract

Objective: Despite growing evidence, the mechanisms connecting adipose tissue (AT) function to type 2 diabetes (T2DM) remain incompletely understood. A detailed analysis of AT transcriptomes could offer valuable insights into this relationship. Here, we examined gene expression patterns in bulk subcutaneous

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AT, focusing on biological pathways and cellular composition associated with glycated haemoglobin (HbA1c) levels.

Methods: A transcriptomic dataset was obtained from subcutaneous AT samples of 901 adults collected during elective surgical procedures. We characterized cellular composition within subcutaneous AT in association with blood HbA1c levels by performing bulk adipose transcriptomes cell deconvolution analysis. We also conducted differential gene expression and overrepresentation analyses. We validated our cross-sectional study using two independent validation cohorts, performing further downstream analyses.

Results: Subcutaneous AT from subjects with increased HbA1c had lower adipocytes, smooth muscle, pericytes and other endothelial cell numbers. Pathways associated with HbA1c levels included cellular senescence and telomere-related pathways and extracellular matrix organisation. We identified the expression of RHO GTPases associated with HbA1c not previously linked to glucose homeostasis, with a possible sexual dimorphism shaped by the obesity state. The findings were confirmed in both longitudinal cohorts. At the gene level, HLA-DR, CCL13, and S100A4 mRNA levels were strongly correlated with HbA1c levels.

Conclusions: This study underscores the utility of AT transcriptome analysis in unravelling T2DM complexities. Our findings enhance knowledge of glucose homeostasis' molecular and cellular underpinnings, paving the way for potential therapeutic targets to mitigate the impact of AT dysfunction in metabolic diseases.

KEYWORDS
glycated haemoglobin, immune system, Rho GTPases, subcutaneous adipose tissue, type 2 diabetes mellitus

1 | INTRODUCTION

Adipose tissue (AT) is a major endocrine and complex organ that plays a significant role in metabolic homeostasis.[1–8] Transcriptomic studies on AT and diabetes have recently shed light on its role in type 2 diabetes mellitus (T2DM) development.[9–13] However, much of the diabetes literature relies on blood cells or pancreatic islets samples, rather than AT.[12,14,15] Moreover, most studies have focused on visceral AT (VAT), while subcutaneous AT (SAT) has been less studied. This perspective has overlooked the critical significance of SAT as an essential indicator of metabolic changes within the body, generating a gap in the current research landscape of diabetes.[16,17] In addition, while Next-Generation Sequencing (NGS) offers high throughput and untargeted analysis, valuable for complex diseases, many studies on diabetes-associated AT transcriptomes still rely on microarrays and RT-qPCRs.[12,14–17] We identified only one SAT transcriptome study performed with the RNA-Seq technique in subjects with T2DM, which analysed the SAT's whole transcriptome of Asian Indians through RNA-Seq analysis, linking T2DM with altered lipid, glucose and protein metabolism, adipogenesis defects, and inflammation.[18] However, this study only involved fat biopsies from 10 individuals. We did not find studies focusing on RNA-Seq SAT transcriptome associated with serum glucose and blood HbA1c levels in humans involving a large sample size (see Appendix S1).

It is essential to deepen our understanding of AT biology and its changes along the progression of diabetes and its complications. The current study focused on the SAT transcriptome and its links to serum glucose and blood HbA1c levels. Our results reinforce current knowledge and provide insights that might aid in better understanding the underlying pathophysiological mechanisms. We also identify several potential expression markers of metabolic control.

2 | RESEARCH DESIGN AND METHODS

2.1 | Clinical study. recruitment of study subjects

The study sample involved adults from the ADIPOMIT cohort ($n = 901$), who had a surgical intervention scheduled between 2009 and 2020 that allowed the removal of SAT and had given written informed consent. Exclusion criteria included: diagnosis of infectious diseases or inflammation within a month prior to the study; liver diseases, thyroid dysfunction or systemic diseases such as lupus or rheumatoid arthritis; undergoing cancer treatment; or being pregnant or in the breastfeeding period. Further details on the sample can be found in Table S1A,B and in the cohort descriptive analysis in Appendix S1.

Data collection included the gathering of SAT sample biopsies for their RNA-Seq analysis; serum samples were extracted in a postabsorptive state before surgery. All actions have received appropriate approvals from the Ethics, External Scientific and Fatbank Internal Scientific Committees CEIm Code: 2019.062. Please refer to Research Design and Methods in Appendix S1 for additional information on data collection.

Adipose tissue collection and handling. AT samples were obtained from SAT depots during elective surgical procedures (mainly gastric-by-pass and gynaecology-related surgeries; see Table S1A). Samples were immediately transported to the laboratory (5–10 min) under strictly aseptic conditions, washed in PBS, dissected (150 mg pieces), flash-frozen in liquid nitrogen and stored at −80°C.

RNA extraction and transcriptomics analyses. RNA was extracted from SAT samples and sequenced using Illumina. Information on RNA sample preparation and RNA libraries is available in the Appendix S1.

2.2 | Study design

This cross-sectional study on the ADIPOMIT cohort aimed to characterize cellular composition within SAT in association with blood HbA1c levels, to determine the association between SAT gene expression patterns, serum glucose and blood HbA1c levels, and to identify associated biological pathways based on the analysis of RNA-Seq samples from the adult population. Additionally, the research sought to ascertain whether these SAT gene expression patterns and pathways associated with HbA1c and glycemic levels are modified by particular population characteristics such as sex, obesity or antidiabetic medication. With this aim, different analyses were performed in the exploratory cohort and further validated in two different cohorts.

2.3 | Statistical analysis

Figure S1 illustrates the statistical analyses performed. Statistical analyses were performed using R version 2024.04.2 (https://www.r-project.org/).

2.3.1 | Spearman correlations and stepwise multiple regression models associating SAT gene expression and glycemic markers

Aiming to perform an initial study of the association between SAT gene expression patterns and blood glucose parameters, we performed Spearman partial correlation analyses to explore the relationship between blood HbA1c and serum glucose levels and SAT gene expression across all subjects, stratified by sex and by obesity status. When necessary, the correlations were adjusted for sex, age, sample origin and BMI.

Subsequently, we applied stepwise multiple linear regression analyses to further investigate the association between HbA1c and the top 10% of genes with the strongest positive correlation in all subjects, in men, in women and in subjects with or without obesity. Again, the regressions were adjusted for glucose, age, sex and BMI.

2.3.2 | Differential expression analyses and pathway characterization

The first analysis included all 901 RNA-Seq samples. The association with RNA-Seq expressed genes was independently assessed for both glycemia (as a continuous variable) and for HbA1c as a categorical variable (i.e. HbA1c above and below 5.7% (39 mmol/mol), following the Centers for Disease Control and Prevention criteria for T2DM). Sensitivity analyses were then performed, stratified by sex, by obesity status and including only nonmedicated patients.

A differential gene expression (DGE) analysis was conducted to identify genes associated with glycemic or HbA1c levels. The statistical analyses were conducted using the 'limma' R package, performing robust linear regression models adjusting for the sex, BMI, age and sample origin covariables, performing complete case

analyses. *p*-values were adjusted for multiple comparisons based on the Sequential Goodness of Fit metatest (SGoF), establishing an adjusted *p*-value (pSGoF) < .05 as a threshold for significance. The pathways related to differentially expressed genes (DEGs; pSGoF < .05) associated with glycemia or HbA1c levels in the SAT were identified through an over-representation analysis (ORA) based on the Reactome database using the ConsensusPathDB tool. A *q*-value < .05 was used as a threshold for statistical significance.

