Metabolism 144 (2023) 155576

Contents lists available at ScienceDirect

Metabolism

journal homepage: www.journals.elsevier.com/metabolism

Neutrophil aging exacerbates high fat diet induced metabolic alterations

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ARTICLE INFO

Keywords:
Neutrophils
Metabolic syndrome
Adiposity
CXCR2
CXCR4

ABSTRACT

Background: High fat diet (HFD) chronically hyper-activates the myeloid cell precursors, but whether it affects the neutrophil aging is unknown.
Purpose: We characterized how HFD impacts neutrophil aging, infiltration in metabolic tissues and if this aging, in turn, modulates the development of metabolic alterations.
We immunophenotyped neutrophils and characterized the metabolic responses in physiology (wild-type mice, WT) and in mice with constitutively aged neutrophils (MRP8 driven conditional deletion of CXCR4; herein CXCR4fl/flCre+) or with constitutively fresh neutrophils (MRP8 driven conditional deletion of CXCR2; CXCR2fl/flCre+), following 20 weeks of HFD feeding (45 % kcal from fat).
Findings: After 20 weeks HFD, the gluco-metabolic profile of CXCR4fl/flCre+ mice was comparable to that of WT mice, while CXCR2fl/flCre+ mice were protected from metabolic alterations.
CXCR4fl/flCre+ infiltrated more, but CXCR2fl/flCre+ neutrophils infiltrated less, in liver and visceral adipose tissue (VAT). As consequence, while CXCR4fl/flCre+ resulted into hepatic "suicidal" neutrophils extracellular traps (NETs) and altered immune cell architecture in VAT, CXCR2fl/flCre+ promoted proresolutive hepatic NETs and reduced accumulation of pro-inflammatory macrophages in VAT.
In humans, higher plasma levels of Cxcl12 (CXCR4 ligand) correlated with visceral adiposity while higher levels of Cxcl1 (the ligand of CXCR2) correlated with indexes of hepatic steatosis, adiposity and metabolic syndrome.
Conclusions: Neutrophil aging might contribute to the development of HFD induced metabolic disorders.

1. Introduction

Metabolic alterations induced by unhealthy lifestyles, including obesity and insulin resistance, are epidemic conditions leading to diabetes and cardiovascular diseases [1]. In particular, nutrient excess and loss of metabolic homeostasis are frequently associated to systemic low-grade inflammation [2] and to the hyper-activation of immune-inflammatory pathways in metabolic tissues, including the liver and the visceral adipose tissue (VAT). These pathological conditions are also reflected in the reprogramming of innate myeloid cell precursors within the bone marrow (BM), an event so far mechanistically linked to the activation of the inflammasome system. The inflammasome is physiologically activated to respond to pathogens [3]. However, its supra-physiological activation, which occurs during chronic exposure to unhealthy dietary patterns, promotes the expansion of innate immune cell subsets and perpetuates a long term state of inflammation [4,5]. This mechanism is critical not only for the expansion of the precursors of the myelomonocytic lineage [6], but has been recently proposed to be also relevant for the priming of the progenitors of the granulocytic arm, the granulocytes-monocytes progenitor Cells ("GMPs"), which selectively give rise to mature circulating neutrophils [3,7].

Beyond their canonical role against foreign invaders, neutrophils are today recognized as key players in chronic inflammatory diseases [8]. Indeed, these immune cells were shown to respond to high fat diet (HFD) feeding [8,9] by supporting systemic inflammation and oxidative stress [10], thus contributing to the development metabolic disturbances and to non-alcoholic steatohepatitis [11]. Proteins typically present in neutrophil granules have been implicated in inflammatory responses to

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https://doi.org/10.1016/j.metabol.2023.155576
Received 19 January 2023; Accepted 20 April 2023
Available online 26 April 2023

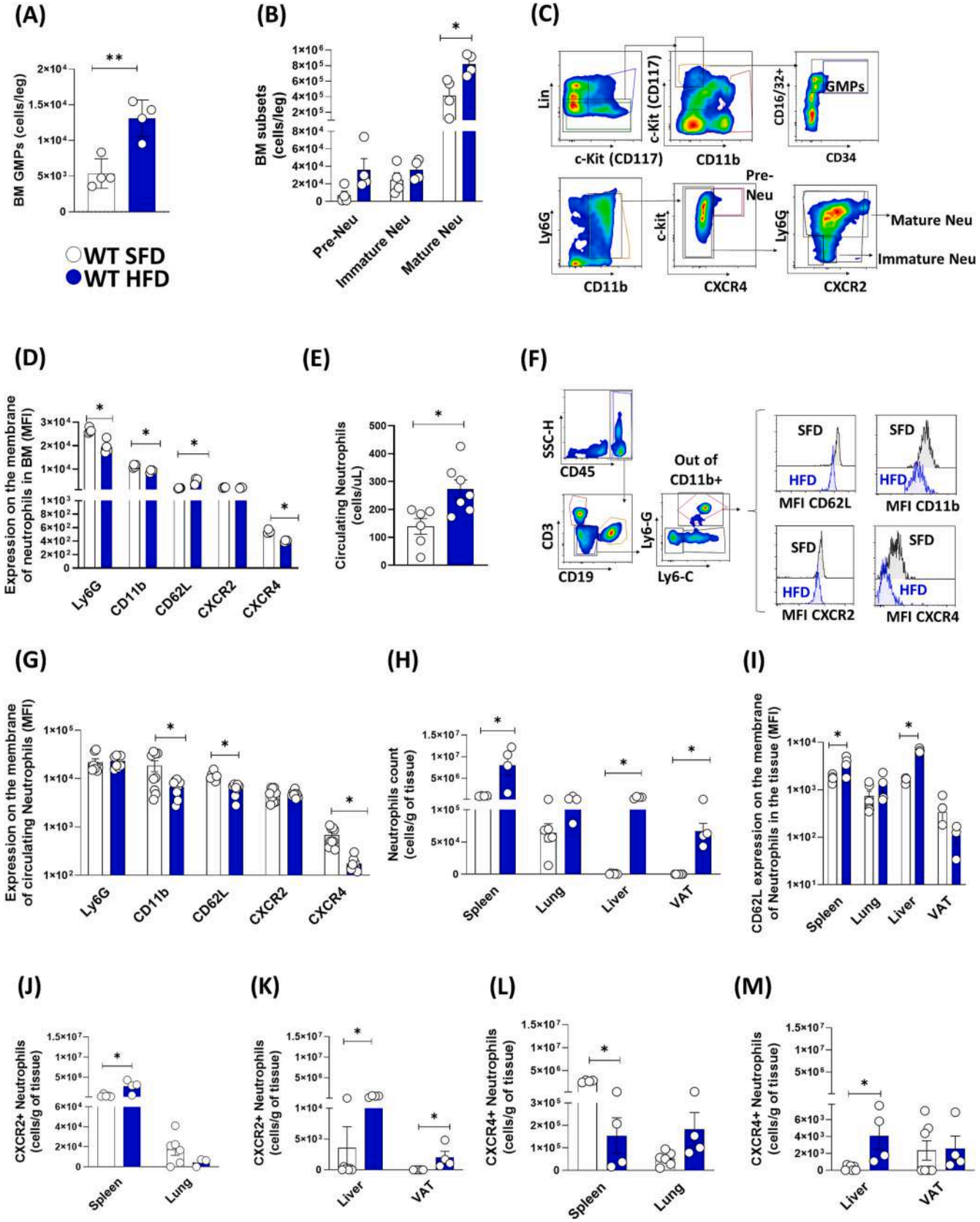

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Fig. 1. High Fat Diet impacts on the aging of neutrophils in liver and adipose tissue.
Wild-type (WT) mice were sacrificed after 20 weeks of Standard Fat Diet feeding (SFD; white bars) or High Fat Diet (HFD) feeding (blue bars). (A) Content of Granulocytes-Monocytes Progenitor Cells (GMPs) in the bone marrow (BM). Data are expressed as absolute count of cells per leg analyzed. (B) Content of pre-neutrophils ("Pre-Neu"), immature neutrophils ("Immature Neu") and mature neutrophils ("Mature Neu") in the bone marrow (BM). Data are expressed as absolute count of cells per leg analyzed. (C) Flow-Cytometry gating strategy for the quantification of GMPs (Lin−/c-Kit+/CD11b-/CD34+/CD16/32+), Pre-Neu (Gr1+/CD11b+/CXCR4+/c-Kit+), Immature Neu (Gr1+/CD11b+/CXCR4-/CXCR2-) and Mature Neu (Gr1+/CD11b+/CXCR4-/CXCR2+) in the bone marrow. (D) Membrane expression of Ly6G, CD11b, CD62L, CXCR2 and CXCR4 on the total number of neutrophils in the BM. Data are expressed as Mean Fluorescence Intensity (MFI). (E) Absolute quantity of neutrophils in circulation (data expressed as number of cells per microliter (μL) of processed blood for Flow-Cytometry analysis). (F) Flow-Cytometry gating strategy for the quantification of total neutrophils (CD45+/CD3−CD19−/Ly6G+/Ly6C−) and their membrane expression of CD11b, CD62L, CXCR2 and CXCR4 in the tissues analyzed. Blue histograms refer to MFI of one sample of WT mice on HFD and white histograms refer to MFI of one sample of WT on SFD. (G) Membrane expression of Ly6G, CD11b, CD62L, CXCR2 and CXCR4 on the total number of neutrophils in circulation. Data are expressed as Mean Fluorescence Intensity (MFI). (H) Absolute quantity of neutrophils in spleen, lung, liver and visceral adipose tissue (VAT) (data expressed as number of cells per gram (g) of tissue processed for the analysis). (I) Membrane expression of CD62L on the total quantity of neutrophils in the spleen, in lung, liver and VAT. Data are expressed as Mean Fluorescence Intensity (MFI). (J) Absolute quantity of CXCR2+ neutrophils in spleen and lung (data expressed as number of cells per gram (g) of tissue processed for the analysis). (K) Absolute quantity of CXCR2+ neutrophils in liver and VAT (data expressed as number of cells per gram (g) of tissue processed for the analysis). (L) Absolute quantity of CXCR4+ neutrophils in spleen and lung (data expressed as number of cells per gram (g) of tissue processed for the analysis). (M) Absolute quantity of CXCR4+ neutrophils in liver and VAT (data expressed as number of cells per gram (g) of tissue processed for the analysis). For Panels (A–D) $N = 4$ WT von SFD were compared to N = 4 WT on HFD; For Panels (E–G) $N = 6$ WT von SFD were compared to $N = 7$ WT on HFD; For Panel (H) N = 6 WT von SFD were compared to N = 4 WT on HFD; For Panels (I–M) N = 4 WT von SFD were compared to N = 4 WT on HFD.
For all the panels, "*" indicates significant difference with p value <0.05 (Kruskal-Wallis test). Grubb's test for the detection of statistical outliers was performed before the Kruskal-Wallis test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chronic unhealthy dietary patterns. This is the case of the neutrophil-derived elastase, which promotes the inflammatory activation of intraperitoneal macrophages of mice fed HFD, dependently to the presence of Toll-like receptor 4 [12]. Also, pentraxin 3 (Ptx3), typically present in neutrophil granules, contributes to inflammatory disorders, and, when absent, results in the reduction of obesity-associated VAT inflammation [13].

