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**IFN $\gamma$ -producing NK cells in adipose tissue are associated with hyperglycemia and insulin resistance in obese women**

Running title: Adipose IFN $\gamma$ -producing NK cells in obesity

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## Abstract

**Background/Objectives:** Innate lymphoid cells (ILCs) play an important role in the maintenance of immune and metabolic homeostasis in adipose tissue (AT). The crosstalk between AT ILCs and adipocytes and other immune cells coordinates adipocyte differentiation, beiging, glucose metabolism and inflammation. Although the metabolic and homeostatic functions of mouse ILCs have been extensively investigated, little is known about human adipose ILCs and their roles in obesity and insulin resistance (IR).

**Subjects/Methods:** Here we characterized T and NK cell populations in omental AT (OAT) from women (n=18) with morbid obesity and varying levels of IR and performed an integrated analysis of metabolic parameters and adipose tissue transcriptomics.

**Results:** In OAT, we found a distinct population of CD56<sup>-</sup>NKp46<sup>+</sup>EOMES<sup>+</sup> NK cells characterized by expression of cytotoxic molecules, pro-inflammatory cytokines, and markers of cell activation. AT IFN $\gamma$ <sup>+</sup> NK cells, but not CD4, CD8 or  $\gamma\delta$  T cells, were positively associated with glucose levels, glycated hemoglobin (HbA1c) and IR. AT NK cells were linked to a pro-inflammatory gene expression profile in AT and developed an effector phenotype in response to IL-12 and IL-15. Moreover, integrated transcriptomic analysis revealed a potential implication of AT IFN $\gamma$ <sup>+</sup> NK cells in controlling adipose tissue inflammation, remodeling, and lipid metabolism.

**Conclusions:** Our results suggest that a distinct IFN $\gamma$ -producing NK cell subset is involved in metabolic homeostasis in visceral AT in humans with obesity and may be a potential target for therapy of IR.

## **Introduction**

The prevalence of obesity has increased drastically during the last decades<sup>1</sup>. The global rise in obesity is associated with IR and the incidence of type 2 diabetes (T2D)<sup>2</sup>. Disruption of energy homeostasis in case of over-nutrition, sedentary lifestyle and ensuing obesity induces metabolic inflammation in AT, thus contributing to dysfunction of glucose metabolism<sup>3</sup>.

The current paradigm for metabolic inflammation is mostly based on data from dietary and genetic mouse models of obesity. The balance between pro- and anti-inflammatory immune cell populations has been shown to control obesity-driven inflammation in white AT<sup>4</sup>. In mice, alternatively activated (M2) macrophages, eosinophils, regulatory T and B cells and Th2 cells maintain tissue homeostasis and prevent inflammation in lean AT. In contrast, obesity results in a shift toward pro-inflammatory M1 macrophages, neutrophils, Th1 cells, cytotoxic CD8 T and effector B cells in AT<sup>5</sup>. However, the roles of these immune cell populations in human adipose tissue are less clear.

Innate lymphoid cells (ILCs) do not express variable antigen-specific membrane receptors characteristic of adaptive immune cells but display antigen-independent responsiveness to immune triggers by producing effector cytokines or eliminating target cells similarly to conventional CD4 and CD8 T cells<sup>6</sup>. ILCs are present in mucosal tissues, tumors and metabolically active organs such as AT and the liver<sup>7, 8</sup>. The ILC family includes three groups of ILCs and natural killer (NK) cells based on differential expression of transcription factors, cytokines and cell surface markers. ILC1 and NK cells express interferon  $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor (TNF), ILC2 express interleukin-4 (IL-4), IL-5, and IL-13, and ILC3 express IL-17 and IL-22. In addition, NK cells express transcription factor Eomesodermin (EOMES) and produce cytotoxic perforin and granzyme<sup>9</sup>. Emerging evidence shows an involvement of ILCs in the pathogenesis of obesity-associated diseases<sup>10</sup>. AT ILC2 promote metabolic fitness of white adipose tissue<sup>7</sup>. In contrast, expansion of AT ILC1 and NK cells has been linked to

obesity and impaired glucose control in various mouse models<sup>11-13</sup>. This is partially mediated by ILC1- and NK-derived IFN $\gamma$  which contributes to expand pro-inflammatory macrophages, in line with the concept that IFN $\gamma$  plays a pro-inflammatory and metabolically deleterious role in AT<sup>14-16</sup>. Nevertheless, the exact immune cell populations that produce IFN $\gamma$  in human AT and their associations with glucose metabolism have not been characterized yet.

Here, we show that IFN $\gamma$  is expressed in CD4, CD8,  $\gamma\delta$ T cells and ILCs, showing phenotypical characteristics of NK cells, in human obese (visceral) OAT. We demonstrate that IFN $\gamma$  expression in AT NK cells, but not in AT T cells, is associated with hyperglycemia and IR in obese individuals. Furthermore, unbiased integrative transcriptomic analysis identified gene modules associated with IFN $\gamma^+$  NK cells that include genes involved in inflammation, adipocyte differentiation, tissue remodeling, glucose and fatty acid metabolism.

## **Research Design and Methods**

### **Subjects**

Among 226 morbidly obese patients who underwent gastric bypass surgery between 2013 and 2014 as part of the A Biological Atlas of Severe Obesity (ABOS) study (ClinicalTrials.gov; NCT01129297), 18 women (9 with T2D) were recruited in this study for immunophenotyping and microarray analyses of AT. Additional 7 women were recruited in 2020 for further immunophenotyping and *ex vivo* activation analysis of AT immune cells. All recruited individuals gave informed consent to participate in this study. Women were selected for this study as, in this cohort of patients, despite morbid obesity, a higher proportion of women had unimpaired glucose metabolism and IR, compared to men, thus allowing to study associations of immune cell populations with a broader spectrum of clinical and biological parameters. 10 mL of peripheral blood and biopsies of visceral OAT were obtained and used for RNA

isolation and flow cytometry. Glucose, insulin, glycated hemoglobin (HbA1c), and C-peptide were measured after overnight fasting and after a 75-g oral glucose tolerance test. C-reactive protein (CRP) was measured in blood after overnight fasting. HOMA2-%S and HOMA2-%B were calculated as described<sup>17</sup>. The Matsuda index was calculated as described<sup>18</sup>. Summary information for all patients included in this study is presented in Table 1 and S1.