For more information on the data collection, RNA extraction and transcriptomics and bioinformatic analyses, see methods in Appendix S1.

2.3.3 | Deconvolution of SAT cellular composition

To delve into SAT cellular composition in association with T2DM, cell deconvolution analysis was performed using our bulk RNA sequencing (RNA-seq) data from 741 subjects grouped by HbA1c levels above ($n = 360$; mean = 6.8%) and below 5.7% ($n = 381$; mean = 5.3%). After bulk RNA-seq count matrix normalization to Counts Per Million, differential expression analysis was performed using the Mann–Whitney *U* test with *p*-values adjustment via Benjamini–Hochberg. Then, cell type marker genes were curated from single-cell RNA sequencing datasets and integrated with marker genes from multiple studies compiled in PanglaoDB, creating a comprehensive reference list of AT-resident cell gene signatures. GSEA[19] assessed cell type composition differences between High and Low HbA1c groups, considering gene-phenotype association, using the 14 cell type-specific marker gene sets as references to generate enrichment scores. An adjusted *p*-value < .05 was considered statistically significant. Positive normalized enrichment scores indicated higher proportions of a cell type in the 'High HbA1c' group compared to the 'Low HbA1c' group, and vice versa. Single-sample Gene Set Enrichment Analysis (ssGSEA)[20] provided enrichment scores for cell type-specific marker genes in the SAT of individual participants. The Mann–Whitney *U* test, followed by Benjamini-Hochberg correction for multiple comparisons, was used to investigate differences in cell type abundances between High and Low HbA1c groups. Spearman's correlation evaluated the relationship between HbA1c levels and cell type enrichment scores. All statistical analyses were performed using R 4.1.0 (https://www.r-project.org/). More information can be found in the bioinformatic analysis section in Appendix S1.

2.3.4 | Validation cohort 1

An independent validation cohort (Table S2) included 16 selected women with obesity, to whom SAT samples were extracted both before and after bariatric surgery (BS) PREVIOUSLY PUBLISHED.[21] All subjects were of Caucasian origin, had stable body weight for at least 3 months before entering the study, and had no infections or systemic diseases other than T2DM or obesity. Liver and thyroid dysfunction were specifically excluded by biochemical work-up, among other exclusion criteria further described in Appendix S1. Serum was collected before surgery and analysed by routine laboratory tests. Total RNA was extracted and purified from AT and cell debris. Microarray RNA expression profiles were obtained from each sample using the Affymetrix GeneChip Human Gene 2.0 ST Array and the miRNA 3.0 Array, respectively. Analyses included gene expression profiles in SAT before and around 2 years after surgery-induced weight loss. More information on the data collection and analyses, RNA extraction and transcriptomic analysis can be found in Appendix S1 and elsewhere.[21]

2.3.5 | Validation cohort 2

A second independent validation cohort (Table S3) included 24 subjects without diabetes from the ADIPOINST cohort. All participants were of Caucasian origin, aged 30–55 years. These subjects had morbid obesity (BMI > 35 kg/m^2), had maintained stable body weight for at least 3 months prior to the study, and were free from other underlying pathology. Exclusion criteria included being treated with medications affecting insulin metabolism; having serious systemic diseases unrelated to obesity, inflammatory systemic conditions or chronic viral infections, liver disease or thyroid dysfunction; following chronic anti-inflammatory treatments; and have suffered any infections within 1 month before the study, were recruited from the Endocrinology Service at Hospital Dr. Josep Trueta. The study protocol (Project Code LBPFGF19, approval number 2016.051) was reviewed and approved by the Ethics Committee of Hospital Dr. Josep Trueta. All subjects provided written informed consent. Further details on the cohort can be found in Table S3 and elsewhere.[22]

Before surgery, serum was collected and analysed by routine laboratory tests. During elective surgery, adipose tissue samples were obtained from SAT depots. Statistical analyses included gene expression profiles in SAT, replicating DGE analyses in the exploratory cohort and

performing enrichment analyses. More information on the data collection and analyses, RNA extraction and statistical analysis can be found in Appendix S1.

3 | RESULTS

3.1 | Adipose tissue expression of genes is associated with HbA1c levels

We performed Spearman partial correlations between HbA1c and gene expression in all subjects within the exploratory cohort (adjusting for age, bmi, sex and sample origin), as well as in subjects with obesity and in women (adjusting for age, bmi and sample origin). We identified multiple gene transcripts significantly correlated with HbA1c levels in all groups, with a level of significance of $p < .001$ (Table S4A).

Following stepwise multiple linear regression models were performed. The dependent variable in these models was HbA1c, while the independent variables included the top 10% of genes correlated with HbA1c in the previously described correlation analyses, along with sex, age, BMI and blood glucose levels. This analysis was conducted in all subjects within the exploratory cohort, as well as on specific subgroups: subjects with obesity (adjusting for the same covariates and using the top 10% of genes correlated with HbA1c in subjects with obesity) and women (adjusting for age, BMI and blood glucose levels, and using the top 10% of genes correlated with HbA1c in women). We identified HLA-DRB1, CCL13, TCF23, TP53I3 and ST14 mRNAs in SAT as significantly and independently associated with HbA1c levels in all patients (Table S4B). Significant genes in the models include HLA-DRA, LAT, PKN2-AS1, TP53I3 and S100A4 in women; HLA-DRB1 and CCL13 in subjects with obesity. In all three models, HLA-DRB1 explains around 2% of the variance of HbA1c, even after controlling for serum glucose levels (Table S4B).

3.2 | Adipose tissue expression of genes belonging to inflammation, immune system and RHO GTPases pathways is associated with serum glucose and blood HbA1c levels

3.2.1 | Association of SAT gene expression with HbA1c status and serum glucose levels in all patients

We observed significant associations of gene transcripts with HbA1c status ($n = 740$) and with glycemia ($n = 856$) (see Table S5A,B, respectively). A total of 4195 genes were differentially expressed in relation to blood HbA1c levels, and 1889 genes related to serum glucose levels.

ORA results show an association of HbA1c status with numerous pathways, mainly the immune system, inflammation-related pathways and RHO GTPases (Figure 1A, Table S5C, Figure 1B). Similar results were observed for serum glucose levels (Figure S2 and Table S5D).

3.2.2 | Sensitivity models

We analysed the association between blood HbA1c and serum glucose levels and SAT gene expression in women ($n = 531$), men ($n = 209$), subjects with simple or morbid obesity ($n = 685$), subjects without obesity ($n = 55$) and nonmedicated subjects ($n = 167$).

3.2.2.1 | *Association of SAT gene expression with HbA1c levels in women and men*

Biological sex subtly yet ubiquitously modulates tissue gene expression, affecting many biological processes. By stratifying, we aimed to control for this variable.

Significant associations of gene transcripts and HbA1c levels were found both in women (Table S6A) and men (Table S6B). The ORA revealed an association between HbA1c levels and numerous pathways in women (Figure 2A and Table S6C) and in men (Figure 2B and Table S6D), including several immune, inflammation-related pathways. In women, associated pathways related to RHO GTPases include: MIRO GTPases, RHOBTB3, RAC1, RAC2, RHOA, CDC42, RAC3, RHOG and RHOD. We did not detect any significant association with RHO GTPases in men.

