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# Lipodystrophy and obesity are associated with decreased numbers of T cells with regulatory function and pro-inflammatory macrophage phenotype.

Running title: Fat excess, fat depletion and immunity.

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## ABSTRACT

**Background/Objectives**: In lipodystrophy adipose tissue function to store lipids is impaired, leading to metabolic syndrome, similar to that found in obesity. Emerging evidence links dysmetabolism with disorders of the immune system (IS). Our aim is to investigate whether T-cells populations with regulatory function and monocyte-derived macrophages (MDMs) are affected by LD and obesity.

**Subjects/Methods**: Blood was collected from 16 LD, 16 obese (OB, BMI>30 Kg/$m^2$) and 16 healthy normal-weight women (CNT). Physical parameters, plasma lipid profile, glucose, HbA1c, leptin levels were determined. Flow cytometry was employed to assess the number of circulating $CD4^+$/$CD25^{hi}$ regulatory T cells (Tregs) and invariant natural killer T (iNKT) cells. Characterization of MDMs included morphology/Oil-Red-O staining that defined 2 main morphotypes: lipid laden (LL) and spindle-like (sp) MDM; gene expression studies; use of conditioned medium (MDM-CM) from MDMs on a human SGBS cells.

**Results**: As compared to CNT, LD and, to a lesser extent obesity, were associated with reduced Tregs and iNKTs (P<0.001 and P<0.01 for LD and OB respectively), higher number of LL-MDMs (P<0.001 and P<0.01 for LD and OB respectively), lower number of sp-MDMs (P<0.001 for both LD and OB), which correlated with increased paracrine stimulation of lipid accumulation in cells (P<0.001 and P<0.01 for LD and OB respectively). LD MDMs showed decreased and increased expression for anti-inflammatory (IL-10 and CD163) and pro-inflammatory (CD68 and CCL20) marker genes respectively. Analysis of correlation indicated that Tregs are directly related with HDL (P<0.01) and inversely related with LL-MDM (P<0.001) and that LL-MDM are directly related with triglycerides (P<0.01) and oxidized-LDL (P<0.01).

**Conclusions**: Lipodystrophy and obesity are associated with changes in the immune system: a significant reduction in the number of T cells with regulatory function and a shift of MDM towards lipid accumulation. Lipid profile of the patients correlates with these changes.

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## INTRODUCTION

Lipodystrophy (LD) syndrome is a group of rare, heterogeneous disorders characterized by selective loss of white adipose tissue, which varies from partial to generalized and occurs as a result of genetic defects or acquired conditions (1). In some forms of LD an association with autoimmunity has been postulated (2).

Independently of the aetiology, LD patients are usually diagnosed with metabolic-related disorders, including insulin resistance, dyslipidemia, non-alcoholic fatty liver disease, reminiscent of a dysmetabolic condition typical of obesity. The severity of the metabolic phenotype is approximately proportional to the degree of WAT loss and tends to be worse in generalised lipodystrophy (1). Fat depletion in LD mainly affects the subcutaneous WAT (SAT), the healthy type of fat where excess lipids should be ideally stored. These data explain why in LD reduced or absent expandability of SAT greatly impairs the capacity of the organism to properly store TGs and leads to ectopic lipid storage, resulting in insulin resistance and severe metabolic alterations (3).

The bidirectional interaction between immune system (IS) and whole-body metabolism is a widely recognized concept supported by: 1) the impaired immune defence and the higher susceptibility to infections found in obese individuals (4); 2) the relationship between immune function and nutritional status, with undernourishment and overfeeding both altering the function of T cells (5); 3) the notion that the altered metabolic state of obesity, being characterized by elevated circulating levels of glucose, cholesterol and fatty acids, can result in Reactive Oxygen Species generation and lipid peroxidation, with a consequent inflammatory phenotype of immune cells (6). Despite this evidence, the impact of systemic immune dysfunction occurring in conditions of excessive or depleted fat mass is far to be completely understood.