### **Cell preparation**

The OAT was collected in 0.9% sterile saline and kept on ice no longer than 2 hr before isolation of the stromal vascular fraction (SVF). 3 g of OAT were minced into small pieces and digested 30 min at 37°C with 10 ml collagenase solution (1 mg/ml collagenase I in DMEM + 0.5% BSA) and SVF cells were isolated by filtration through a 70µm nylon mesh and centrifuged for 10 min at 400g at 20°C. For the preparation of peripheral blood mononuclear cells (PBMCs), blood was separated by a one-step Percoll gradient. SVF cells and PBMC were treated with red blood cell lysis buffer and washed with flow cytometry buffer (1% BSA in PBS). For specific experiments, SVF cells were transferred into RPMI + 1% BSA, and incubated at 37°C and 5% CO<sub>2</sub> in presence of the indicated cytokines during 12 hr, followed by activation with PMA (100 ng/ml), ionomycin (1 mg/ml) and GolgiPlug (555029, BD Biosciences) during 3 hr.

### **Flow cytometry**

Single-cell suspensions were incubated with Fc receptor binding inhibitor antibody (14-9161-73, eBioscience) and stained on ice with antibodies (Table S2). Samples were analyzed using an LSRFortessa cytometer (BD) and the Diva Software. Data were analyzed using the FlowJo software.

### **RNA isolation and microarray analysis**

For RNA isolation, the OAT was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . RNA was extracted with RNeasy Mini Kit (Qiagen). 100 ng of RNA were amplified with GeneChip WT PLUS Reagent Kit and labeled with GeneChip WT Terminal Labeling Kit (Affymetrix). The resulting complementary RNAs were hybridized with Human Gene 2.0 ST array (Affymetrix) according to the manufacturer's protocol. Microarray data were normalized by the robust multi-average (RMA) method<sup>19</sup> by using affy R package<sup>20</sup>.

### **Weighted gene co-expression network analysis**

Highly correlated genes were identified and grouped as gene modules using the weighted gene co-expression network analysis (WGCNA) bioconductor package in R<sup>21</sup>. Normalized, log-transformed expression data were filtered based on expression level  $> 2$ , and then variance-filtered with top 7267 annotated transcripts taken for analysis from a ranked list of median absolute deviation values for all probes. A soft threshold power equal 12 was selected on the basis of the criterion of approximate scale-free topology. Gene modules were identified from the resulting topological overlap matrix, summarized as modular eigengenes and correlated with a matrix of clinical variables and proportions of adipose immune cell populations. To analyze identified gene sets, Gene Set Enrichment Analysis (GSEA) was used as described<sup>22</sup>.

### **Statistical analysis of biological data**

No statistical method was used to predetermine sample size. Non-parametric Spearman's rank-order correlation or parametric Pearson correlations were used. Unpaired and paired Mann-Whitney U tests were used to compare clinical and biological parameters in two groups of individuals. P-values below

0.05 were considered significant. Statistical analyses were performed with R open source software (version 3.5.3) and GraphPad Prism (version 8.1.2).

## **Results**

### **Characteristics of the studied morbidly obese patients from the ABOS cohort**

25 women with morbid obesity and varying levels of IR who gave informed consent to participate in the ABOS research project were enrolled in this study. Clinical and biological characteristics of the patients involved in this study are summarized in Table 1 and S1.

### **Innate and adaptive lymphocytes express IFN $\gamma$ in human adipose tissue**

To study the lymphocyte subset composition of obese OAT, flow cytometry analysis was performed on freshly isolated SVF cells. CD45<sup>+</sup> immune cells expressing CD3, a marker of T cells, were found in OAT, including CD4<sup>+</sup> (12.3  $\pm$  5.6 % of CD45<sup>+</sup> cells), CD8<sup>+</sup> (12.0  $\pm$  4.2 % of CD45<sup>+</sup> cells) and  $\gamma\delta$ T cells (1.8  $\pm$  1.5 % of CD45<sup>+</sup> cells) (Figure 1A-B). Interestingly, when excluding CD3<sup>+</sup> cells and known cell populations expressing lineage markers from gated cells with lymphocyte scatter characteristics, *bona fide* ILCs were identified, representing 2.3  $\pm$  1.1 % of AT CD45<sup>+</sup> cells (Figure 1A-B and S1A).

Because IFN $\gamma$  produced by Th1 cells<sup>14, 15</sup> and NK cells<sup>12</sup> in AT is detrimental in obesity, we assessed whether the individual AT lymphoid immune populations produced IFN $\gamma$  after restimulation *ex vivo*. CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T cells and ILCs were all capable of producing IFN $\gamma$  showing comparable proportions of IFN $\gamma$ <sup>+</sup> cells among these cell populations (Figure 1C-D). Furthermore, IFN $\gamma$  expression in these cells was associated with a high level of expression of the transcription factor T-BET (Figure 1C), a master Th1regulator<sup>23</sup>. Next, we correlated proportions of IFN $\gamma$ <sup>+</sup> cell subsets with mRNA levels of cytokines in OAT. We found that IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells shared a correlation pattern and were positively



linked to expression of IL-6 and, to a lesser extent, TNF and CCL2 (Figure 1E). In contrast, IFN $\gamma$ <sup>+</sup>  $\gamma$  $\delta$ T cells were associated with expression of IFNG and IL10, whereas IFN $\gamma$ <sup>+</sup> ILCs demonstrated a profound and distinct association with expression of the IL12A, IL1B, IFNG and IL23A genes in OAT (Figure 1E). These results indicate that obese OAT contains innate and adaptive lymphocytes expressing IFN $\gamma$ , whose accumulation correlates with a pro-inflammatory profile of the tissue.