3.2.2.2 | *Association of SAT gene expression with HbA1c levels in patients with obesity and morbid obesity, and with blood HbA1c and serum glucose levels in patients without obesity*

Obesity is associated with low-grade chronic inflammation, which contributes to the metabolic syndrome development and its associated complications, such as T2DM.[23] SAT gene expression in individuals with obesity may impact glucose homeostasis.[24] Obesity might thus act as a confounder and/or modifier. We assessed the association between HbA1c levels and SAT gene expression in patients with simple obesity ($30 \leq BMI < 35\,kg/m^2$) and morbid obesity ($BMI \geq 35\,kg/m^2$), on one hand, and in patients without obesity ($BMI \leq 30\,kg/m^2$), on the other hand.

We observed numerous significant associations of SAT gene transcripts and HbA1c levels in subjects with obesity (Table S7A), but only some relevant transcripts in subjects without obesity (Table S7B). HbA1c levels were

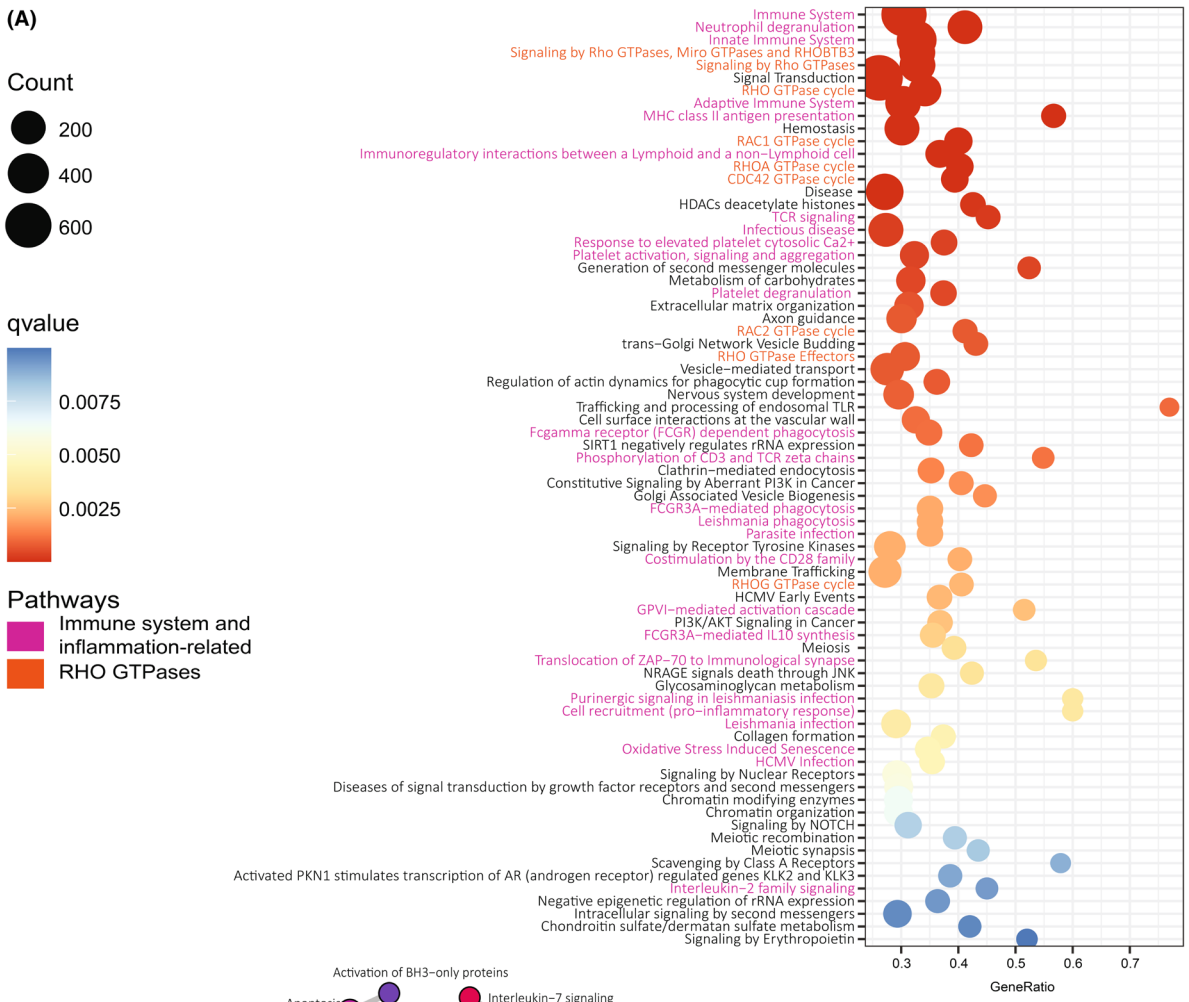


FIGURE 1 (A) Dotplot of significant Reactome pathways that are differentially expressed (q-value < .01) in SAT samples of subjects with HbA1c levels above and below 5.7% ($n = 740$). Significant pathways resulted from an over-representation analysis (ORA) of genes differentially expressed (DEGs, pSGoF < .05) in HbA1c levels above and below 5.7% while controlling for age, sex, BMI and sample origin, identified from the SAT RNA-Seq data in the ADIPOMIT exploratory cohort. The ORA was performed with ConsensusPathDB, considering the Reactome database. Pathways are shown in the y-axis; Counts refer to the number of significantly DEGs that belong to the given pathway (or gene-set); Gene Ratio (x-axis) is calculated as count/set size (being set size the number of genes in a given pathway). Dots are coloured according to pathways' q-value. Pathways related to the immune system, inflammation and RHO GTPases are highlighted in colour. (B) Emapplot of significant pathways that are differentially expressed (Reactome database, q-value < .05) in SAT samples of subjects with HbA1c levels above and below 5.7%. Significant pathways resulted from an ORA of DEGs (pSGoF < .05) in HbA1c levels above and below 5.7%. Dots are coloured according to q-value; dot size represents the number of genes involved in each pathway; nodes link pathways with shared significant genes, with a minimum percentage of overlap genes of .2.

associated with numerous pathways in subjects with obesity (Figure 3A and Table S7C). Associated pathways mirrored those from the full participant set, including the immune system, inflammation-related pathways and signalling by RHO GTPases.

In contrast, in subjects without obesity, HbA1c levels were associated with only two pathways: eicosanoid ligand-binding receptors and prostanoid-ligand receptors (Figure 3B and Table S7D). However, it is important to consider that we evaluated only 55 samples. On the other hand, when assessing the potential association between glycemia and SAT gene expression in patients without obesity ($n = 57$), we identified associations with several genes (Table S7E) and pathways (Figure 3C and Table S7F).

3.2.2.3 | *Association of SAT gene expression with HbA1c levels in nonmedicated patients*

Antidiabetic drug intake is a confounder as it can alter RNA expression profiles in addition to affecting glycemia.[10] Therefore, we performed association analyses of SAT's gene expression with HbA1c levels considering only patients not medicated with antidiabetic drugs, using a robust linear regression model adjusted for age, BMI and sex. More information on this subgroup can be found in Table S1A. Some transcripts were found significant (Table S8A). Again, an ORA based on DEGs with a pSGoF < .1 revealed the association of HbA1c levels with several pathways, including the immune system or RHO GTPases (Figure S3 and Table S8B).

3.3 | Deconvolution analysis in experimental cohort ADIPOMIT

According to cell deconvolution analysis, immune cells, including neutrophils and macrophages were overrepresented in subjects with HbA1c levels above 5.7%, while adipocytes, smooth muscle cells (SMCs), pericytes and other endothelial cells were underrepresented (Figure 4). This finding aligns with correlations shown in Figure 4, where blood HbA1c levels are positively associated with enrichment scores for macrophages and neutrophils and negatively associated with adipocytes and endothelial cells. Multiple regression models further supported these associations between cell type fractions and HbA1c levels, also when accounting for age, sex and BMI covariates (Table S9).