Compelling evidence supports the existence of a population of $CD4^+$ T cells able to suppress immune over-reactivity in various inflammatory conditions, including autoimmune diseases and cancer (7). These cells are characterized by a constitutive and strong expression of the IL2 receptor alpha chain (CD25), which defines a subset of naturally arising CD4 regulatory cells, the $CD4^+$/CD25 high ($CD4^+$/$CD25^{hi}$) T cells (for the purpose of this study defined as Tregs), first identified in animals as a population of suppressor T cells that maintain peripheral immune tolerance by inhibiting the activation and expansion of self-reactive T cells (8).

Another population of immune cells, invariant natural killer T (iNKT) cells, play a role in various pathological conditions, including microbial infection, autoimmune disease, cancer and metabolic syndrome (9). In both mice and humans, circulating iNKT cell number negatively correlate with weight gain (10). Interestingly, it was shown that iNKTs accumulation in adipose tissue promotes an anti-inflammatory phenotype in macrophages and stimulates proliferation and function of Tregs (11). Thus, iNKT cells are thought to play a protective role against obesity-induced inflammation and reduced glucose tolerance (10).

Monocyte-derived macrophages (MDMs) are commonly accepted as a good *in vitro* model recapitulating macrophages that infiltrate tissues. MDMs can be polarized *in vitro* into pro-inflammatory M1 or anti-inflammatory M2 phenotypes, by exposure of monocytes to cytokine/chemokine cocktails, or colony stimulating factors (CSFs). These two phenotypes represent the extremes of a continuous spectrum of macrophage subtypes, which can be found in human and animal tissues, indicating the high degree of phenotypic plasticity of these cells (12, 13). Infiltration of WAT by macrophages is an example of this plasticity: in lean individuals M2 macrophages predominate, whereas in obese patients a shift towards the pro-inflammatory M1 subtype prevails (14). Besides M1 and M2, another subtype of macrophages has been described, which constitute a prominent feature of inflammation in atherosclerotic plaques, the lipid laden macrophages (LL-MDMs), also known as foam

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cells. LL-MDMs develop by internalization of modified lipoproteins, in particular oxidized form of LDL, which plays a critical role in foam cell formation both *in vitro*, and *in vivo*. Foam cells are characterized by expression of multiple scavenger receptors and the presence of cytosolic lipid droplets (15, 16). In addition, it was reported that foam cells can develop *in vitro* when MDMs are cultivated with autologous human serum, whereas rarely when monocytes are grown in foetal bovine serum (17).

The function of the IS in lipodystrophy has been investigated only in the context of the effects of leptin replacement (18): Oral and colleagues report that in basal conditions the number of B lymphocytes is reduced with respect to normal range values, whereas no difference were observed for various subsets of T cells. In the present study, we focused on the populations of immune cells known to be regulated by the metabolic state and on the phenotype of monocyte-derived macrophages (MDMs). As a second aim, we investigated whether these changes are due to fat depletion *per se* or to dysmetabolic condition resembling those of obese subjects. To this end a group of obese individuals, matched for sex and age with LD patients, was studied.

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## MATERIALS AND METHODS

### Study Population

The study group included 16 women diagnosed with different types of lipodystrophy, including 6 patients with Dunnigan (DUN). All DUN patients were heterozygous for already reported (19,20) nucleotide missense mutation in the LMNA gene (see supplementary information Table 2, SI T2). Clinical characteristics of DUN are reported in SI T1 and there is no significant difference compared with the remaining 10 non DUN. 16 obese women (OB, BMI≥30 Kg/$m^2$) and 16 female normal weight controls (CNT, BMI<25 Kg/$m^2$) were recruited at the Endocrinology Unit/University Hospital of Pisa. LD and OB patients donated blood during routine lab test. CNTs were recruited from the general population. The study, approved by the local Ethical Committee (Prot. 21752), was conducted according to the Helsinki declaration; all participants signed an informed consent.