### **Human AT ILCs show phenotypical and functional properties of NK cells**

IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in AT contributes to IR in mouse models of obesity<sup>24-26</sup>. Moreover, in human AT, the relative abundance of Th1-like cells is highly associated with systemic inflammation and IR<sup>27</sup>. By contrast, little is known about IFN $\gamma$  expression in AT ILCs and their characteristics in humans. Unlike the majority of blood ILCs, AT ILCs did not express detectable levels of membrane CD56, a marker of human circulating NK cells (Figure 2A and S1A). Both NK cells and ILC1 are capable to produce type 1 cytokines, but EOMES and cytotoxic molecules, such as granzymes and perforin, are preferentially expressed by NK cells<sup>28</sup>. To elucidate whether the AT ILCs are phenotypically similar to NK cells or represent ILC1-like cell populations, we systematically studied multiple molecular markers of those cell types. The majority of AT ILCs expressed the NK cell-specific transcription factor EOMES and NK cell marker NKp46 (but not the ILC1 marker NKp44) (Figure 2B-C). Moreover, these cells co-expressed CD94, a C-type lectin receptor that recognizes non-classical MHC class I glycoproteins, and T-BET (Figure 2A, D-E), both markers of activated NK cells. Expression of CD94, also known as NKG2D, has been detected in both classical NK cells and ILC1 from intestinal *lamina propria* in mice<sup>29</sup>. AT ILCs expressed an additional marker of mature NK cells CXCR3 (Figure 2A).

In line with the NK cell-like phenotype of the AT ILCs, a majority of these cells co-expressed cytotoxic molecules such as granzymes A (GZMA) and B (GZMB) as well as perforin (PRF) (Figure 2F). The percentage of GZMA and GZMB expression was the highest in activated CD94-expressing NK cells (Figure 2G).

Next, we further analyzed expression of markers of all three types of ILCs in the AT ILCs. Neither expression of the ILC1 marker CD127, nor the ILC2 marker CRTH2, nor the ILC3 markers ROR $\gamma$ t and c-KIT/CD117 were detected in AT ILCs (Figure 2A). In addition, expression of IL-5 and IL-13 (ILC2 cytokines) was not detected in AT ILCs (data not shown). Moreover, only a minor part of the NK-like cells in OAT express IL-17A (ILC3 cytokine), whereas a larger proportion of these cells expressed TNF (cytokine expressed by NK and Th1-like cells) (Figure 2H-I). These data show that AT ILCs represent a specific population of mature CD56<sup>-</sup>EOMES<sup>+</sup>CD94<sup>+</sup> NK cells with a high capacity to express cytotoxic factors and pro-inflammatory cytokines including IFN $\gamma$ .

In line with previous reports showing that NK cells are activated by IL-12 and IL-15<sup>30</sup>, *ex vivo* activation of AT NK cells with IL-12 or IL-15 resulted in an increased expression of IFN $\gamma$  with CD94<sup>+</sup> cells as the main responders (Figure 2J-K). These results further indicate that most AT ILCs in obese patients show a broad NK cell phenotype and are sensitive to inflammatory stimuli. Thus, this cell population will be further referred as NK cells.

### **AT T and NK cells correlate with local and systemic inflammation**

To assess whether accumulation of lymphoid cells in OAT is linked to inflammation, their numbers were correlated with different markers of inflammation. The numbers of CD4 and CD8 T cells and NK cells (but not  $\gamma\delta$ T cells) in OAT positively correlated with the numbers of total AT macrophages but not with M1 macrophages (Figure 3A and Figure S1B and data not shown). Moreover, AT CD8 T and NK

cells strongly correlated with plasma levels of CRP, a marker of systemic inflammation (Figure 3B). Interestingly, IFN $\gamma$  expression in these lymphoid cells did not correlate with AT macrophage numbers nor CRP levels in blood (data not shown). These results show that accumulation of CD4 and CD8 T cells and NK cells in OAT, rather than their capacity to produce IFN $\gamma$ , is linked to macrophage infiltration in OAT and systemic inflammation in obese humans.

### **IFN $\gamma$ expression in AT NK cells correlates with glucose levels and IR**

Next, we assessed whether IFN $\gamma$  expression in AT cell populations correlates with impaired glucose metabolism. The proportions of AT IFN $\gamma$ <sup>+</sup> lymphoid cells did not correlate with BMI (Figure S2A). However, the proportions of IFN $\gamma$ <sup>+</sup> NK cells, but not IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup> or  $\gamma\delta$ T cells, significantly and positively correlated with fasting and postprandial blood glucose levels and HbA1c (which integrates long-term average glycemia) (Figure 4 and S2B). Similarly, IFN $\gamma$ <sup>+</sup> NK, but not the IFN $\gamma$ <sup>+</sup> T cells, negatively correlated with the homeostatic insulin sensitivity index (HOMA2-%S) and with the Matsuda index, indicators of insulin sensitivity taking into account dynamic changes in blood glucose and insulin levels after an oral glucose tolerance test (Figure 4). By contrast, no significant correlations were found between IFN $\gamma$ <sup>+</sup> NK cells and HOMA2-%B, which estimates steady state  $\beta$ -cell function (data not shown). These results indicate that AT IFN $\gamma$ <sup>+</sup> NK cells are specifically linked to elevated blood glucose and IR in obese individuals.