3.4 | Validation cohort 1 results

HbA1c levels decreased significantly after BS, as well as the expression of selected genes related to RHO GTPases and associated with HbA1c levels in our findings (Figure 5). An ORA based on DEGs (pSGoF < .05) revealed that the immune system, inflammation and RHO GTPase-related pathways were differentially expressed before and after bariatric surgery (Figure 5 and Table S10A,B).

Robust linear regression models showed that HbA1c significantly explains variation in RAC2, ARHGAP22 and ARHGAP4 gene expression, while BMI is significantly associated with ARHGAP30 and ARHGAP4 expression (Table S11).

3.5 | Validation cohort 2 results

The overrepresentation analysis with the KEGG database, based on DEGs (pSGoF < .1) (Table S12A), confirmed again that immune system-related pathways were significantly associated with HbA1c levels in obese subjects without diabetes (Table S12B).

4 | DISCUSSION

While VAT has long been acknowledged for its significant contribution to the onset of metabolic disorders, recent evidence suggests that both SAT and VAT experience disruptions in their homeostasis in environments conducive

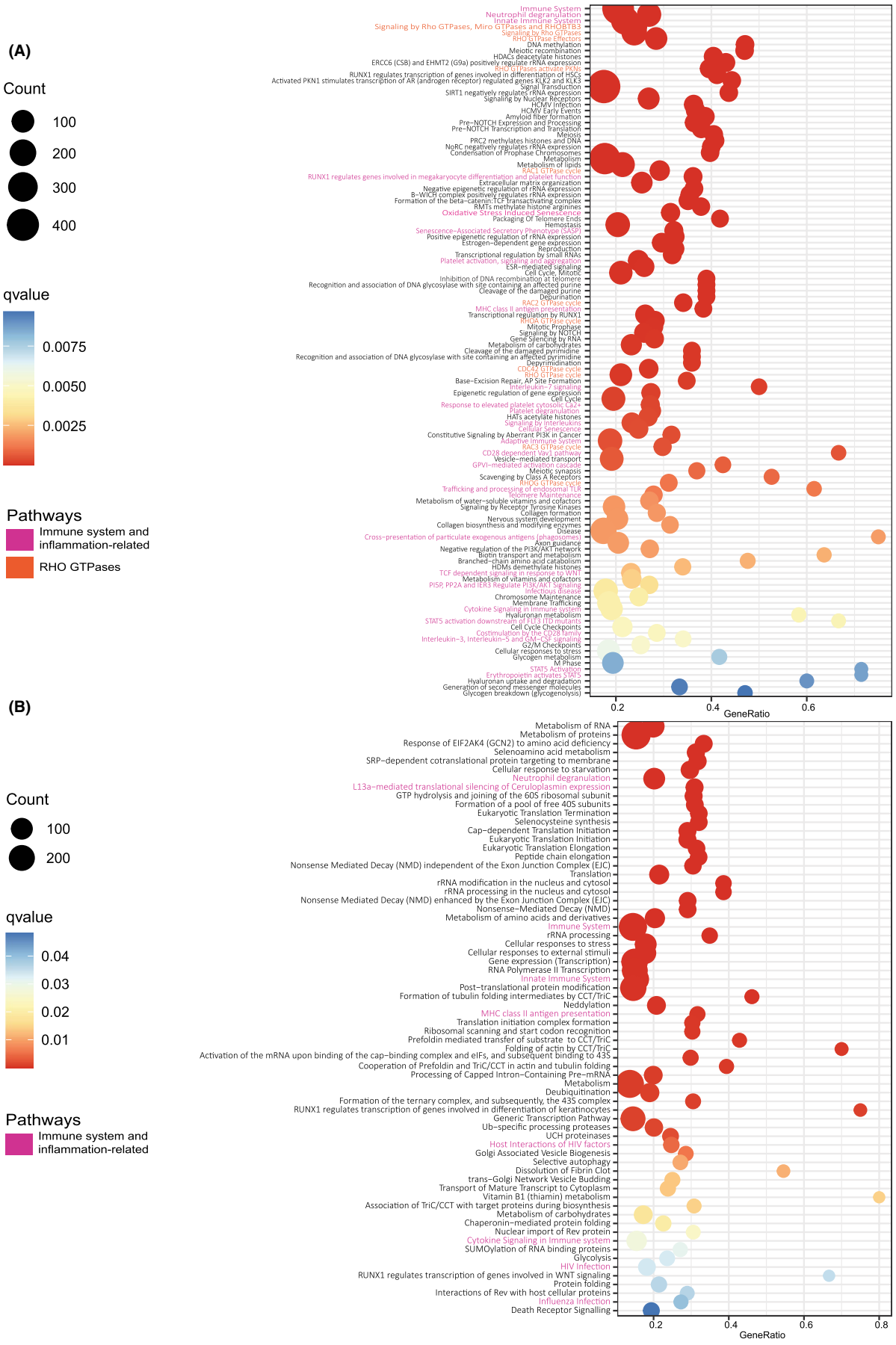


FIGURE 2 (A) Dotplot of Reactome pathways that are significantly associated (q-value < .01) with HbA1c levels in women's SAT samples ($n = 531$). Significant pathways resulted from an ORA of DEGs (pSGoF < .05) significantly associated with HbA1c levels while controlling for age, BMI and sample origin, identified from the women's SAT RNA-Seq data in the ADIPOMIT exploratory cohort. Pathways related to the immune system, inflammation and RHO GTPases are highlighted in colour. (B) Dotplot of Reactome pathways that are significantly associated (q-value < .05) with HbA1c in men's SAT samples ($n = 209$). Significant pathways resulted from an ORA of DEGs (pSGoF < .05) significantly associated with HbA1c levels while controlling for age, BMI and sample origin, which were identified from the men's SAT RNA-Seq data in the ADIPOMIT exploratory cohort. Pathways related to the immune system and inflammation are highlighted in colour.

to metabolic disturbances.[25] Cutting-edge research has associated proinflammatory macrophages in SAT with insulin resistance (IR), and reduced body weight with reduced SAT inflammation.[26]

Studies have revealed a notable increase in macrophage infiltration in SAT depots among lean individuals with metabolic syndrome compared to their metabolically healthy counterparts, whereas differences in VAT infiltration were minimal.[26] This suggests that the condition of SAT more accurately reflects an individual's overall metabolic state than VAT. Thus, monitoring the condition of SAT provides valuable insights into an individual's metabolic well-being.

This study strengthens evidence linking HbA1c levels to SAT inflammation across cellular populations, pathway and gene expression levels, confirming again the value of SAT reflecting metabolic state, particularly glycemic control.

We identified several cellular populations that may reflect SAT inflammation in high blood HbA1c conditions. Our analyses also identified several genes and numerous pathways associated with HbA1c and glycemia, detailed in the Tables S5–S12. Immune system and inflammation-related pathways are associated with HbA1c across all subgroup analyses. Common pathways associated with HbA1c in all subjects, in men, women and individuals with obesity include the immune system, neutrophil degranulation, the innate immune system, MHC class II antigen presentation and cytokine signalling in the immune system. RHO GTPases are also among the most significantly associated pathways, including signalling by RHO GTPases, and Miro, RHOBTB3, RAC1, RHOA, CDC42 and RhoG GTPases. Analyses in women and in subjects with obesity show highly similar results. The differences between men and women are primarily RHO GTPases pathways, present in women but not in men. Furthermore, RHO GTPases are not associated with HbA1c in subjects without obesity. In individuals without obesity, pathways associated with HbA1c include eicosanoid ligand-binding receptors and prostanoid ligand receptors, both of which are related to the immune system and inflammatory response.