### Blood collection and laboratory analysis

Venous blood samples were obtained after an overnight fasting for measurement of serum glucose, triglycerides, total cholesterol, low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, C reactive protein (CRP). Serum leptin was measured by ELISA (Mediagnost, Germany). HbA1c was analyzed by HPLC (Tosoh/G7) with a CV% of 1.79. Oxidized low density lipoprotein (ox-LDL) levels in serum were measured by commercially available ELISA kits (Mecordia, Sweden). Details in SI M1.

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### Evaluation of circulating Tregs and iNKTs by flow cytometry

$CD4^+/CD25^{hi}$ Tregs and iNKTs levels were detected by direct staining of whole blood of CNT (n=16), OB (n=16) and LD (n=16) patients, as described (21-23). Briefly, 100 µl of fresh peripheral blood were stained with anti-CD4-FITC and anti-CD25-PE antibodies for labelling $CD4^+/CD25^{hi}$ Tregs, and with anti-CD3-FITC and anti-iNKT-APC antibodies (Miltenyi Biotec, Germany) for labelling iNKTs. Then, cells were incubated in the dark at room temperature (RT) for 30 min following red blood cells lysis with 2 ml of FACS Lysing Solution, according to the manufacturer instructions (BD Biosciences Pharmigen, CA). The cells were washed twice in PBS by centrifuging at 200xg for 5 min and the pellet resuspended in 200 ul of PBS with 0.5% formaldehyde. FACS analysis was performed immediately using the FACS Calibur Cytometer. Data were analysed using Summit software (Beckman Coulter, CA). 15,000 and 30,000 events were counted for Tregs and iNKTs respectively. Details of FACS data analysis and gating strategy in SI M2 and SI F4.

### Isolation and culture of monocyte-derived macrophages (MDMs)

Total peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Histopaque 1.077 (Sigma Diagnostics, MO) according to the manufacturer's instructions. Recovered cells were washed with PBS and suspended in RPMI-1640 medium supplemented with 2% heat inactivated foetal bovine serum (HI-FBS). To eliminate lymphocyte contamination and mature circulating endothelial cells, an initial pre-plating step was performed by plating $1.5x10^6$ cells/well in 6 wells of 24-well plates. After 1 hour, non-adherent cells were removed and the plates were washed 4 times with RPMI-1640+2% HI-FBS. Adherent cells were grown in 1ml of RPMI-1640 supplemented with 10% fresh autologous human serum (SI M1). Growth medium was replaced every 3 days, and morphology of the monocyte-derived macrophages (MDMs) evaluated after 7

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days using a light microscope (LeicaDMIL, Germany). MDM conditioned media (MDM-CM) were collected, centrifuged at 700xg for 15min at 4˚C to remove cells and debris and stored at -80 C.

**Oil Red O staining (ORO)**

Cells were fixed with 10% formaldehyde, stained for 10' at RT in freshly diluted ORO solution (6:4 parts of 0.5% ORO stock in isopropanol and double distilled water) and washed 5 times (SI M3 for details).

**Quantitative Real Time PCR**

Total RNA was isolated from the MDMs cultured for 7 days, using combined TRIzol reagent (Life Technologies, CA) and Rneasy kit (Qiagen, Germany) method according to manufacturer's instructions (SI M4 for details). cDNA was synthesized from 1μg of total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad, CA). Quantitative real-time PCR (RT-PCR) was performed using iTaq™ Universal Probes Supermix (BioRad, CA) with CFX96TM Real-Time System (BioRad, CA) instrument, and the relative mRNA expression for *Tumour necrosis factor α (TNFα), CD68, Monocyte Chemoattractant Protein 1 (MCP1), Interleukin 1b (IL1b), C-C motif ligand 20 (CCL20), Interleukin 10 (IL10), CD206* and *CD163* (Applied Biosystem, CA) genes was calculated using ΔΔ-Ct method. *GAPDH* gene expression was used for normalization of gene expression.