To determine whether IFN $\gamma$  expression in AT NK cells is affected by T2D, individuals with available AT NK cell data were further analyzed. As expected, patients with diagnosed T2D had significantly decreased insulin sensitivity (Figure S2C). Interestingly, the proportions of AT IFN $\gamma$ <sup>+</sup> NK cells were significantly higher in T2D versus non T2D individuals (Figure S2C). However, the proportions of AT IFN $\gamma$ <sup>+</sup> NK cells were not associated with insulin sensitivity parameters in T2D individuals (Figure

S2D). These results suggest that accumulation of IFN $\gamma^+$  NK cells in OAT might precede the development of clinical stage T2D. Finally, we found that treatment of T2D individuals with insulin, metformin or statins did not significantly alter the proportions of IFN $\gamma^+$  NK cells in OAT (Figure S2E), although the limited number of observations in this study did not allow to definitely conclude about the impact of these medications.

### **AT IFN $\gamma^+$ NK cells correlate with specific transcriptional program alterations in OAT**

The association of IFN $\gamma^+$  immune cell populations with AT transcriptional programs was studied to gain insight into their potential roles in the development of IR. Microarray analysis identified 7267 genes exhibiting maximal average expression and variability levels in OAT from the 18 obese patients. Weighted gene co-expression network analysis (WGCNA) allowed to identify in an unbiased manner 16 gene modules of co-expressed genes in OAT (Figure 5A and Supplementary Table 3) which were further studied for associations with clinically relevant phenotypes. Correlation of module eigengenes (1<sup>st</sup> principal component of gene expression in gene module) with fasting and postprandial glucose levels and HOMA2-%S revealed specific gene modules positively associated with impaired glucose control (gene modules Blue, Cyan and Green) or negatively linked to hyperglycemia (gene modules Salmon and Turquoise) (Figure 5B). Then we performed a correlation analysis of module eigengenes with the proportions of AT IFN $\gamma^+$  T cells and AT IFN $\gamma^+$  NK cells from the same patients (Figure 5B). Interestingly, a distinct correlation pattern was found, including positive associations of IFN $\gamma^+$  CD4 $^+$  and CD8 $^+$ , but not  $\gamma\delta$ , T cells with the gene expression modules Brown, Green, Greenyellow, and Midnightblue (Figure 5B), whereas the proportions of IFN $\gamma^+$  NK cells positively correlated with gene modules Blue, Cyan, and Green (Figure 5B). Notably, these latter three gene modules were also linked to impaired glucose metabolism. Next, an in-depth analysis of the genes within each of these modules

was performed to explain associations between IFN $\gamma^+$  cells and IR. Gene module Green was enriched in genes related to tissue development and regulation of cell proliferation as revealed by gene set enrichment analysis (GSEA). Interestingly, expression levels of several collagen genes from gene module Green correlated with hyperglycemia and IFN $\gamma^+$  T and NK cells (Figure 5C). Moreover, leptin (LEP) was part of gene module Green (Figure 5C) that appears to regulate pathways involved in AT fibrosis and inflammation. Gene module Blue was enriched by genes involved in insulin and PPAR signaling. It contained several metabolically important genes such as the leptin receptor (LEPR) and PPAR $\gamma$ , controlling adipogenesis, as well as genes regulating insulin sensitivity (FOXO1), glycolysis (PK4), lipid metabolism (CD36, LPL, FABP4, DGAT1) and fatty acid oxidation (ACADS and ACADL) (Figure 5C). Again, expression levels of this gene module not only positively correlated with high glucose levels, but also with increased proportions of IFN $\gamma^+$  NK cells. Finally, gene module Cyan was enriched in genes involved in intracellular signal transduction and inflammation and was associated with both hyperglycemia and IFN $\gamma^+$  NK cells (Figure 5C). Furthermore, multiple genes within modules Blue, Cyan, and Green showed maximal positive correlation with proportions of IFN $\gamma^+$  NK cells (Figure 5D). Overall, this unbiased integrated analysis shows that increased AT IFN $\gamma^+$  NK cells are linked to specific and metabolically relevant gene modules.

## **Discussion**

Obesity is one of the main risk factors for metabolic diseases, such as T2D, dyslipidaemia, and non-alcoholic fatty liver disease<sup>31, 32</sup>. In obesity, excessive fat deposition and accumulation of dysfunctional adipocytes contribute to IR and an increased risk of T2D<sup>33</sup>. Adipocyte hyperplasia is usually not linked to IR. In contrast, unhealthy expansion of white AT due to adipocyte hypertrophy accompanied by

cellular stress, fibrosis, hypoxia and accumulation of pro-inflammatory M1 macrophages and NK cells may result in metabolic dysfunction<sup>34</sup>.

IFN $\gamma$  produced by Th1 cells has been associated with IR in mouse models of obesity<sup>35</sup>. However, cellular sources of IFN $\gamma$  in human AT have not been well-studied. Here we characterize IFN $\gamma$ <sup>+</sup> lymphoid cell populations in human OAT. These original results show that, in morbidly obese patients, T and NK cell subsets co-express high levels of T-BET and IFN $\gamma$  indicating that morbid obesity is associated with AT inflammation biased towards Th1-like immunity.

Recent studies using mouse models of HFD-induced obesity have shown that ILC1 and NK cells producing IFN $\gamma$  and TNF seem to participate in the promotion of AT inflammation and IR<sup>12, 13, 36</sup>. Our new results in humans show that obese OAT is populated by specific NK cells expressing IFN $\gamma$  and  $\alpha$  activation and maturation markers but low levels of CD56. In a recent study, using a broad panel of antibodies and a high dimensional analysis approach, conventional ILC1 were not identified in human OAT, while NK cells were the dominant innate cell type<sup>37</sup>. We show that some lineage-negative lymphoid cells express CD56 in OAT, requiring appropriate gating to identify *bona fide* NK cells in human AT. Despite the fact that key markers of ILC1 and NK cells have been used in this study, a broader and more complex analysis of the molecular identity of AT ILCs in obese individuals is required to better classify these cell populations. Although low numbers of ILC2 and ILC3 have been reported in human AT<sup>7, 38</sup>, we found virtually no ILC2 and ILC3 in OAT, probably due to an effect of morbid obesity on homeostasis of these ILC populations. Taken together, these novel results indicate that, despite the differences between studies in obese OAT, the majority of the ILCs can be described as NK cells.