Associated genes and pathways identified align with existing knowledge on glucose homeostasis, diabetes, metabolic diseases and SAT's physiological and pathophysiological processes involved in these conditions. For instance, pathways such as PI3K, PI3K/Akt2 and phosphoinositides, which we identified, are known to participate in glucose homeostasis.[27] Results are also consistent with prior transcriptomic studies, which have revealed that the immune system, inflammation, cancer signalling, cell cycle pathways, ubiquitin-proteasome systems, altered lipid, glucose and protein metabolism and IR are associated with diabetes, all pathways that we have encountered in this analysis.[9,10,12] These findings reinforce the validity of our results, obtained in a tissue whose transcriptome has not been extensively studied.

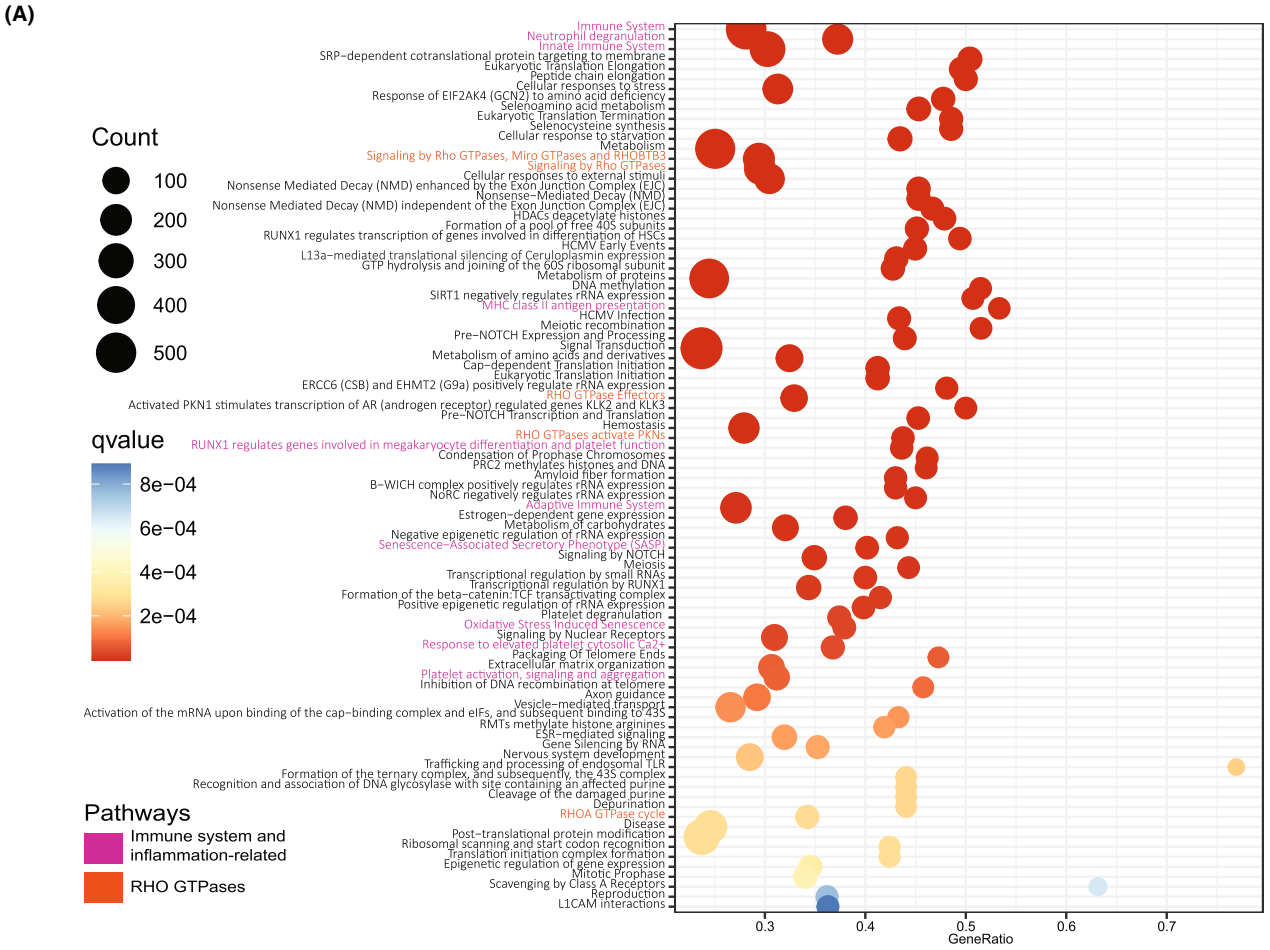
4.1 | Immune cells are overrepresented in subjects with HbA1c levels above 5.7, while adipocytes, smooth muscle cells (SMCs), pericytes and other endothelial cells are underrepresented

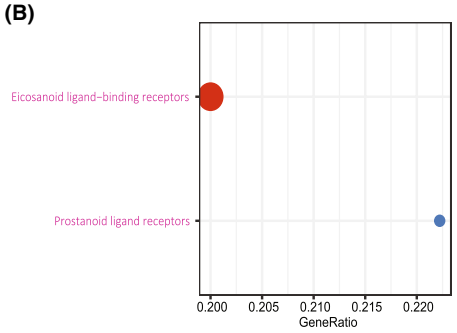
The positive strong and highly significant association between macrophages and neutrophils and HbA1c levels, being overrepresented in AT of subjects with HbA1c levels over 5.7%, was consistent across the different deconvolution analysis of AT cellular composition (Figure 4). This result was also coherent with our previous results indicating an association between glucose HbA1c levels and AT expression of genes related to inflammation and immune system pathways and aligned with existing literature, as already discussed.

The negative association between HbA1c levels and endothelial cells and adipocytes was strong and highly significant. Pericytes and SMCs representation in AT was also underrepresented in subjects with HbA1c levels over 5.7%.

All these cell types—macrophages, neutrophils, endothelial cells, adipocytes, pericytes and SMCs—have been identified as canonical in human WAT.[28]

Adipocytes, smooth muscle, pericytes and endothelial cells are crucial cell types for AT functioning and its irrigation. Endothelial cells and pericytes are part of the blood vessels structure and regulate the vascular system, while smooth muscle cells in AT regulate blood flow,