**Effect of MDM-CM on SGBS cells proliferation and lipid accumulation**

Simpson-Golabi-Behmel syndrome (SGBS) fibroblasts were grown as previously described (24) and seeded at concentration of $2x10^3$ cells/well in 96 wells plates with Dulbecco Modified Medium (DMEM) containing 10% of Fetal Bovine Serum (FBS): 2hrs later, after cells were attached, culture medium was replaced with 100 μl of MDM conditioned medium (MDM-CM) from CNT, OB and LD. Control cultures were kept in standard culture medium (DMEM, 10% FBS). After 96 hours of incubation, SGBS cells were ORO stained, digital images were acquired and lipid accumulation was measured using light microscope and Image J software (NIH).

Cell proliferation was monitored by WST-1 reagent kit (Roche Diagnostics GmbH, Germany), according to manufacturer's instructions (details in SI M5).

**Statistics**

Statistical power analysis using G*Power 3.1 (Heinrich-HeineUniversität Düsseldorf, Germany) showed that a total sample size of 45 patients enabled an 80% power to detect of differences in regulatory T cells distribution between CNT, LD, and OB patients at a significance level of 0.05 while assuming a large effect size (f = 0.5). Parametric tests were used when data were normally distributed and variances were not statistically different. Non parametric tests were used in all the other cases, as appropriate and indicated in figure legends.

Non-parametric correlation analysis (Spearman) was performed considering the 3 groups as a single population. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., CA). Data in bar graphs are expressed as mean ± SEM. P<0.05 was considered statistically significant.

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# RESULTS

## Circulating T cells with regulatory function and MDMs morphological phenotype are modified in patients with Dunnigan syndrome

To determine whether LD is associated with changes in immune parameters, we first considered 6 female patients with Dunnigan (DUN) syndrome, the most common monogenic form of partial lipodystrophy (25) and 16 healthy women (CNT). As assessed by flow cytometry, the percentage of circulating Tregs ($CD4^+/CD25^{hi}$) and iNKTs ($CD3^+/iNKT^+$) in peripheral blood was significantly lower in the DUN as compared with the CNT group (FIG 1A, 1B).

We then focused on the morphological phenotype of monocyte-derived macrophages (MDMs). After 7 days of cell culture in the presence of autologous serum, MDMs from DUN showed a distinct morphology compared with MDMs from healthy controls. Indeed, cells from CNT were markedly heterogeneous in size and shape, and included a significant number of spindle-like MDMs (Sp-MDMs), while cells from DUN were far more homogeneous, being mainly composed of large round lipid laden macrophages (see SI M3 for details) (FIG 1C top panel). Oil Red O (ORO) staining further amplified the difference because the number of ORO positive cells was markedly higher in MDMs from DUN as compared with controls (FIG 1C, bottom panel). To transform this descriptive, eye-based observation into quantitative data we counted only those phenotypes that were clearly divergent, i.e. cells that were either big (>30 uM) round ORO positive LL-MDMs or ORO negative spindle-like MDMs (Sp-MDMs) (see SI M3). Cell cultures from DUN showed greater numbers of LL-MDMs and lower number of Sp-MDMs (FIG 1D).

## Circulating T cells with regulatory function and MDMs morphological phenotype are modified in LD of various aetiology and in obesity

We next asked whether changes in immune parameters observed in DUN patients could be extended to LD in general by investigating 10 additional women with different types of LD (SI T2). To disentangle the effect of fat depletion from that of altered metabolism, we also recruited an age matched group of obese women (OB). The clinical characteristics of all patients and controls are presented in Table 1 and 2, in which DUN and non-DUN LD patients were combined. Clinical data show an altered glucose disposal for LD and dyslipidemia for both OB and LD. OB patients display a higher inflammation burden with respect to the other 2 groups. While there was no significant differences in total LDL levels between the groups, the circulating levels of oxidized form of LDL (ox-LDL), previously reported to be higher in patients with HIV related lipodystrophy (26), were increased in both LD and OB patients as compared to CNT (Table 1).