Although these findings highlight a negative impact of neutrophils on obesity and metabolic syndrome, an open question remains on how neutrophils, the shortest-living myeloid cell subsets, acquire such long-term pro-inflammatory behavior [14]. Indeed, a large quantity of mature neutrophils ("Mat Neu") is released from the BM daily, as "fresh" neutrophils, develops form the immature ("Immat Neu"), pre-neutrophil ("Pre-Neu") stages [15,16] and migrates to different tissues (mainly the lung and the liver), undergoing "aging" within hours/days, both under homeostatic and inflammatory conditions, gradually releasing their protein content, to cope with the different tissue requirements, and being finally eliminated in the spleen. In homeostatic conditions a fraction of "aged neutrophils" returns to BM, they are phagocytosed by local macrophages which, in turn, instruct the niches to promote neutrophils egress [17,18]. This system is finely regulated also during systemic inflammation; macrophages, indeed phagocytose aged neutrophils that have reacted against pathogens or have responded to an inflammatory activation and, as a consequence, dampen neutrophil egress from the BM [17,18].

The egression from BM and the homing toward tissues is the result of the expression of two key molecules CXCR2 and CXCR4 (C-X-C-motif chemokine receptor 2 and 4), known to coordinate neutrophil plasticity and extravasation toward tissues [15,19]. CXCR4 anchors neutrophils to BM niches and supports granulocytes proliferation, while CXCR2 is involved in neutrophil activation via the engagement of the inflammasome [20]. In turn, its activation also promotes CXCR4 expression [21], thus establishing a loop able to control the activity of neutrophils during infections [19], where a continuous balance between fresh (CXCR4 positive) and aged (CXCR2 positive) neutrophils is critical.

Whether this scenario is relevant during diseases associated with chronic inflammation such as metabolic disorders is poorly explored. To address this aspect, we profiled the immunoinflammatory and the metabolic response of an experimental model of constitutively aged neutrophils (MRP8/CRE+ crossed to CXCR4flox/flox mice, herein: CXCR4fl/flCre+) and of a model of constitutively "fresh" neutrophils (MRP8/CRE+ crossed to CXCR2flox/flox mice, herein: CXCR2fl/flCre+), which developed metabolic syndrome and obesity following chronic HFD feeding [19,22].

Our data indicate not only that metabolic alterations induced by HFD impact the balance and the homing of fresh and aged neutrophils but also that the presence of constitutively aged neutrophils contributes to insulin resistance, hepatic steatosis, inflammation and to the accumulation of inflammatory macrophages in VAT. On the contrary the presence of constitutively fresh neutrophils improves the response to insulin and limits hepatic steatosis. In humans, we observed that higher plasma levels of Cxcl12, which binds to CXCR4, correlated with visceral adiposity while higher levels of Cxcl1, the ligand of CXCR2, correlated with indexes of hepatic steatosis, adiposity and metabolic syndrome. Altogether these findings suggest that neutrophil aging might contribute to the inflammatory consequences of high fat diet induced metabolic disorders.

2. Methods

An extensive description of all experimental procedures and materials is provided in the Supplemental Material section.

2.1. Ethic statements

All animal procedures performed conform to the guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Ethical Committee (Progetto di Ricerca 5247B.52, Autorizzazione Ministeriale 594/2019-PR). Neutrophil count and neutrophil aging marker profiling were performed in samples of the PLIC study (Progressione delle Lesioni Intimali Carotidee) [23,24]. The study was approved by the Scientific Committee of the Università degli Studi di Milano (SEFAP/Pr.0003). Each subject signed the informed consent for the collection of blood samples and clinical data for research purpose prior to the enrolment in the study. The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.2. Mice experiments

In order to study the physiological effect of the neutrophil aging mediated by the CXCR4/2 axis on the development of metabolic alterations induced by HFD, we compared the gluco-metabolic profile of MRP8cre+/CXCR4flox/flox and MRP8cre+/CXCR2flox/flox mice. As the profile of each flox/flox model is similar to that of wild type mice (not shown), data are presented as compared to this group in agreement with other publications with the same experimental models [15,19].

Eight-week old mice were housed four per cages and kept in a temperature-controlled environment (20 ± 2 °C, 50 ± 5 % relative humidity) with a 12 h of light/12 h of dark cycle in an air-conditioned