Because both obesity and IR are associated with adipose tissue inflammation in humans<sup>39</sup>, it is possible that accumulation of IFN $\gamma$ <sup>+</sup> NK cells may be driven by pro-inflammatory signals in OAT. We show that

both IL-12 and IL-15 enhance IFN $\gamma$  expression in AT NK cells. IFN $\gamma$  and TNF, secreted by NK cells, are associated with AT inflammation and IR in mouse models of diet-induced obesity<sup>12, 13</sup>. We found that IFN $\gamma$  expression in AT NK cells, but not in CD4<sup>+</sup>, CD8<sup>+</sup> or  $\gamma\delta$  T cells, positively correlates with glucose levels and IR in obese individuals. This might be due to the fact that IFN $\gamma$  expression in NK cells is triggered by cytokine signals linked to IR in OAT whereas the T cells may require activation through their T cell receptors to up-regulate IFN $\gamma$  expression. Intriguingly, unbiased gene expression analysis in OAT identified distinct gene modules whose expression activity was linked to proportions of IFN $\gamma$ <sup>+</sup> NK cells. These gene modules were enriched in genes implicated in multiple pathways already described in controlling AT inflammation and IR. For instance, AT IFN $\gamma$ <sup>+</sup> NK cells positively correlated with elevated expression of collagen genes in OAT. This suggests an involvement of NK cells into AT remodeling and fibrosis, which aggravate obesity-associated IR in humans<sup>40</sup>. These results also suggest that a crosstalk between IFN $\gamma$ -producing AT NK cells and adipocytes might be involved in the development of IR in obese patients.

This study has several limitations. First, lean individuals were not included because of the difficulty to obtain samples of OAT in these individuals. Second, only women with morbid obesity were enrolled, which are known to have a different pattern of adipose tissue deposition compared to men potentially affecting the prevalence of metabolic diseases among these groups<sup>41</sup>. In a large cohort of individuals with obesity, we found a higher proportion of women, compared to men, with normal glucose levels and non-impaired insulin sensitivity, which prompted the enrollment of women into this study to address associations between immune cell populations and a broad spectrum of biological and clinical parameters. Further study is needed to test whether our results are also relevant to men. In addition, in this study of cells from visceral fat, we did not directly evaluate visceral obesity using PET/CT Scan<sup>42</sup> but relied on less specific measurement of BMI. Finally, because of the difficulty to obtain OAT

biopsies suitable for both preparation of live immune cells, only 18 patients (and a supplementary cohort of 7 patients) were analyzed. Nevertheless, our results demonstrate that IFN $\gamma$ <sup>+</sup> NK cells exist in human OAT and are significantly associated with hyperglycemia and IR.

In conclusion, this study is the first example of simultaneous analysis of IFN $\gamma$  expression in AT innate and adaptive lymphoid cells and an integrated gene expression analysis in humans in the context of obesity and IR. We show that AT ILCs express features of activated NK cells, are capable to produce IFN $\gamma$  in response to inflammatory stimuli and are associated with IR. These results help to better understand the development of obesity-associated IR in humans and may pave the way therapeutic targeting of IFN $\gamma$  in obesity.

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**Author contributions:** DAM, BS, FP, and DD conceived the study and designed the experiments. DAM, BS, and DD interpreted data and wrote the manuscript. RC, VR, and JN collected human biopsies. BD performed microarray assay. DAM, LP, and LL performed flow cytometry, DAM performed transcriptomic analysis and WGCNA.

**Competing interests:** The authors declare no conflict of interest.



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**Table 1**

<b>Mean (Min; Max)</b>	<b>Flow cytometry and transcriptomics cohort</b>	<b>Additional flow cytometry patients</b>
N	18	7
Men/Women	0/18	0/7
Age, years	44 (19; 63)	44 (27; 63)
BMI, kg/m <sup>2</sup>	45.9 (37.3; 55.2)	46.8 (39.2; 58.8)
Fasting glucose, mg/dL	124.6 (82.2; 288.5)	105.9 (82.1; 136.1)
2 h postprandial glucose, mg/dL	184.1 (78.0; 487.8)	128.1 (70.0; 162.2)
Fasting insulin, $\mu$ U/mL	24.7 (3.2; 192.1)	22.2 (12.5; 33.9)
2 h postprandial insulin, $\mu$ U/mL	69.3 (7.9; 253.1)	69.3 (5.4; 194.5)
Fasting C-peptide, ng/mL	3.3 (0.2; 8.2)	3.7 (2.3; 5.8)
2 h postprandial C-peptide, ng/mL	9.0 (0.2; 19.9)	9.0 (4.4; 13.1)
HOMA2-%S	77.2 (13.4; 231.7)	39.8 (21.8; 59.3)
% HbA1c	6.2 (4.6; 9.2)	6.0 (4.5; 7.7)
HOMA2-%B	107.4 (20.9; 357.2)	153.9 (85.2; 235.3)
Matsuda index	3.7 (0.2; 13.3)	NA
T2D	9/18	3/7
Metformin treatment	5/18	
Statin treatment	6/18	
Insulin treatment	6/18	

## Table legends

**Table 1. Summary information on subjects included in this study.** 18 women with morbid obesity was included for flow cytometry and gene expression analyses. Additional 7 women with morbid obesity were included for detailed flow cytometry and functional analyses. Data are shown as mean, minimal (Min) and maximal (Max) values, or as proportion of patients.