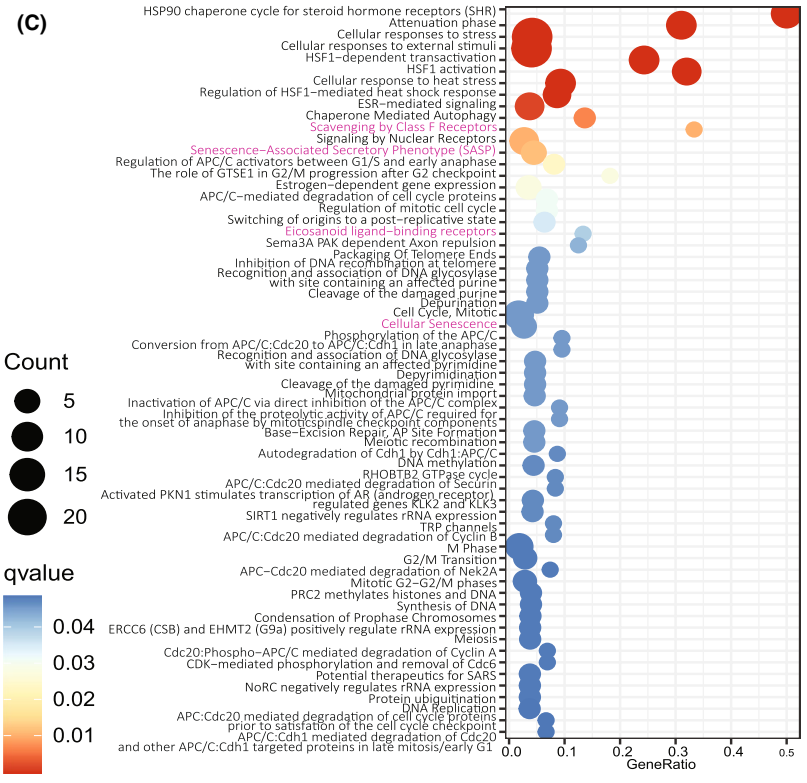


FIGURE 3 (A) Dotplot of Reactome pathways that are significantly associated (q-value < .001) with HbA1c levels in SAT samples from patients with obesity ($n = 685$). Significant pathways resulted from an ORA of DEGs (pSGoF < .05) significantly associated with HbA1c levels while controlling for age, sex, BMI and sample origin, identified from the SAT RNA-Seq data. Pathways related to the immune system, inflammation and RHO GTPases are highlighted in colour. (B) Dotplot of Reactome pathways that are significantly associated (q-value < .05) with HbA1c levels in SAT samples from patients without obesity ($n = 55$). Significant pathways resulted from an ORA of DEGs (pSGoF < .05) significantly associated with HbA1c levels while controlling for age, sex, BMI and sample origin, identified from the SAT RNA-Seq data. Pathways related to the immune system and inflammation are highlighted in colour. (C) Dotplot of Reactome pathways that are significantly associated (q-value < .05) with serum glucose levels in SAT samples from patients without obesity ($n = 57$). Significant pathways resulted from an ORA of DEGs (pSGoF < .05) significantly associated with serum glucose levels while controlling for age, sex and BMI, identified from the SAT RNA-Seq data. Pathways related to the immune system and inflammation are highlighted in colour.

tissue structure, support adipocyte function and ongoing research suggests that they could participate in signalling pathways that influence AT.[29] Imbalance in these cell populations can lead to a loss of AT's vascular integrity.[30] Their underrepresentation in AT of subjects with high HbA1c can be explained due to the complex interplay between SAT dysfunction and vascular complications in diabetes. Hyperglycaemia induces oxidative stress, contributing to vascular dysfunction by reducing nitric oxide bioavailability, while vascular dysfunction impairs insulin delivery to SAT, exacerbating insulin resistance and worsening hyperglycaemia. Vascular dysfunction can also lead to hypoxia and can impair angiogenesis, resulting in adipocyte necrosis. Both hyperglycaemia and vascular dysfunction promote inflammation in SAT, which contributes to vascular dysfunction and hyperglycaemia, creating a vicious cycle. Together with hyperglycaemia, low-grade inflammation induced by proinflammatory immune cells and released cytokines is another hallmark underlying endothelial dysfunction in diabetic vascular complications.[31]

ASPCs were positively associated with HbA1c levels. Different studies have observed an ASPCs expansion—as a reaction to maintain homeostasis and the death of adipocytes, or to excess circulating lipids and glucose in hyperglycemia or insulin resistance development—while showing impaired function in altered metabolic conditions, failing to enter adipogenic lineage.[32] In fact, hyperglycemic conditions may foster senescence of ASPCs, affecting lineage commitment.[33,34] Therefore, even if ASPCs increase with higher HbA1c levels, adipogenesis is often impaired, leading to AT dysfunction instead of effectively counteracting hyperglycemia.

Moreover, Emont et al. 2022 performed a deconvolution analysis to estimate cell type proportions in bulk SAT RNA-sequencing data in men, finding the adipocytes' relative abundance negatively correlated with BMI, and ASPCs' and myeloid cells' positively correlated.[28] Given the positive correlation between BMI and HbA1c, these findings are consistent with ours. Figure 4 shows positive and significant Spearman's correlations between BMI and HbA1c levels in our cohort, suggesting again the potential confounder role of BMI in the association between HbA1c and cell types proportion. Nonetheless, the multiple regression models assessing cell types overrepresented in relation to HbA1c levels were adjusted for BMI. After adjustment, adipocytes and ASPCs appeared to be more strongly, positively and significantly associated with HbA1c levels, while endothelial cells were more strongly, negatively and significantly associated. Immune cells remained strongly and significantly positively associated compared to the same analyses without adjusting for BMI (Table S9). We therefore hypothesise that these cell types' relation with diabetes or altered insulin sensibility is independent of BMI confounding effect.

4.2 | Immune system and inflammation pathways expression in SAT is associated with blood HbA1c and serum glucose levels

Immune system and inflammation-related pathways consistently emerged in all stratified analysis. Among them, we consistently found: innate and adaptive immune systems, neutrophil degranulation, cytokine signalling in the immune system, interleukins and MHC antigen presentation. This association aligns with existing knowledge. Further information can be found in supplemental information on immune system and inflammation pathways associated with HbA1c levels in SAT and their link with T2DM in Appendix S1.

4.3 | RHO GTPase expression in SAT is associated with HbA1c in women with obesity, but not in men or in subjects without obesity

RHO GTPases expression in SAT was associated with HbA1c and glycemia in the analysis of all patients, and with HbA1c in women, in patients with obesity, and in nonmedicated subjects. It should be noted that most ADIPOMIT participants were women and had simple or