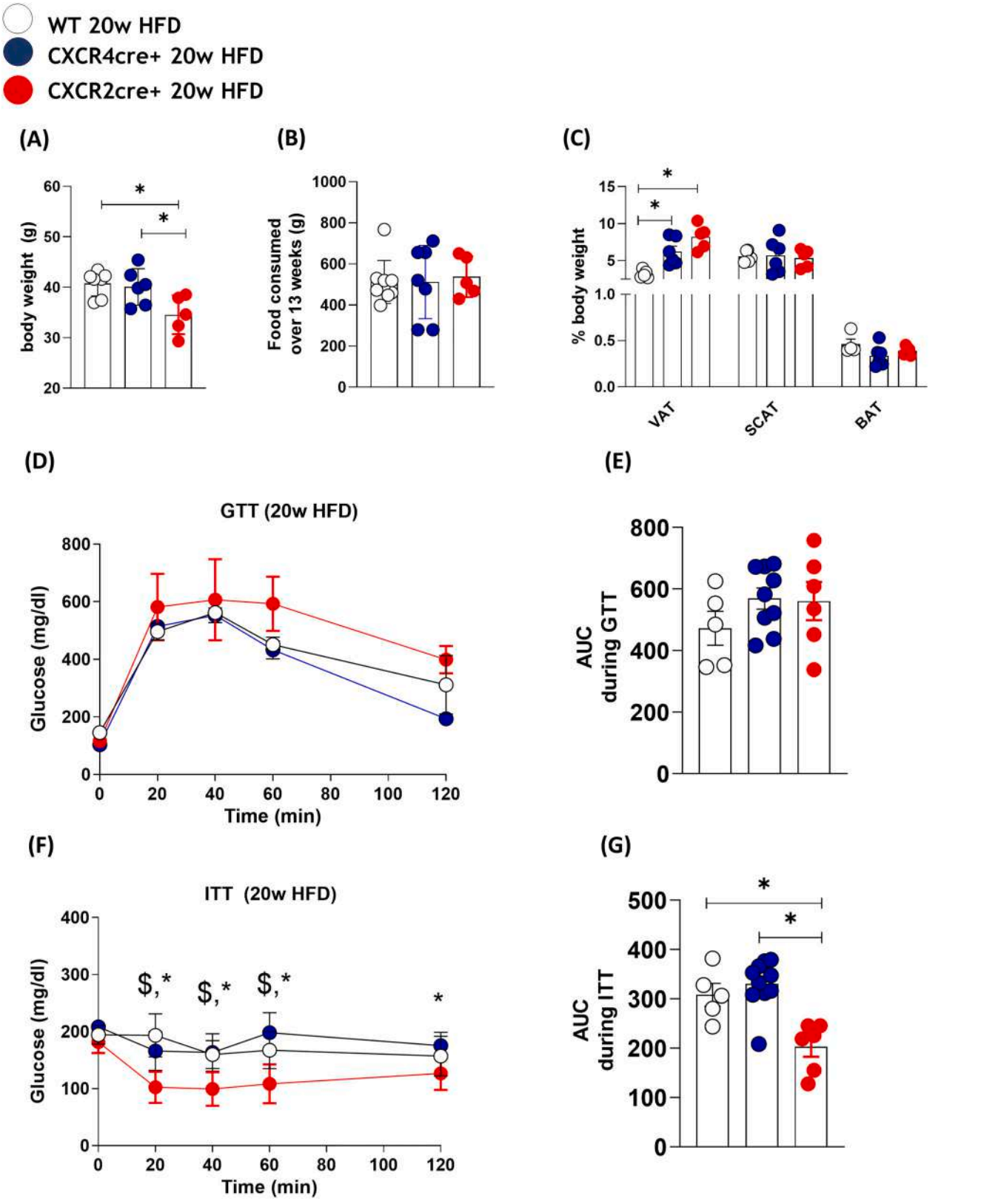

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Fig. 2. Preventing the aging of neutrophils reduces obesity and improves the sensitivity to insulin.
Wild-type (WT) (white dots), CXCR4fl/flCre+ (dark blue dots) and CXCR2fl/flCre+ (dark red dots) mice were sacrificed after 20 weeks of High Fat Diet (HFD) feeding. (A) Total body weight of the mice at sacrifice. Data are expressed as grams (g). (B) Total amount of food consumption. Data was registered over the first 13 weeks of HFD feeding and expressed as the sum of three determinations of the residual food left in the rack per week. Data are expressed as grams (g). (C) Weight of Visceral Adipose Tissue (VAT), Subcutaneous Adipose Tissue (SCAT) and inter-scapular Brown Adipose Tissue (BAT). Data are expressed as percentage (%) out of the total body weight of each mouse sacrificed. For Panels (A–C) N = 7 WT vs N = 6 CXCR4fl/flCre+ vs *N* = 5 CXCR2fl/flCre+. (D) Glucose Tolerance Test (GTT) at 20 weeks HFD. Glycaemia was measured before i.p. glucose injection ("0") and after 20, 40, 60 and 120 min. N = 5 WT; *N* = 9 CXCR4fl/flCre+; N = 6 CXCR2fl/flCre+. (E) Glucose changes during the GTT (data are expressed Area Under the Curve (AUC)). (F) Insulin Tolerance Test (ITT) at 20 weeks HFD. Glycaemia was measured before i.p. insulin injection ("0") and after 20, 40, 60 and 120 min. (G) Glucose changes during the ITT (data are expressed Area Under the Curve (AUC)). For Panels (D–G), *N* = 5 WT; *N* = 10 CXCR4fl/flCre+; N = 5 CXCR2fl/flCre+ were compared. "$" indicates significant difference between WT and CXCR2fl/flCre+; "*" indicates significant difference between CXCR4fl/flCre+ and CXCR2fl/flCre+. For all the panels, "*" indicates significant difference with p value <0.05 (Kruskal-Wallis test). Grubb's test for the detection of statistical outliers was performed before the Kruskal-Wallis test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

room and free access to food and water.

In the quest to study the impact of neutrophil aging on metabolic alterations induced by dietary fats, starting from 8 weeks of age, we fed either a HFD or a standard fat diet (SFD) male littermates' mice from the three experimental groups. Food intake and weight gain were measured thrice weekly (Monday, Wednesday and Friday).

Mice had free access to food and water, except when fasting was required for procedures. After sacrifice and the explant of one leg, muscle and fibrous tissues were removed from the femur; then, both femur ends were cut with sharp scissors and the BM content was flushed out with PBS, 5 % BSA solution using 25G 1mL syringe; after filtering the cell suspension with a 70 μm cells strainer and after removal of the excess of red blood cells, BM was finally stained for immunophenotyping. The preparation other organs for immunophenotyping as like as the other methodological aspects, including dietary compositions are detailed in Supplemental Material.

3. Results

3.1. High fat diet feeding increases aged neutrophils in liver and adipose tissue

The effect of HFD diet on neutrophil plasticity in BM, in the circulation, in peripheral and in metabolic tissues was tested in mice fed 20 weeks HFD versus mice fed 20 weeks SFD. As expected, HFD feeding resulted in insulin resistance and accumulation of adiposity versus SFD feeding (Supplemental Fig. 1A, B), in spite of a similar food intake (Supplemental Fig. 1C). The amount of GMPs and of Mat Neu was increased in the BM of HFD fed mice versus SFD fed mice (Fig. 1A–C). Interestingly, HFD also reduced the expression of Ly6G, CD11b and CXCR4, increased that of CD62L, but it did not modify the expression of CXCR2 on neutrophils residing in BM versus SFD (Fig. 1D); These changes are suggestive for the development of "fresh" neutrophils in the BM [16,17]. HFD feeding, also increased the amount of total myeloid CD11b+ cells and Ly6C+ monocytes in the BM (Supplemental Fig. 1D, E) and increased their surface expression of CD62L, while reduced that of CXCR4 versus SFD feeding (Supplemental Fig. 1F). These changes in BM subsets translated into an increased number of circulating CD11b+ myeloid cells (Supplemental Fig. 1G), both neutrophils (Fig. 1E), and Ly6C+ monocytes (Supplemental Fig. 1H), while HFD feeding did not affect the counts of other non-myeloid cells subsets (Supplemental Fig. 1I–K). Circulating neutrophils in HFD fed mice, although increased in number, expressed significantly less CD11b, CD62L, and CXCR4 on their membrane versus neutrophils in SFD fed mice (Fig. 1F, G). These findings suggest that HFD feeding accelerates neutrophil aging in the circulation, an effect mirrored by the presence of more fresh neutrophils in the BM.

To further address whether HFD feeding also induces different distribution of neutrophils in peripheral tissues, we next quantified neutrophils in classical disposal sites (spleen and lung) as well as in tissues responsive to HFD feeding, such as the liver and VAT. As expected, neutrophil counts increased in the spleen in mice fed HFD versus those fed SFD (Fig. 1H), together with Ly6C+ monocytes and other non-myeloid cell subsets (Supplemental Fig. 1N–Q), in line with the inflammatory response associated to HFD feeding. However, the largest increase of neutrophils was observed in the liver and in VAT (six and ten folds respectively versus SFD) (Supplemental Fig. 1M), and the expression of CD62L increased on the surface of splenic neutrophils but, more relevantly, in that of neutrophils infiltrating the liver in mice fed HFD (Fig. 1I).

Given that splenic neutrophils largely mirror the profile of their circulating counterpart, the latter observation suggests that HFD could increase neutrophil aging in the circulating-liver-VAT axis under dysmetabolic conditions. To further explore this aspect, we characterized neutrophil profile with a focus on the expression of a specific marker of neutrophil aging, CXCR2, and of neutrophil freshness, CXCR4 (Supplemental Fig. 2). Similarly to the changes in CD62L expression, also the amount of CXCR2 positive ("aged") neutrophils increased in the spleen, in the liver and in VAT of mice fed HFD compared to that of mice fed SFD (Fig. 1J, K). Notably, CXCR4 positive ("fresh") neutrophils, were also reduced in the spleen (Fig. 1L), but increased in the liver (Fig. 1M), while no differences were observed in lung and in VAT of mice fed HFD as compared to mice fed SFD (Fig. 1L, M).

3.2. Preventing the aging neutrophils reduces obesity and improves the sensitivity to insulin

We next investigated the impact of the 20 weeks HFD feeding on glucose response and development of obesity in an experimental models of constitutively fresh (crossing MRP8/CRE+ to CXCR2flox/flox mice; herein CXCR2fl/flCre+) or aged neutrophils (generated by crossing MRP8/CRE+ to CXCR4flox/flox mice; herein CXCR4fl/flCre+) [19,22]. These two models were compared to WT mice.

After 20 weeks of HFD feeding, GMPs increased in the BM of CXCR2fl/flCre+ mice (Supplemental Fig. 3A), while BM pre-Neu, Immat-Neu and Mat-Neu (Supplemental Fig. 3A–D), as like as circulating neutrophils, did not change versus WT mice after 20 weeks of HFD (Supplemental Fig. 3E). After 20 weeks of HFD feeding, only Mat-Neu increased in the BM of CXCR4fl/flCre+ mice, GMPs were reduced versus CXCR2fl/flCre+ mice, while the amount of Pre-Neu, Immat-Neu in the BM was comparable (Supplemental Fig. 3B–D). The increased number of Mat-Neu neutrophils in the BM of CXCR4fl/flCre+ mice was mirrored by a robust increase of circulating neutrophils versus WT and CXCR2fl/flCre+ mice (Supplemental Fig. 3E).

Of note, after 20 weeks of SFD feeding, despite significant neutrophilia (Supplemental Fig. 3E), CXCR4fl/flCre+ mice did not present higher amount of Mat-Neu in BM (Supplemental Fig. 3D) and, on the opposite, CXCR2fl/flCre+ mice did no longer display increased GMP content (Supplemental Fig. 3A). These findings suggest that HFD differently impact BM neutrophils according to their aging status. This mechanism appears to also affect the infiltration and activation of neutrophils in the periphery. While CXCR4fl/flCre+ neutrophils display less CD62L membrane expression, both on SFD or HFD, and increased CD11b expression when mice were fed HFD, CXCR2fl/flCre+