## Figure legends

**Figure 1. IFN $\gamma$ -expressing lymphoid cells in human OAT.** (A) Gating strategy to identify CD4<sup>+</sup>, CD8<sup>+</sup>, and  $\gamma\delta$ T cells and ILCs in human OAT. ILCs were detected as FSC-A<sup>lo</sup> SSC-A<sup>lo</sup> CD45<sup>+</sup> lineage<sup>-</sup> CD3<sup>-</sup> cells. Lineage: CD1a, CD11c, CD14, CD19, CD34, CD123, BDCA2, Fc $\epsilon$ R1, TCR $\gamma\delta$ . (B) Proportions of cell populations are shown as per cent of CD45<sup>+</sup> cells. (C-D) Flow cytometry and proportions of AT IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>, and  $\gamma\delta$ T cells and ILCs that express IFN $\gamma$  and T-BET. (E) Spearman's rank correlations of proportions of AT IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T cells and ILCs with expression levels of cytokine genes in OAT. n = 15-18 patients per group. Data are shown as mean  $\pm$  SD (A, C) or SEM (B, D). \*P < 0.05 by Spearman's correlation test.

**Figure 2. Characteristics of AT and blood ILCs.** (A) Flow cytometry analysis of ILC1, ILC2, ILC3 and NK cell markers in AT and blood ILCs. Results are reproducible in 3-6 individuals. (B) Expression of EOMES, NKp46, and NKp44 in AT ILCs. (C) Proportions of EOMES<sup>+</sup>NKp46<sup>+</sup> and EOMES<sup>-</sup>NKp44<sup>+</sup> cells among AT ILCs (n = 4). (D) Co-expression of CD94 and T-BET in AT ILCs. (E)

Proportions of T-BET<sup>+</sup> cells among AT ILCs (n = 3 patients per group). (F) Representative flow cytometry of granzymes A and B and perforin in AT ILCs. (G) Flow cytometry of co-expression of granzymes A and B in subsets of AT ILCs. (H) Flow cytometry of IFN $\gamma$ , IL-17, and TNF expression in AT NK cells. (I) Proportion of TNF<sup>+</sup> and IL-17A<sup>+</sup> cells among AT NK cells (n = 9-14 patients per group). (J-K) Flow cytometry of IFN $\gamma$  expression and proportions of IFN $\gamma$ <sup>+</sup> cells in AT ILCs stimulated *ex vivo* with IL-12 or IL-15 in the presence of IL-2 during 12 h (n = 6-9 patients per group). Data are shown as median (C, E) or mean  $\pm$  SEM (I). P values by paired Mann–Whitney U test.

**Figure 3. AT NK cells are associated with local and systemic inflammation.** (A-B) Spearman's rank correlations of numbers of lymphoid cell populations with numbers of macrophages and blood levels of CRP (n = 15 patients per group).

**Figure 4. AT IFN $\gamma$ <sup>+</sup> NK cells are associated with perturbed glucose control.** Spearman's rank correlations of AT IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T and NK cells with fasting and 2 h postprandial glucose, HOMA2-%S and Matsuda index (n = 15-18 patients per group).

**Figure 5. Integrated analysis of gene expression in OAT and immune and metabolic parameters.** (A-D) WGCNA of 7267 transcripts in human OAT from the 18 obese women. (A) Gene modules containing genes with similar patterns of expression among patients were identified and coded by colors. (B) Gene module eigengenes were correlated by Pearson correlation test with fasting and 2 h postprandial glucose levels, HOMA2-%S, and proportion of AT IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T and NK cells. (C) Expression levels of selected genes from gene modules Green, Blue, and Cyan were correlated with fasting and 2 h postprandial glucose levels, HOMA2-%S, and proportion of AT IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>,



$\gamma\delta$ T and NK cells. (D) All transcripts from gene modules Green, Blue, and Cyan were correlated with proportion of AT IFN $\gamma$ <sup>+</sup> NK cells and genes with maximal positive correlation coefficients are shown. n = 15-18 patients in each group.