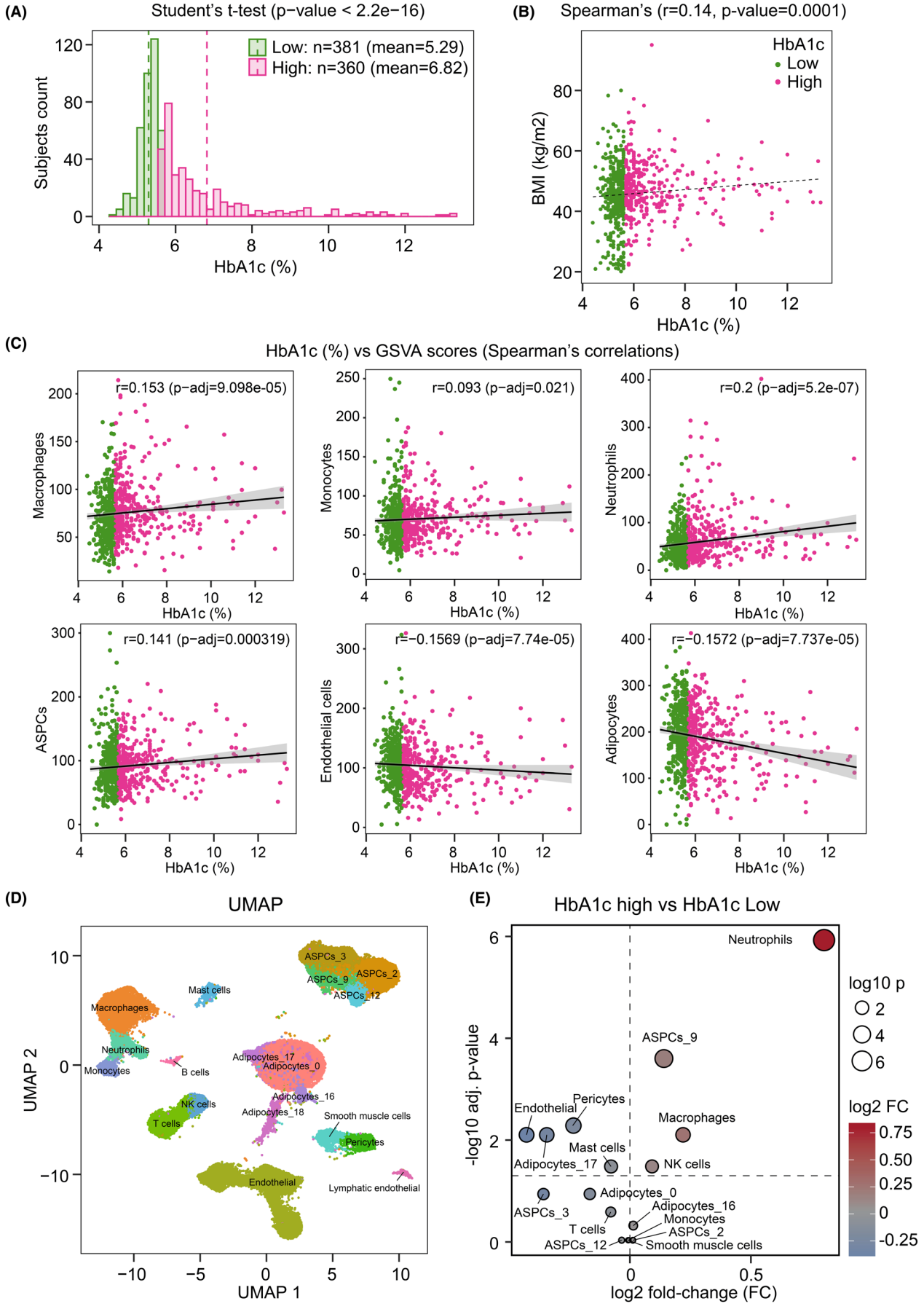


FIGURE 4 (A) Histogram showing subjects' count and blood HbA1c (%) levels, including Student's *t*-test assessing differences between the high HbA1c group (above 5.7%) and the low (below 5.7%). (B) Spearman's correlation between BMI (kg/m^2) and HbA1c (%). (C) HbA1c (%) versus GSVA scores regression analysis (β), after correcting for age, sex and BMI. (D) UMAP of cell type composition. Clustering indicates similarities in expression patterns based on cell deconvolution analysis. (E) Volcano plot of cell types from deconvolution analysis, showing fold change (FC) and *p*-values comparing high versus low HbA1c levels.

morbid obesity. When assessing subjects without obesity, even considering the vast majority were women, RHO GTPases were not associated with HbA1c levels. Similarly, RHO GTPases were not associated with HbA1c or glycemia in men (even considering a p*SGoF* < .1, results not shown). Note also that most men had obesity or morbid obesity.

Differences between men and women, potentially interacting with obesity, could explain these results. However, we could not rule out that they might be caused by the composition and characteristics of the different subgroups or due to different sample sizes (*n* men = 209; *n* women = 531): further studies are needed to clarify it. Few studies have focused on the sexual dimorphism in RHO GTPases expression in relation to diabetes and glucose homeostasis. A study found male-biased expression of RHO GTPases at transcriptomic, lipidomic and metabolomic levels in mice neutrophils.[35] Another publication also identifies sexual dimorphism in RHO GTPase expression in pluripotent stem cell-derived myoblasts of nondiabetic and diabetic insulin-resistant individuals.[36]

The RHO family of GTPases are key regulators of the actin cytoskeleton and vesicle traffic, modify axon outgrowth and growth cone motility, have crucial roles in triggering inflammation and multiple immune functions, and are now emerging as regulators of metabolic homeostasis.[37] They seem to have an essential function on regulating glucose metabolism in health and disease, but this is still an emerging field. It is known they participate in serum glucose control via actions in metabolically active tissues such as AT. They are involved in the pancreatic release of insulin and the resulting insulin stimulation of glucose into AT, and they likely also play insulin-independent roles in maintaining glucose homeostasis.[38] For example, evidence suggests key functions for RAC1, CDC42 and RHOA in maintaining glucose homeostasis. RAC1 and CDC42 are considered important players in glucose-stimulated insulin secretion via actin reorganization, enabling insulin-containing vesicle translocation to the plasma membrane in response to increased serum glucose concentration.[38] Evidence is more limited regarding MIRO, RHOG, RHOD, RAC2, RAC3 and RHOBTB3 link with glucose homeostasis. Recently, RAC2 has been associated with IR in epiploic AT.[39] Our results support the role of RHO GTPases in glucose homeostasis. Further discussion can be found in the Appendix S1 on RHO GTPases and glucose homeostasis in Appendix S1.

4.4 | CCL13 and S100A4 and HLA-DR gene expression in SAT is associated with HbA1c and could potentially serve as T2DM biomarkers

We have identified several genes whose expression may be indicative of high HbA1c levels, as well as inflammation and related comorbidities, and could be valuable in early diabetes diagnosis. Nonetheless, further research is needed to elucidate how these genes and their variants may influence the risk of T2DM.

S100A4, CCL13, HLA-DRB1 and HLA-DRA were among the genes most strongly and positively correlated with HbA1c levels in our analyses and were included in the regression models as significant variables in explaining HbA1c levels, following a stepwise analysis of various factors (Table S4A,B). Given the consistency of these associations in the literature, these genes are potential biomarkers for further complications of T2DM. More information on the possible links between SAT gene expression and T2DM is described in the supplemental information on HbA1c-associated genes in Appendix S1.

S100A4 association with inflammation and T2DM has been considerably described.[40] S100A4 has been identified as a novel SAT adipokine linked to IR, inflammation and hypertrophy independently of BMI, although its function needs to be further studied.[41] Higher levels of S100A4 have been suggested as a marker of IR in adults with obesity.[42] An association of S100A4 with IR and white adipose tissue (WAT) dysfunction in prepubertal populations has also been reported.[43]

CCL13 has been involved in many chronic inflammatory diseases.[44] It has been associated with islet injury and inflammation in T2DM, as there is a two- to three-fold increase in islet concentrations of chemokines, including CCL13, and cytokines compared with the normoglycemic state.[45] CCL13 expression in human islets has also been shown to correlate negatively with insulin secretion and positively with HbA1c, coherently with our results.[46]