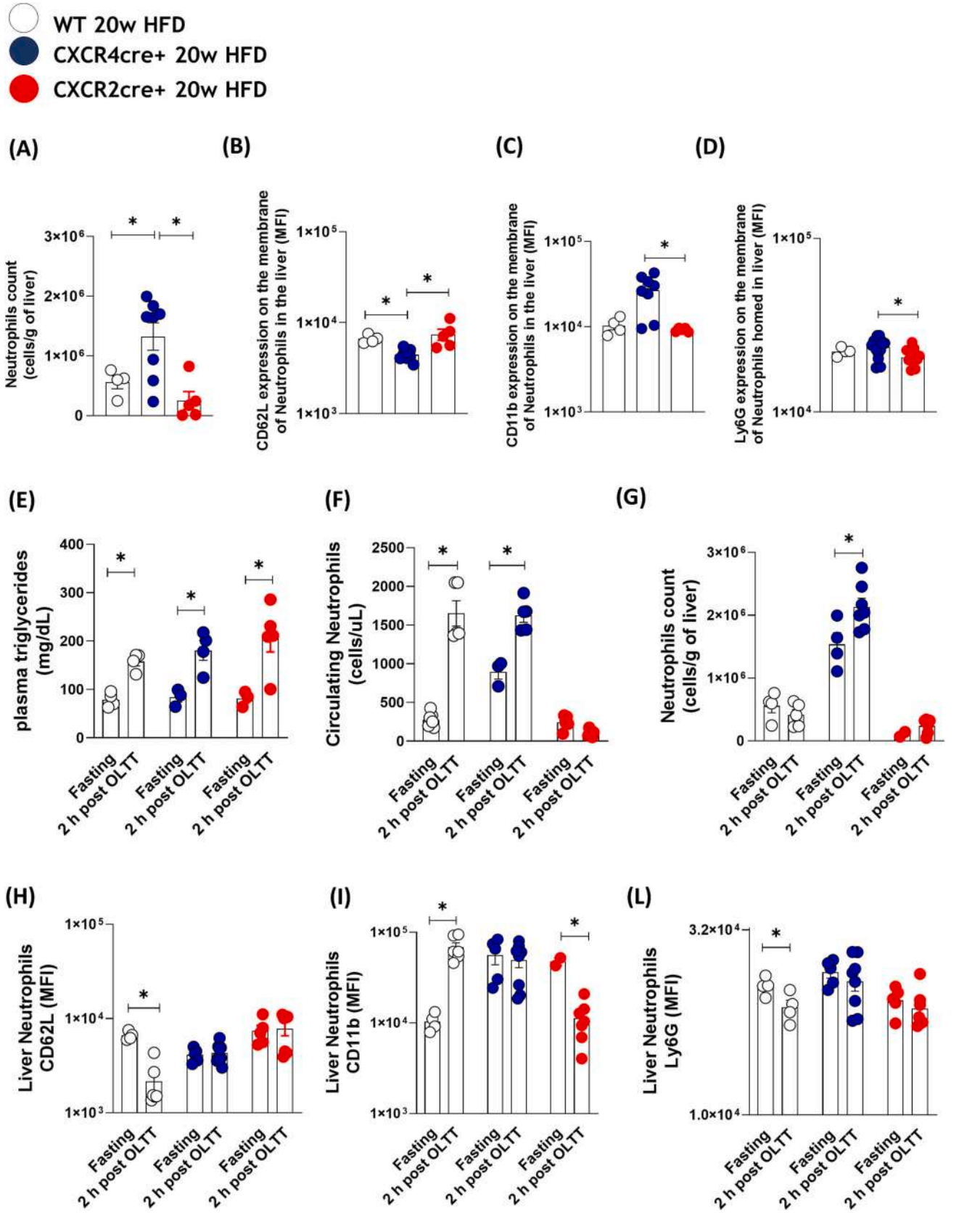

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Fig. 3. High Fat Diet primes aged, but not fresh neutrophils, to infiltrate in the liver.
Wild-type (WT) (white dots), CXCR4fl/flCre+ (dark blue dots) and CXCR2fl/flCre+ (dark red dots) mice were sacrificed after 20 weeks of High Fat Diet (HFD) feeding. (A) Absolute quantity of total neutrophils in liver (data expressed as number of cells per gram (g) of liver processed for the analysis). (B–D) Membrane expression of CD62L (B), CD11b (C) and Ly6G (D) on the total number of neutrophils in liver. For panels B to D data are expressed as Mean Fluorescence Intensity (MFI). (E) Triglycerides levels measured in the plasma before (fasting) and 2 h post OLTT (data are expressed as mg/dL). (F) Absolute quantity of neutrophils in circulation before (fasting) and 2 h post OLTT (data expressed as number of cells per microliter (μL) of processed blood for Flow-Cytometry analysis). (G) Absolute quantity of neutrophils in liver before (fasting) and 2 h post OLTT (data expressed as number of cells per gram (g) of liver processed for the analysis). (H–L) Membrane expression of CD62L (H), CD11b (I) and Ly6G (L), on the total quantity of neutrophils in the liver before (fasting) and 2 h post OLTT. For panels H to L data are expressed as Mean Fluorescence Intensity (MFI). For Panels (A–D), $N = 5$ WT; $N = 9$ CXCR4fl/flCre+; N = 5 CXCR2fl/flCre+ were compared; Panels (E–L) include $N = 4$ WT fasting, N = 5 WT post OLTT, N = 5 CXCR4fl/flCre+ fasting, $N = 7$ CXCR4fl/flCre+ post OLTT, $N = 3$–5 CXCR2fl/flCre+ fasting, $N = 6$–7 CXCR2fl/flCre+ post OLTT. For all the panels, "*" indicates significant difference with p value <0.05 (Kruskal-Wallis test). Grubb's test for the detection of statistical outliers was performed before the Kruskal-Wallis test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neutrophils present a different trend in Ly6G and CD11b expression when mice were fed either SFD or HFD (Supplemental Fig. 3F, G). No major differences were observed in other immune cell subsets in the blood of CXCR2fl/flCre+ and CXCR4fl/flCre+ mice after 20 weeks HFD, apart from increased Ly6C+ monocyte counts in the latter group both on HFD and SFD (Supplemental Fig. 4A–H).

On these premises, we next analyzed how neutrophil aging might impact the progression of HFD induced metabolic disturbances. To this aim, the development of metabolic syndrome and obesity was investigated in CXCR2fl/flCre+ mice, CXCR4fl/flCre+ and WT mice fed HFD for 20 weeks.

CXCR2fl/flCre+ mice gained less body weight compared to CXCR4fl/flCre+ and WT mice (Fig. 2A), despite comparable daily food intake (Fig. 2B). Interestingly, VAT accumulation (as % of body weight) was significantly increased in CXCR2fl/flCre+ mice and to a minor extent in CXCR4fl/flCre+ mice compared to WT mice (Fig. 2C) while this was not the case for SCAT and interscapular BAT (Fig. 2C). CXCR4fl/flCre+ mice also presented splenomegaly (Supplemental Fig. 4I) while the weight of liver and lung as well as the length of tibia and femur (long axes) were comparable between the three experimental groups (Supplemental Fig. 4I, J).

To then assess whether the impact of the aging of neutrophils on the distribution of adiposity relates to the impairment of glucose metabolism, we profiled glucose tolerance (intraperitoneal injection of 2 mg of glucose per grams of body weight "GTT"; see Methods) and insulin sensitivity (intraperitoneal injection of 1 mU insulin per gram of body weight "ITT"; see Methods) in HFD fed mice. Fasting plasma glucose levels were comparable between the three experimental groups and a similar increase in glycemia after glucose injection was observed (Fig. 2D, E). Of note CXCR2fl/flCre+ mice presented an improved insulin response compared to CXCR4fl/flCre+ and WT mice (Fig. 2F, G).

We next profiled the liver proteome to investigate whether these findings might highlight a different contribution of aged vs fresh neutrophils on systemic metabolism. HFD fed CXCR2fl/flCre+, CXCR4fl/flCre+ or WT mice presented a different hepatic proteomic signature (Supplemental Fig. 5A) and the aging of neutrophils overall resulted into an enrichment of proteins involved in lipids and fatty acid metabolism (see Supplemental Fig. 5B). Indeed, liver from CXCR4fl/flCre+ mice presented higher abundance of proteins involved in oxidative metabolic pathways as compared to WT (Supplemental Fig. 5C) and CXCR2fl/flCre+ mice (Supplemental Fig. 5D). Moreover, the proteins belonging to these pathways were less abundant in the liver of CXCR2fl/flCre+ compared to WT mice (Supplemental Fig. 5E). By hierarchical clustering of most significant proteins involved in these pathways, we found that the metabolism of FAs, beta-oxidation and tri-carboxylic acids cycle (TCA) were overall downregulated in CXCR2fl/flCre+ as compared to CXCR4fl/flCre+ mice (Supplemental Fig. 5F, G). In agreement with these findings, the liver from CXCR4fl/flCre+ mice presented increased steatosis compared to CXCR2fl/flCre+ and WT mice after 20 weeks of HFD feeding (Supplemental Fig. 6A, B). Moreover, also the presence of fibrosis (Supplemental Fig. 6C, D) and plasma aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio were increased in CXCR4fl/flCre+ mice compared to CXCR2fl/flCre+ and WT mice (Supplemental Fig. 6E).

Therefore, neutrophil aging might promote increased lipid accumulation in the liver, coupled to the alteration in oxidative metabolism, while constitutive neutrophil freshness contributes to maintain a proper insulin response together with a preserved liver function.

3.3. High fat diet primes aged, but not fresh neutrophils, to infiltrate in the liver

We next analyzed whether the changes in the metabolic profiles of CXCR4fl/flCre+ and CXCR2fl/flCre+ mice after 20 weeks HFD feeding could be the consequence of a different ability of fresh or aged neutrophils to patrol the liver.

We observed more neutrophils in the liver of CXCR4fl/flCre+ mice compared to that of CXCR2fl/flCre+ and WT mice after 20 weeks of HFD feeding (Fig. 3A), which also expressed less CD62L but more CD11b on their cell surface (Fig. 3B, C). CXCR4fl/fl Cre+ neutrophils homed in the liver presented also a higher membrane expression of Ly6G, marking increased homing capacity [25], compared to CXCR2fl/flCre+ neutrophils (Fig. 3D).