Figure 2

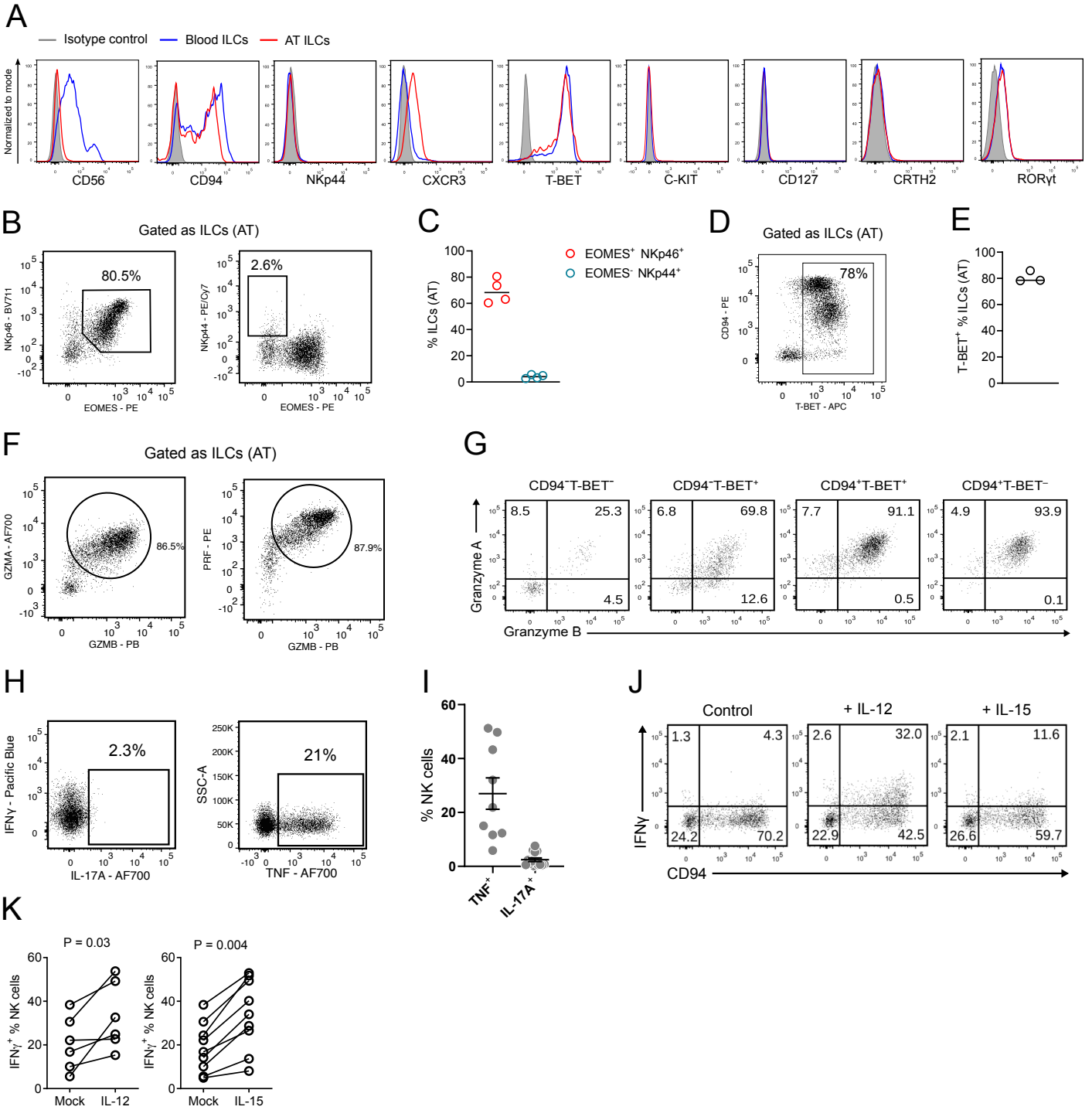


Figure 3

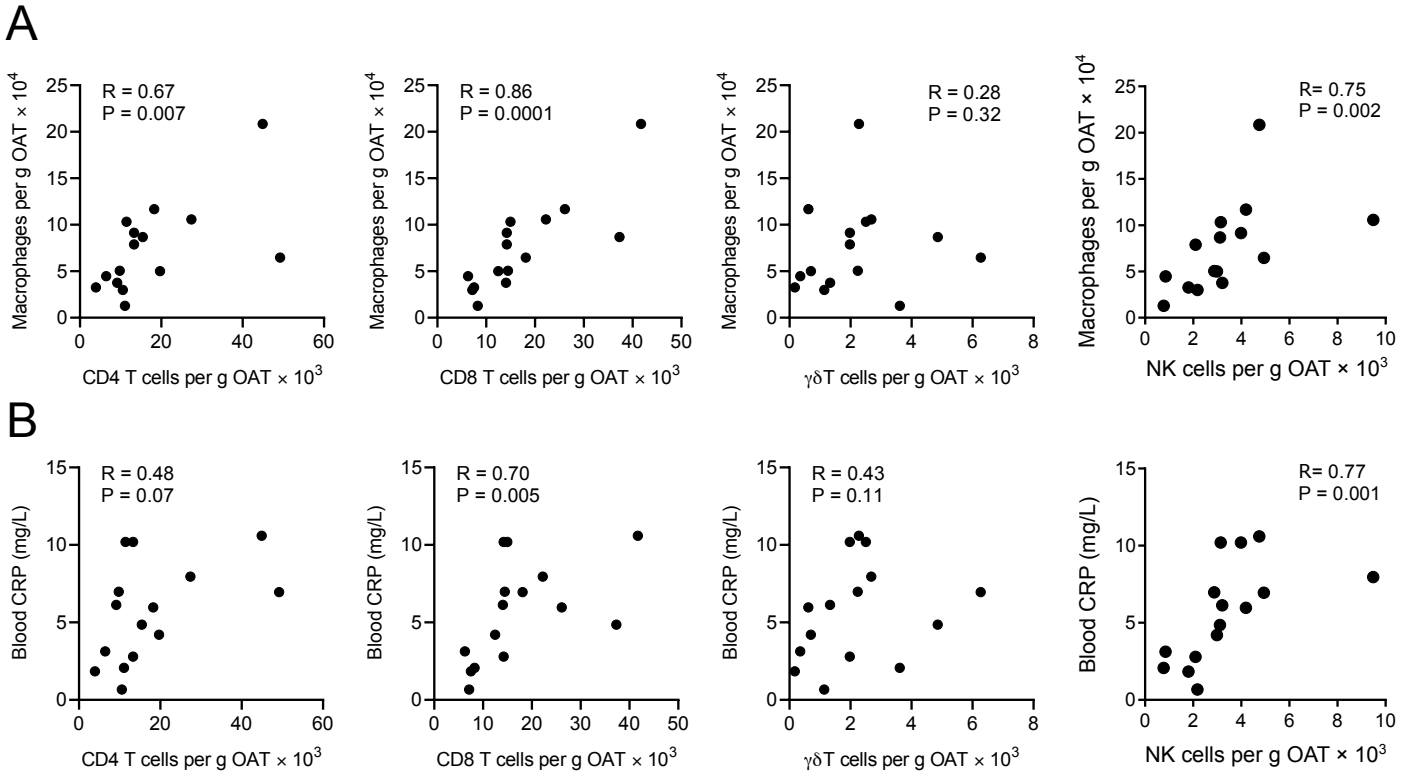
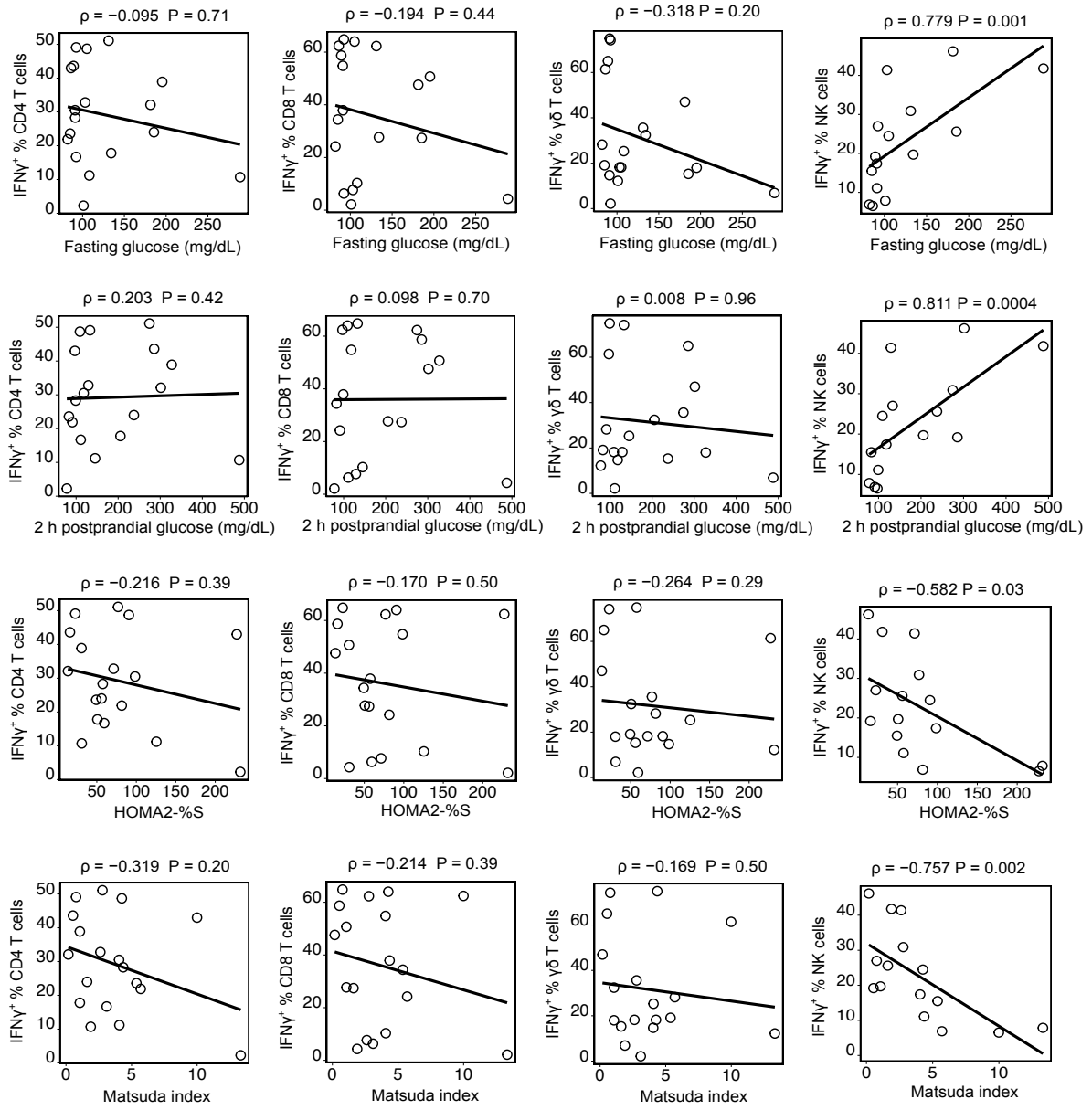
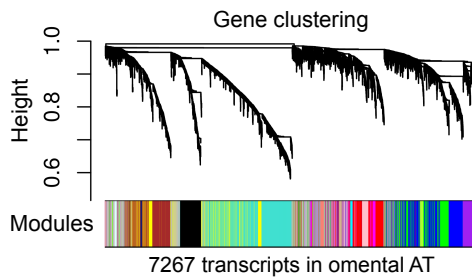