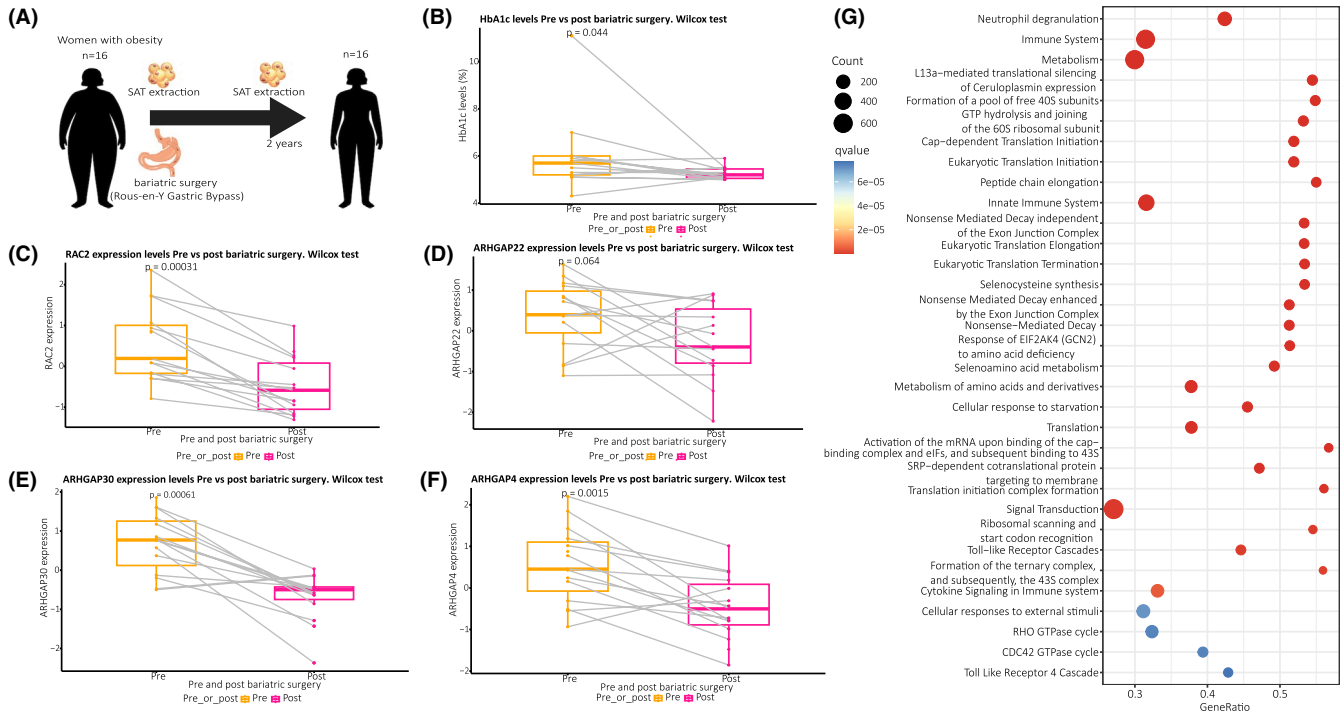


FIGURE 5 (A) Validation cohort 1 study design. Women with obesity were recruited and SAT samples were obtained during bariatric surgery after informed consent. SAT was again extracted around 2 years after from the same patients. $N = 30$ from 16 patients with pre- and post-BS data. (B–F) Boxplots showing change in HbA1c levels (%) (B), RAC2 (C), ARHGAP22 (D), ARHGAP30 (E) and ARHGAP4 expression levels as normalized intensities (F) between pre- and post-bariatric surgery states. (G) Dotplot of significant Reactome pathways that are differentially expressed (q-value<EXP-05; 33 most significant pathways) in SAT samples taken before and after bariatric surgery from women within the validation cohort 1 ($n = 16$). Significant pathways resulted from an over-representation analysis (ORA) of genes differentially expressed (DEGs, pSGoF < .05). No covariables were considered. The ORA was performed with ConsensusPathDB, considering the Reactome database.

4.5 | Results from the validation cohorts are consistent with our experimental cohort

Subjects within the validation cohort 1 had undergone bariatric surgery and therefore were expected to have both lower BMI and HbA1c levels. Consistently, differentially expressed pathways between pre-and post-surgery states were very similar to differentially expressed pathways associated with HbA1c levels in the ADIPOMIT cohort. These include the immune system, inflammation and RHO GTPase-related pathways. Results are also consistent with existing studies. For instance, recent findings suggest that surgery-induced weight loss may lead to changes in inflammatory response in AT.[21] On the other hand, a decrease in HbA1c levels was observed after bariatric surgery in our validation cohort 1, along with a decrease in expression levels of genes related to RHO GTPases, such as RAC2, ARHGAP22, ARHGAP30 or ARHGAP4, suggesting a positive correlation between these RHO GTPases expression and HbA1c levels (Figure 5). These results complement and are in line with those arising from the ADIPOMIT cohort, as RHO GTPase-related pathways were associated with HbA1c levels in women with obesity. Robust linear regression models further suggested that HbA1c and BMI levels may shape these changes in RHO GTPases expression (Table S11), coherently with our results showing that obesity status may play a role. Overall, this validation cohort 1 enabled us to suggest that HbA1c levels might shape RHO GTPase expression, as well as inflammation and immune system pathways.

An additional validation cohort 2 validated the findings from the enrichment analyses in the exploratory cohort, again revealing multiple immune system–related pathways significantly associated with HbA1c levels in morbidly obese subjects without diabetes.

4.6 | Limitations

Five percent of samples (44 patients out of a total of 901) lacked data on glucose, and a seventeen percent lacked data on HbA1c (160 patients out of a total of 901). Missing data can introduce bias into a study when the missingness is not random. Consequently, only complete cases were used in our analyses to ensure consistency and

comparability across variables. If the data is Missing Completely at Random (MCAR), this approach does not introduce bias.

5 | CONCLUSION

In summary, we observed an association between inflammation-related cellular populations, pathways and gene expression with HbA1c and glycemic levels in SAT. At the cellular population level, macrophages and neutrophils overrepresentation in SAT at high HbA1c conditions reflects tissue inflammation, while the underrepresentation of crucial cell types such as adipocytes, smooth muscle, pericytes and endothelial cells suggests their dysfunction and imbalance at hyperglycemic conditions, related to vascular and adipogenesis complications and leading to AT dysfunction.

At the biological pathway level, immune system and inflammation pathways were consistently associated with hyperglycemic conditions across different sensitivity analyses and in the validation cohorts. Interestingly, RHO GTPase expression was associated with HbA1c only in women with simple or morbid obesity, but not in men or in women without obesity.

In line with these results, the expression of HLA-DR, CCL13 and S100A4 genes in SAT is strongly correlated and appears to explain HbA1c levels. Considering SAT's importance in reflecting general metabolic status, these genes hold potential as early biomarkers for T2DM complications.

Further investigation is needed to infer the causality of the associations found. Nonetheless, our results bring added value in the field by focusing on less-studied SAT transcriptomes using Next-Generation Sequencing techniques based on a large number of SAT samples, which is necessary and scarce in existing literature.

AUTHOR CONTRIBUTIONS

Sara Pauli was involved in data curation, formal analysis, validation, visualization and writing–original draft. Núria Oliveras-Cañellas, Anna Castells-Nobau, Jose Ignacio Rodriguez-Hermosa and Ernesto Castro were involved in investigation. José Maria Moreno-Navarrete was involved in investigation and writing—review and editing. Francisco José Ortega was involved in formal analysis, methodology, software, supervisión, validation and writing—review and editing. Birong Zhang and You Zhou were involved in formal analysis, methodology, software and validation. Javier Gómez-Ambrosi, Ana Belén Crujeiras, Oriol Alberto Rangel-Zuñiga, Lourdes Garrido-Sanchez, Sara Becerril, María Pardo, Juan Luis Romero-Cabrera, Carolina Gutierrez-Repiso, Marcos C. Carreira, Manuel Macias-Gonzalez and Miguel Ángel Martinez-Olmos were involved in investigation, data curation and writing—review and editing. Gema Frühbeck, Luisa Maria Seoane, José López Miranda, Carlos Diéguez and Francisco José Tinahones were involved in funding acquisition and writing—review and editing. Jordi Mayneris-Perxachs* was involved in conceptualization, methodology, formal analysis, software, supervision and writing—review and editing. José Manuel Fernández-Real* was involved in conceptualization, methodology, investigation, funding acquisition, supervision and writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no personal or financial conflicts of interest that could potentially bias the results or interpretation of the findings presented in these manuscripts.

DATA AVAILABILITY STATEMENT

Data will be available upon personal request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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