To then study whether the chronic exposure to dietary lipids primes aged, but not fresh neutrophils, to infiltrate the liver in acute conditions, we profiled neutrophil behavior following oral lipid tolerance test (OLTT) in CXCR4fl/flCre+, CXCR2fl/flCre+ and WT mice. 2 h post OLTT, although plasma triglyceride levels increased in all the experimental groups (Fig. 3E), neutrophil blood levels rapidly increased in CXCR4fl/flCre+, in WT but not in CXCR2fl/flCre+ mice fed HFD (Fig. 3F). These changes in the circulation were mainly paralleled by an increase in liver neutrophil counts only in CXCR4fl/flCre+, but nor in WT neither in CXCR2fl/flCre+ mice (Fig. 3G). Of note, while WT neutrophils reduced the membrane expression of CD62L, Ly6G but increased that of CD11b 2 h post OLTT, CXCR4fl/flCre+ neutrophils retained the expression of both markers compared to the fasting state (Fig. 3H, L). On the contrary, only a reduction in CD11b was observed in CXCR2fl/flCre+ neutrophils (Fig. 3H, L). This phenotype was characteristic of mice fed HFD; indeed, these differences were not appreciated in SFD fed mice (Supplemental Figs. 7A–D and 8A–F).

This latter information suggests that aged neutrophils accumulate to a larger extent in the liver under metabolic-induced inflammatory conditions while this is not the case for fresh neutrophils and, to further confirm this possibility, we profiled liver proteome investigating the functional pathways associated with neutrophil responses. Notably, we notice that the abundance of proteins involved in the formation of neutrophils extracellular traps (NETs) ("NETosis", that is the spouting of their chromatin, radical oxygen species (ROS), enzymes and proteins in response to pathogens/inflammatory stimuli), was upregulated in liver samples from both CXCR4fl/flCre+ and WT compared to CXCR2fl/flCre+ mice (hierarchical clustering in Fig. 4A, B). By contrast, samples from CXCR2fl/flCre+ mice compared to WT displayed higher abundance of proteins involved in the activation of the G-protein coupled N-formyl-methionyl-leucyl-phenylalanine receptor (fMLPR), which is

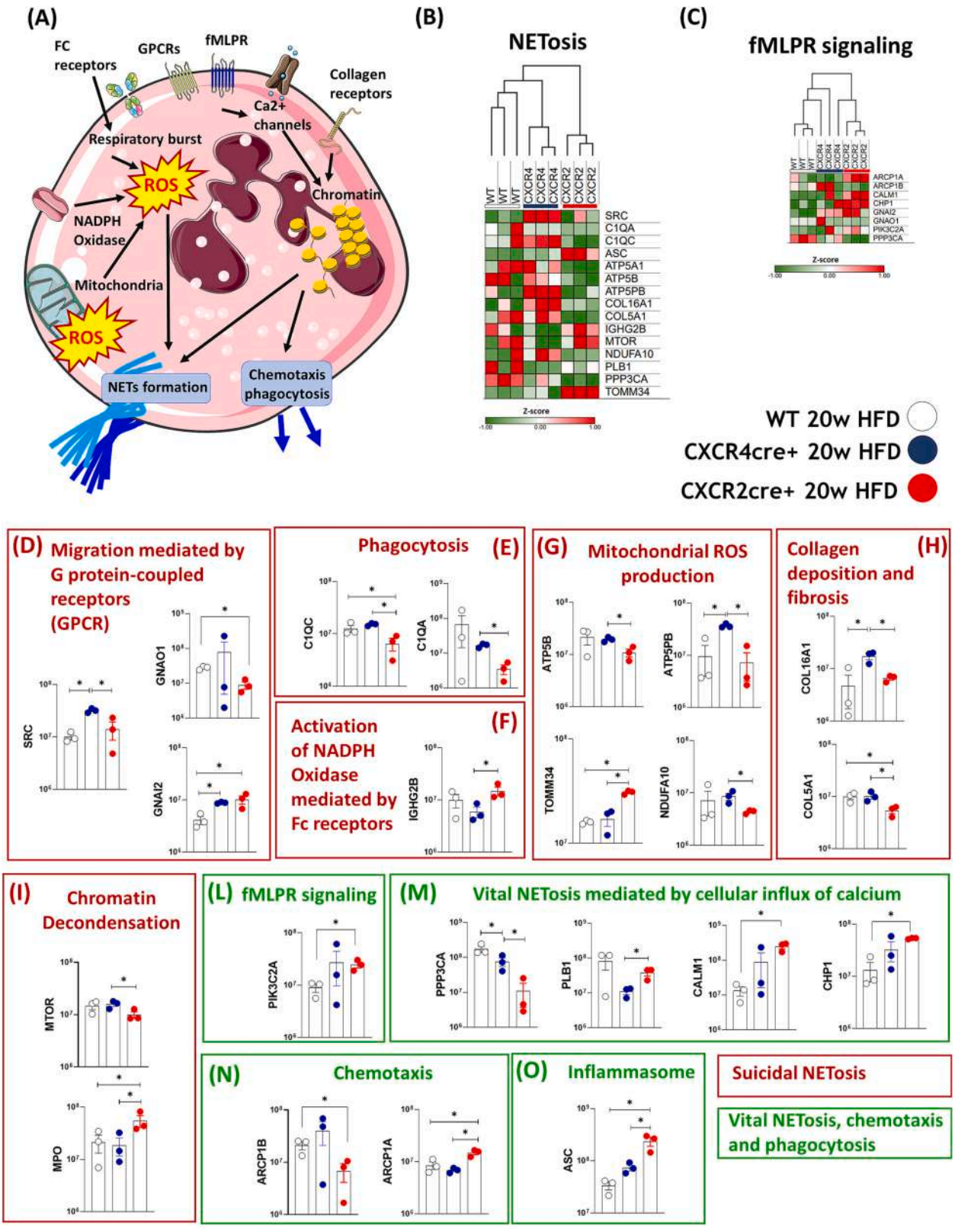

Figure 4.

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Fig. 4. High Fat Diet alters the cellular activation of aged, but not fresh neutrophils.
Liver from N = 3 WT, N = 3 CXCR4fl/flCre+ and N = 3 CXCR2fl/flCre+ mice were pooled up to 20 mg and homogenized for the proteomics analyses. (A) Graphical resume of the principal receptors and mechanisms involved for the formation of neutrophils extracellular traps (NETs). ROS = reactive oxygen species; NADPH = Nicotinamide adenine dinucleotide phosphate; GPCRs = G protein-coupled receptors; fMLPR = G-protein coupled N-formyl-methionyl-leucyl-phenylalanine receptor; Ca2+ = calcium ion. (B, C) Heatmaps and hierarchical clustering of the hepatic proteome of CXCR4fl/flCre+, CXCR2fl/flCre+ and WT mice showing relative protein expression values (*Z*-score transformed LFQ protein intensities) for NET signaling (C) and fMLPR signaling (D); data are presented as triplicate for mouse model. (D–O) Absolute abundances of the proteins indicated in that are involved either in suicidal NETosis (red squares) or in vital NETosis, chemotaxis and phagocytosis (green squares). For all the panels, "*" indicates significant difference with p value <0.05 (Kruskal-Wallis test). Grubb's test for the detection of statistical outliers was performed before the Kruskal-Wallis test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crucial for neutrophils chemotaxis (Fig. 4A, C). In detail, in samples from CXCR4fl/flCre+ mice compared to both CXCR2fl/flCre+ and WT mice we appreciated the enrichment of SRC, a component of the CXCR2-mediated aging signaling for migration toward inflammatory stimuli [26] (Fig. 4D), and of proteins actively involved in phagocytosis (C1QC, C1QA) (Fig. 4E). Also, samples from CXCR4fl/flCre+ mice compared to CXCR2fl/flCre+ presented different enrichment in proteins involved in mitochondrial ROS production and NAPDH Oxidase activity, with ATP5B, ATP5PB, NDUFA5 resulting more enriched while IGH2B and TOMM34 reduced (Fig. 4F, G). Also, in specimens from CXCR4fl/flCre+ compared to CXCR2fl/flCre+ mice we found an increased expression of proteins involved in collagen deposition or fibrosis (COL5A1, COL16A1) (Fig. 4H) and of mTOR (Fig. 4I), which is engaged in the decondensation of chromatin, the final step for NETosis (Fig. 4A). By contrast, samples from CXCR2fl/flCre+ mice compared to WT displayed higher abundance of the Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha polypeptide (PIK3C2A) (Fig. 4L), a downstream effector of the fMLPR signaling, together with an enrichment of proteins involved in the cellular calcium influx (CHP1, CALM1, PLB1) and of proteins crucial for neutrophils actin remodeling and chemotaxis (ARCP1A) (Fig. 4M, N). These findings, coupled with a higher abundance of ASC, a key coordinator of inflammasome assembly with pro-resolutive potential [27] (Fig. 4O), and of myeloperoxidase (MPO) in the liver of CXCR2fl/flCre+ compared to both CXCR4fl/flCre+ and WT (Fig. 4I), suggest the possibility that aged neutrophils are more prone to infiltrate in the liver and to induce liver damage following "suicidal NETosis" [28], while fresh neutrophils promote proresolutive patrolling ("vital NETosis") of the liver even during HFD induced inflammatory conditions.

3.4. Aged, but not fresh neutrophils, promote inflammation and alter cell immune architecture in visceral adipose tissue during obesity

Considering these findings in the liver, we analyzed whether the aging of neutrophils affects their infiltration in VAT and how it relates to the development of obesity following 20 weeks HFD feeding.

Both CXCR4fl/flCre+ and CXCR2fl/flCre+ mice presented increased quantity of VAT, but no hypertrophy of the adipocytes (as mean adipocytes area by histochemistry) versus WT (Fig. 2C, Supplemental Fig. 9A, B) and we next investigated whether this could depend on a different ability of aged or fresh neutrophils to infiltrate the adipose tissue during the development of obesity. CXCR4fl/flCre+ neutrophils infiltrated VAT to a larger extent versus the other groups (Fig. 5A), expressing more CD62L, Ly6G, but not CD11b, on their membrane (Fig. 5B–D). The increased infiltration of aged neutrophils associated with the reduction of total leukocytes, including T and B lymphocytes in the VAT from CXCR4fl/flCre+ mice (Fig. 5E–G).