Figure 4

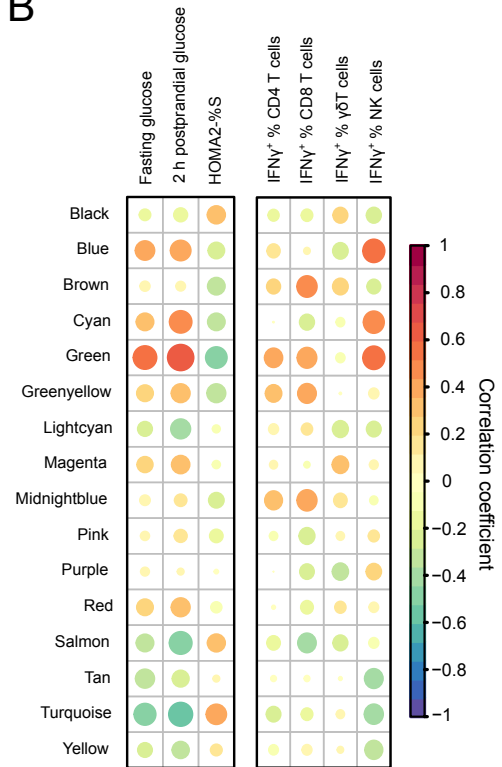


# Figure 5

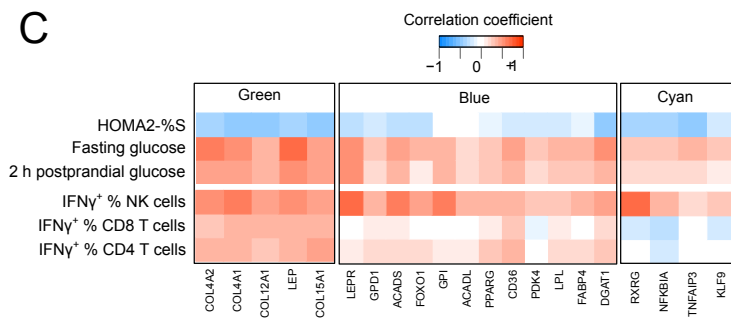
**A**



**B**



**C**



**D**