This phenotype was characteristic of mice fed HFD as it was not observed in SFD fed mice (Supplemental Fig. 9C–E and F–I) suggesting, similar to what observed in the liver, that the impact of metabolic impairment on neutrophils is different according to their aging status.

Furthermore, Ly6C+ monocytes and macrophage were also less in the VAT from CXCR4fl/flCre+ mice (Fig. 5H, I) and a detailed analysis of macrophage subsets in VAT (Supplemental Fig. 10A) showed an increased amount of pro-inflammatory macrophages (CD11c+) in CXCR4fl/flCre+ mice versus CXCR2fl/flCre+ and WT mice (Fig. 5L). By contrast, the accumulation of proresolutive macrophages (CD206+) was unchanged (Fig. 5M).

Finally, different expression profile of multiple inflammatory chemokines (Cxcl11, Cxcl2, Ccl3, Ccl4) and molecules involved in macrophages/neutrophils interaction (Il23, Il17, Ptx3) characterized the VAT from CXCR4fl/flCre+, compared to that of CXCR2fl/flCre+ or WT mice after 20 weeks HFD feeding (Fig. 5N). Notably, in VAT from CXCR2fl/flCre+ mice only the expression of MPO was different when compared to WT (Fig. 5N). Neutrophils NETosis, marked as Histone2B and elastase content, was also increased only in VAT from CXCR4fl/flCre+ mice (Supplemental Fig. 10B–E).

Together these findings indicate that neutrophil aging influences the immune cell architecture of visceral adiposity supporting the development of a pro-inflammatory environment.

3.5. Markers of neutrophils aging marks the development of ectopic adiposity and metabolic syndrome in human

To finally translate this evidence in humans, we quantified blood neutrophils in 188 lean subjects (body mass index, BMI < 25 kg/m^2) without metabolic syndrome (MetS) ("BMI−/MetS−"), in 430 overweight/obese subjects (BMI 25–30 kg/m^2) without MetS ("BMI+/MetS−") and in 84 overweight/obese subjects with MetS ("BMI+/MetS+") (Table 1, Supplemental Table 1).

Blood neutrophils increased in BMI+/MetS−, but not in BMI+/MetS+, versus BMI−/MetS− (Fig. 6A, Table 1). To rule out that this trend is consequence of the increase of other immune cells in the hematocrit formula of the BMI+/MetS+ group (Supplemental Table 1), and to relate this observation with the aging of neutrophils, we evaluated plasma levels of Cxcl1 (a CXCR2 ligand which is produced by neutrophils to promote "aging") and of Cxcl12 (ligand of CXCR4, produced by stromal cells to anchor neutrophils to BM niches and to support granulocytes proliferation) in the three metabolic phenotypes.

Although no differences in Cxcl1 plasma levels were observed between the different groups- (Supplemental Fig. 11A–C) they were directly correlated with neutrophil count in BMI+/MetS+ (rho = 0.324, p = 0.003) and BMI+/MetS− (rho = 0.113, p = 0.020) subjects but not in BMI−/MetS− subjects (rho = −0.092, p = 0.213) (Fig. 6B). By contrast, the Cxcl12 plasma expression increased in BMI+/MetS+ subjects compared to BMI+/MetS− and BMI−/MetS− subjects (Supplemental Fig. 11A–C) and it directly correlated with neutrophils count only in BMI+/MetS+ subjects (rho = 0.233, p = 0.033) (Fig. 6B). Finally, the expression of Cxcl1 was associated with both abdominal fat percentage and Fatty Liver Index (FLI) (marker of visceral adiposity and surrogate score of hepatic steatosis respectively, and both increased in obesity and MetS (Supplemental Fig. 11D, E and Table 1), only in BMI+/MetS− (Fig. 6C). Vice versa, the expression of Cxcl12 was associated with abdominal fat percentage, but not with FLI, both in BMI+/MetS− and BMI+/MetS+ (Fig. 6D). Finally, these correlations were independent from neutrophils count, which correlated with FLI, but not with abdominal fat percentage, in all the three groups.

All these associations were not explained by age, or differences in gender or differences in pharmacological therapies, indicating that Cxcl1 and Cxcl12 appears as markers of the aging of neutrophils

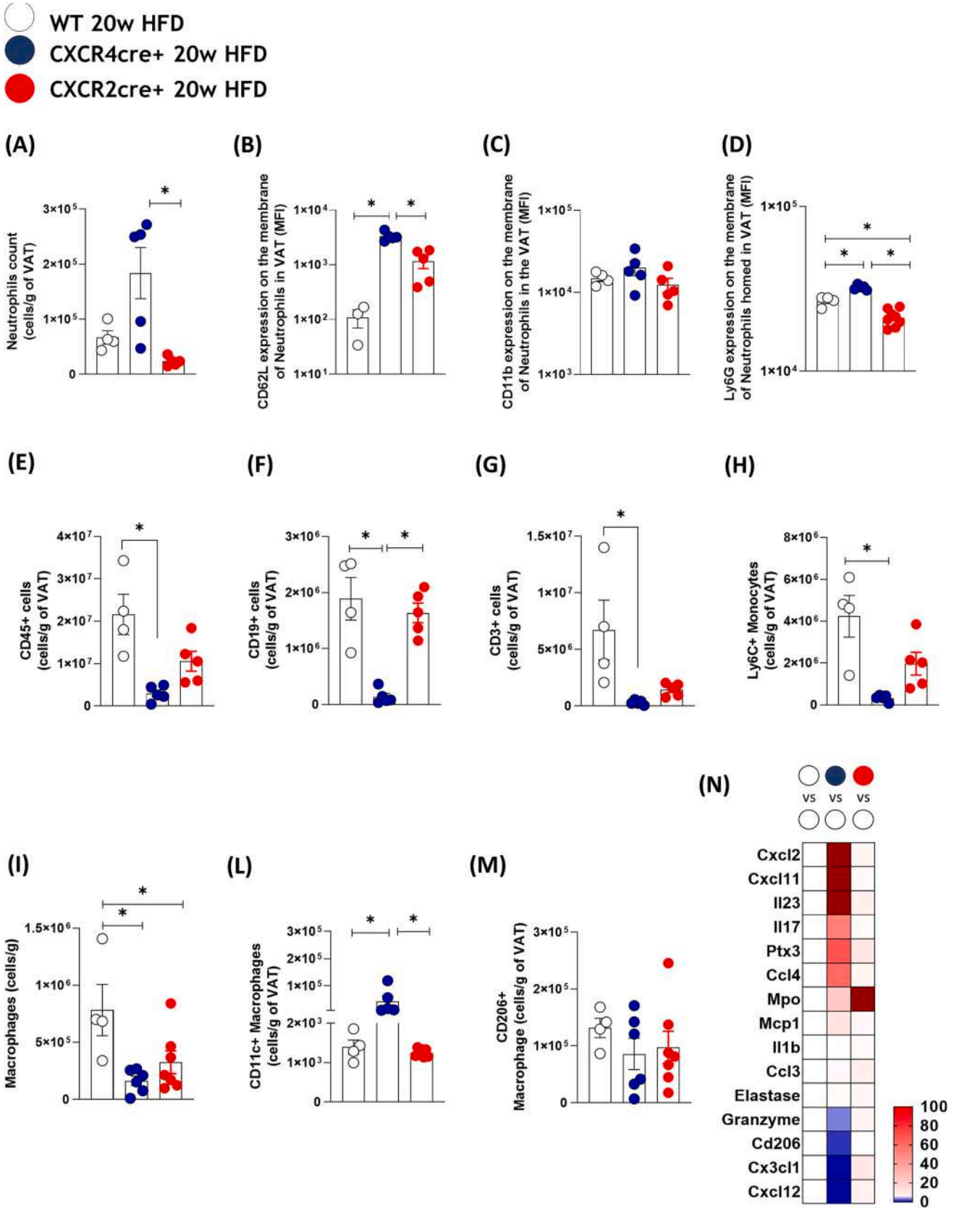

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Fig. 5. Aged, but not fresh neutrophils, promote inflammation and alter cell immune architecture in visceral adipose tissue during obesity.
Wild-type (WT) (white dots), CXCR4fl/flCre+ (dark blue dots) and CXCR2fl/flCre+ (dark red dots) mice were sacrificed after 20 weeks of High Fat Diet (HFD) feeding. (A) Absolute quantity of neutrophils in VAT (data expressed as number of cells per gram (g) of VAT processed for the analysis). (B–D) Membrane expression of CD62L (B), CD11b (C) and Ly6G (D), on the total quantity of neutrophils in the VAT. For panels B to D data are expressed as Mean Fluorescence Intensity (MFI). (E–M) Absolute quantity of CD45+ leukocytes (E), CD19+ B cells (F), CD3+ T lymphocytes (G), Ly6C+ monocytes (H), total macrophages (I), CD11c+ inflammatory macrophages (L) and CD206+ macrophages (M) in the VAT. For panels E to M data expressed as number of cells per gram (g) of VAT processed for the analysis. For all the panels, "*" indicates significant difference with p value <0.05 (Mann-Whitney *U* test). Grubb's test for the detection of statistical outliers was performed before the Mann-Whitney U test. (N) Heatmap showing expression of principal inflammatory genes and chemokines in the VAT of CXCR4fl/flCre+, CXCR2fl/flCre+ and WT. Data are expressed as log2Fold of changes ($2^{-\Delta\Delta T}$) in the expression of each gene in each group setting WT as a reference. For Panels (A–M) $N = 5$ WT; N = 5–7 CXCR4fl/flCre+; N = 5–7 CXCR2fl/flCre+ are compared. For Panel N, gene expression was compared in the VAT of N = 5 WT, N = 5 CXCR4fl/flCre+ and N = 5 CXCR2fl/flCre+. "*" indicates significant difference with *p* value <0.05 (Kruskal-Wallis test). Grubb's test for the detection of statistical outliers was performed before the Kruskal-Wallis test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Description of the population according to metabolic syndrome and adiposity.
Table reports the clinical history, the metabolic parameters and the markers of adiposity of the three groups. For linear variables, median and interquartile ranges (in brackets) are reported. "*" indicates $P < 0.05$ between "BMI−/MetS−" vs "BMI+/MetS−"; "**" indicates P < 0.05 between "BMI−/MetS−" vs "BMI+/MetS+"; "$" indicates P < 0.05 between "BMI+/MetS−" vs "BMI+/MetS+".

	BMI−/MetS− ($n = 188$)	BMI+/MetS− (n = 430)	BMI+/MetS+ (n = 84)
Age (y-old) (mean, IQR)	66 (59–71)	67 (62–71) *	65 (59–71) $
Gender (n, women)	149	282	37
BMI (kg/m^2) (mean, IQR)	22.8 (21.5–24.1)	28.7 (26.5–30.8) *	28.4 (25.7–31.7) **
Waist circumference (cm) (mean, IQR)	80 (75–86)	97 (91–102) *	97 (90–105) **
Abdominal fat mass (%) (mean, IQR)	39.5 (32.9–44.0)	50.6 (45.4–55.3) *	49.4 (43.2–55.6) **
Systolic blood pressure (mm Hg) (mean, IQR)	120 (116–130)	130 (120–140) *	130 (120–145) **
Diastolic blood pressure (mm Hg) (mean, IQR)	80 (70–80)	80 (70–85) *	80 (80–90) **,$
Anti-hypertensive users (n, yes)	48	222	40
Fasting glucose (mg/dL) (mean, IQR)	88.0 (82.0–94.0)	93.0 (86.0–102.0) *	97.5 (90.0–108.7) **,$
Glucose lowering treatments users (n, yes)	3	29	11
Triglycerides (mg/dL) (mean, IQR)	80.0 (62.0–103.0)	95.0 (73.5–125.0) *	105.0 (74.0–140.7) **,$
HDL-C (mg/dL) (mean, IQR)	69.0 (57.0–81.0)	58.0 (51.0–70.0) *	54.0 (43.2–68.0) **,$
Lipid lowering treatments users (n, yes)	47	161	34
Fatty Liver Index (%) (mean, IQR)	11.20 (5.94–18.81)	48.64 (30.82–66.77) *	56.52 (26.65–76.08) **
Total leukocytes (cells * 10^3/μL) (mean, IQR)	5.56 (4.84–6.67)	6.24 (5.36–7.18) *	6.32 (5.40–7.43) **
Neutrophils (cells * 10^3/μL) (mean, IQR)	3.27 (2.60–3.83)	3.44 (2.81–4.16) *	3.50 (2.78–4.31)

independently of other factors associated with the development of obesity and metabolic syndrome.

4. Discussion

The aging of neutrophil starts already during medullary development and influences its trafficking, co-option, activation in peripheral tissues and their immune competency upon pathophysiological demands [16,17,19]. Our data indicate that accelerated aging, as it is observed during chronic exposure to HFD, contributes to the development of metabolic alterations. Notably, the metabolic consequences induced by HFD feeding were attenuated when the aging of neutrophil was prevented by programming a constitutive "fresh" neutrophil phenotype. Indeed, upon HFD feeding, mice with constitutively "aged" neutrophils present obesity, liver steatosis, markers of liver inflammation/damage and accumulation of pro-inflammatory macrophages in VAT and, on the opposite, mice with constitutively "fresh" neutrophils are protected toward obesity and the development of liver steatosis, insulin resistance and VAT inflammation. CXCR4fl/flCre+ and CXCR2fl/flCre+ mice presented similar accumulation of VAT which, given a comparable glucose tolerance, could mark the remodeling of the ectopic adiposity in CXCR2fl/flCre+ mice to compensate the insulin-resistance induced by HFD feeding.

Our findings rely on specific experimental models of neutrophil freshness or aging and go beyond data accumulated so far, where the impact of changes in specific proteins related to neutrophil function, including those involved in the migration and activation of neutrophils against pathogens (MPO [29], inflammasome [3], elastase [12], pentraxins [13] or nitric oxidase synthase 2 [10]), were tested on obesity and insulin-resistance. Indeed, our experimental models interfere selectively with the medullary egress and the tissue distribution of neutrophils [16] as neutrophils are not only released in large quantity from the BM when CXCR4 (physiologically expressed in pre-Neu, Immat-Neu and Mat-Neu [16]) is lacking, but they also age faster in the circulation, being less efficient to patrol peripheral tissues, and they accumulate more in the spleen. On the opposite, the specific deletion of CXCR2 (CXCR2fl/flCre+) renders neutrophils "fresh" which, although less abundantly released from BM, display a "constitutive effectiveness" to rapidly infiltrate in tissue in response to invading pathogens [19]. Hence, by either ablating CXCR4 or CXCR2, we were able to study, for the first time, a cell-intrinsic and non-redundant system actively coordinating the reactivity of neutrophils facing the development of metabolic and inflammatory consequences induced by HFD feeding. Our data indicate that 20 weeks HFD completely reverted the distribution of neutrophils over tissues in the CXCR4fl/flCre+ mice, as compared to the 20 weeks SFD feeding condition, with which lower quantities of medullary developmental aged neutrophil fractions, neutrophilia, splenomegaly and higher amounts of neutrophils homed in liver and in VAT were observed versus CXCR2fl/flCre+ animals. Several aspects could be responsible for this switch.

Firstly, HFD feeding significantly increased the amount of pre-Neu, Immat-Neu and Mat-Neu in the BM of CXCR4fl/flCre+ mice while only increased that of GMPs in BM of CXCR2fl/flCre+ animals. Hence, our data suggest that HFD induced systemic metabolic alterations, impact hematopoiesis favoring the release of aged while constraining that of fresh neutrophils from their medullary sites of production. Whether this is the consequence of local mediators that impact the medullary environment during HFD feeding (e.g. the adiposity in BM [5], local innervation [30], endothelial integrity [31,32] or the medullary macrophages [18]) requires to be further explored.

Secondly, neutrophilia persisted in CXCR4fl/flCre+ compared to

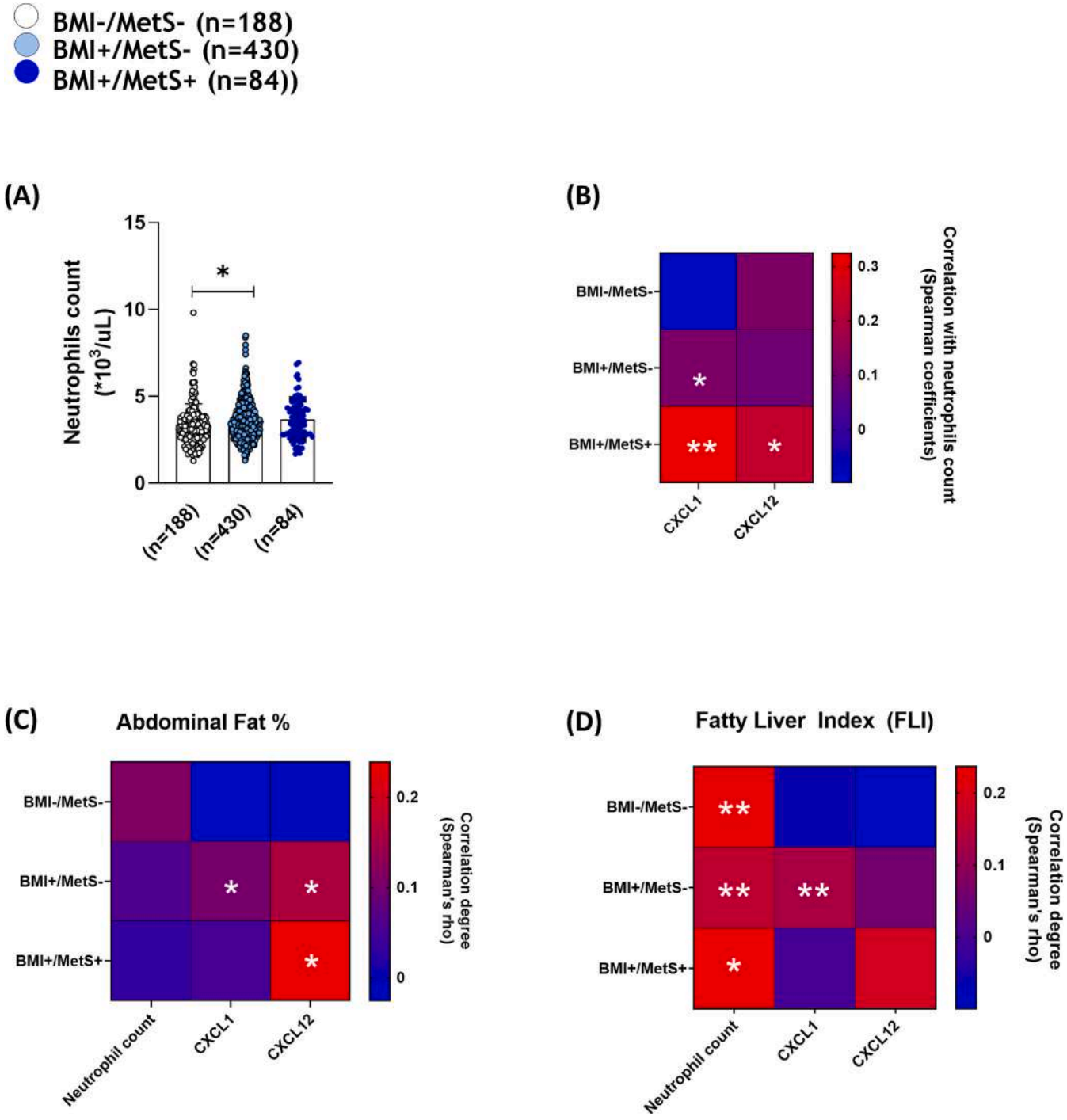

Fig. 6. Markers of neutrophils aging marks the development of ectopic adiposity and metabolic syndrome in humans.
(A) Blood neutrophils in subjects with BMI−/MetS− (n = 188; white dots), in subjects with BMI+/MetS− (n = 430; light blue dots) and in subjects with BMI+/MetS+ (n = 84; dark blue dots). Data are expressed in decimal scale $\times 10^3$ cells/microliter (μL) of blood processed for the hematocrit analysis. (B-D) Heatmap reporting degrees of univariate correlation between the three metabolic phenotypes (BMI−/MetS−; BMI+/MetS−; BMI+/MetS+) with Cxcl1 and Cxcl12 plasma levels (B), with abdominal fat % in each metabolic phenotype (C) and with Fatty Liver Index in each metabolic phenotype (D). Correlations are derived from non-parametric Spearman's analysis. Red intensity indicates positive correlation; blue intensity indicates negative correlation. Grubb's test for the detection of statistical outliers in the distribution of these parameters in the population was performed before the Spearman's correlation analysis. "*" indicates significant difference with p value <0.05 (Kruskal-Wallis test for (A) and non-parametric Spearman's test for B–D). Grubb's test for the detection of statistical outliers was performed before the analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CXCR2fl/flCre+ mice fed 20 weeks HFD, but it is attenuated as compared to what can be appreciated after 20 weeks SFD (Supplemental Fig. 3E). As aged neutrophils infiltrating in the liver and VAT were constantly elevated with both dietary regimens, these observations suggest that neutrophilia upon HFD feeding less likely marks prolonged half-life of aged neutrophils in circulation but perhaps might indicate an acquired ability to infiltrate in peripheral tissues. In line with this hypothesis, we observed an increased infiltration of aged, but not fresh neutrophils, 2 h after an oral olive oil gavage (OLTT), only when mice were fed 20 weeks HFD. Furthermore, aged neutrophils infiltrating more

in the liver did not present an increased expression neither of CD62L (involved in tissue infiltration) nor of CD11b (marking cell activation). In contrast, CXCR2fl/flCre+ mice, presented a reduction in CD62L membrane expression upon HFD, and the expression of CD11b was markedly reduced 2 h post OLTT only in HFD fed mice. This different infiltrating ability calls for the investigation of the downstream effects in metabolic tissues, as the conditional ablation of CXCR2 activates, while that of CXCR4 disarms, the intracellular granular proteomic armamentarium and organization of neutrophil [15]. Moreover, as CXCR4fl/flCre+ mice, which present less tissue infiltration of "disarmed" neutrophils, are protected from fatal acute infections while CXCR2fl/flCre+ mice, which display hyper-reactive "fresh" neutrophils, are not protected because of their impaired anti-inflammatory resolution potential [19], it could be intriguing to understand whether the systemic metabolic alterations induced by HFD feeding aggravate or adapt the cellular behavior of fresh/aged neutrophils.

The analysis of the liver proteome in mice fed HFD highlighted an increased presence of "suicidal NETosis" network [28] in the liver of CXCR4fl/flCre+, while underscored proteins associated with pro-resolutive chemotaxis (fMLPR signaling) in the liver of CXCR2fl/flCre+ mice. Neutrophils suicidal NETosis occurs following the activation of constitutive G-coupled receptors (including the CXCR2 receptor), that lead to chromatin decondensation, but can also be induced by mitochondria derived oxygen species production [28]. Neutrophils also coordinate non-lytic ("vital") NETosis, mainly by MPO activation [28,33]. Notably, we observed an increase in the abundance of proteins involved in suicidal NETosis in CXCR4fl/flCre+ compared to CXCR2fl/flCre+ mice, suggesting that neutrophil aging could be an intrinsic cellular feature against inflammatory stimuli that occur during the development of metabolic alterations. Furthermore, the possibility that the CXCR2 signaling promoting suicidal NETosis could act as a pathway engaged against dietary fats, alternatively or in addition to other neutrophil-specific pathways sensitive to lipids [34], including G protein coupled receptors such as GPR40 and GPR120 [35] or receptors of the resolvins or maresins [36], requires future and in-depth assessments.

In addition to liver, neutrophil aging impacted also on cell architecture and inflammatory profile of VAT, as CXCR4fl/flCre+ mice presented increased accumulation of CD11b+ inflammatory macrophages and rarefied density of other immune cell subsets in this tissue. By contrast, VAT from CXCR2fl/flCre+ mice presented an immune cell profile similar to that of WT in spite of a reduced infiltration of CD11+ macrophages compared to CXCR4fl/flCre+ mice. This also reflected in a different array of VAT inflammatory gene expression, including the Il23/Il17 axis, known to modulate the phagocytosis of apoptotic neutrophils mediated by local macrophages [17,37], and Cxcl1, recapitulating the active role of neutrophils in activating the inflammasome in adipose tissue residing macrophages [38]. Obviously other factors might contribute to the infiltration of immune competent cells in HFD [39].

4.1. Strengths and weaknesses

We demonstrated that the aging of neutrophils contributes to the development of metabolic alterations induced by fats-enriched diet, thanks to the use of validated neutrophil specific CXCR4 or CXCR2 knock-out mice models. Although epigenetic sensing of dietary fats within the BM has been proposed, the intracellular molecular pathways connecting this effect with neutrophil aging within the bone marrow remain to be elucidated.

Finally, the human cohort represents another strength of our study, by which we observed that higher plasma levels of Cxcl12, which binds to CXCR4, correlated with visceral adiposity while higher levels of Cxcl1, the ligand of CXCR2, correlated with indexes of hepatic steatosis, adiposity and MetS. While genetic determinism of CXCR4/CXCR2 appears strictly evolutionary controlled in humans, with few and very rare causal loss-of-function variants [40], thus limiting the possibility to specifically address the impact of these defects in humans on metabolic traits, this mere association is ancillary of the findings in the experimental models and reinforces recent observations in independent cohorts [41,42].

4.1.1. Translational perspective

The translational relevance of these findings is supported by the recent observations showing that targeting the neutrophil aging with selective CXCR2 antagonist prevented the development of insulin resistance and liver pathology in HFD-fed mice [43]. Given the increasing interest for neutrophil aging in multiple therapeutic areas, including neutropenias [44] or to treat advanced liver diseases [45,46], future studies on the same line should be designed to also investigate the modulation of neutrophil response in the context of metabolic disorders.

Funding sources

The work was supported by: Telethon Foundation [GGP19146], Progetti di Rilevante Interesse Nazionale [PRIN 2017 K55HLC], Ricerca Finalizzata, Italian Ministry of Health [RF-2019-12370896], PNRR NextGenerationEU [M4C2-Investimento 1.4-CN00000041 National Center for Gene Therapy and Drugs based on RNA Technology] and PNRR NextGenerationEU [Multilayered Urban Sustainability Action – MUSA] to GDN. The work of A. Baragetti is supported by: "Cibo, Microbiota, Salute" by "Vini di Batasiolo S.p.A" AL_RIC19ABARA_01; a research award (2021) from "the Peanut Institute"; PRIN 2017H5F943; ERANET ER-2017-2364981; "PNRR M4C2-Investimento 1.4-CN00000041", European Union—NextGeneration EU. A. Baragetti has been supported in part by Ministry of Health - Ricerca Corrente to IRCCS MultiMedica. The work of A.L. Catapano is supported by Ministry of Health - Ricerca Corrente - IRCCS MultiMedica, PRIN 2017H5F943, ERANET ER-2017-2364981, and Fondazione SISA.

CRediT authorship contribution statement

Conceptualization (formulating the idea and evolution of overarching research goals and aims of the study): AB, GDN. Supervision: GDN. Funding acquisition and Project administration: GDN, ALC. Methodology: LDD, AM. Investigation: AB, LDD, AM, OT, EM, PU, LND, ALC. Validation: LDD, AM. Data curation and Formal analysis: AB, MS. Writing – original draft: AB. Writing - review & editing of the manuscript: AB, GDN. Visualization: AB, LDD, OT, MS.

Declaration of competing interest

Prof. Catapano has received honoraria, lecture fees, or research grants from: Sigma-Tau, Manarini, Kowa, Recordati, Eli Lilly, Astrazeneca, Mediolanum, Pfizer, Merck, Sanofi, Aegerion, Amgen, Genzyme, Bayer, Sanofi, Regeneron, Daiichi-Sankyo The Corpus

Data availability

All data needed to understand and assess the conclusions of this research are available in the main text and supplementary materials. Raw datasets supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

The authors would like to thank Prof. Andés Hidalgo Alonso, Dr. Ivan Ballesteros Martìn and Dr. Jose Adrover Montemayor for the experimental mice models and the help for interpretation of the phenotype. Authors also thank Prof. Giangiacomo Beretta for the help in processing the LC-MS raw file.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2023.155576.

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