Similar to what found in DUN patients considered separately, the number of Tregs and iNKTs was significantly reduced in LD patients as compared with CNT (FIG 2A, B). OB patients showed an intermediate condition with a significant decrease as compared to CNT (FIG 2A, B). Interestingly, number of Tregs was significantly associated to HDL levels (FIG 2C) when subjects were considered as a single population. The results of MDMs analysis were similar to those found in DUN patients. Indeed, the number of LL-MDMs was significantly greater, while Sp-MDMs were depleted in LD patients, as compared with controls (FIG 3A). MDMs from OB subjects showed numbers of LL-MDMs and Sp-MDMs similar to those observed in LD, even if the difference with

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controls was slightly less pronounced. Differences in the IS parameters mentioned in this paragraph remained statistically significant when diabetic patients (OB and LD) were removed from the analysis (SI F1).

To investigate deeper in MDM function we evaluated expression of the M1, M2 phenotypes in the three groups using gene expression by quantitative real-time PCR. M1 markers included TNFα, CD68, MCP1, IL1b and CCL20 while the M2 markers were IL10, CD206 and CD163 (27, 28). We could observe an increased expression for the M1 markers CD68, CCL20 and MCP1 in the LD group with significant and borderline with significance differences for CD68, CCL20 respectively (FIG 3B). M2 markers tended to be lower in the LD group with IL10 and CD163 displaying statistically significant differences (FIG 3B).

Next, we investigated whether the number of T cells with regulatory function and the MDM morphotype were interrelated. Indeed, the number of Sp-MDMs was positively related to the circulating levels of Tregs and iNKTs, which on the other hand, were negatively related to the number of LL-MDMs (FIG 3C).

We then asked if morphology and lipid content of MDMs were associated with the clinical characteristics of patients. We observed that the number of Sp-MDMs was negatively related to TG and ox-LDL plasma levels and positively related to HDL plasma levels (FIG 4A). On the other hand, LL-MDMs were positively related with TG and ox-LDL levels and negatively related with HDL (FIG 4B).

Taken together these data indicate that in lipodystrophy and in obesity Tregs and iNKTs are less represented with respect to healthy controls, this being associated with a higher number of LL-MDMs and a reduced number of Sp-MDMs. Interestingly, these changes are significantly associated with the lipid profile of the subjects.

**Cross-talk between MDMs and SGBS cells**

We next wanted to investigate whether the changes observed in MDMs from LD and OB corresponded to differences in their function. To this purpose, we examined the effect of conditioned medium (MDM-CM) from the cells of CNT, LD and OB on primary stromal fibroblasts isolated from WAT of a subject with Simpson-Golabi-Behmel syndrome (SGBS cells). We observed that MDM-CM from CNT displayed a greater capacity to induce proliferation of SGBS cells as compared with standard culture conditions in DMEM medium (FIG 5A). However, this growth inducing effect was not reproduced by MDM-CM from LD that had a significantly weaker proliferating effect as compared to MDM-CM of CNT. The MDM-CM of cells from OB had a growth promoting effect that was intermediate between that of cells from LD and CNT (FIG 5A).

Lipid droplets accumulation was also observed inside the MDM-CM treated SGBS cells. ORO allowed quantifying this phenomenon: MDM-CM from LD induced a significantly greater lipid accumulation in SGBS cells as compared with MDM-CM from CNT or OB, the latter exhibiting an intermediate phenotype (FIG 5B, C). Similar differences in lipid accumulation were obtained when diabetic patients (OB and LD) were removed from the analysis (SI F2). To rule out the possibility that lipid accumulation was induced by the presence of autologous human serum in MDM-CM (see Methods), SGBS cells were incubated for 96 hours with DMEM containing 10% of human serum from CNT or LD patients. ORO staining analysis confirmed that there was no significant difference in the lipid accumulation induced by the human serum between LD and CNT (FIG SI F3). Regression analysis revealed that lipid accumulation by SGBS cells was positively related with plasma levels of TG and ox-LDL and negatively related with HDL (FIG 5D).

These data indicate that MDMs from LD patients inhibit cell proliferation and induce lipid accumulation in a different cell type.

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# DISCUSSION

A comprehensive understanding of how fat depletion impacts various functions of the organism is yet to be reached. Because the IS is closely related to metabolism, the new term immuno-metabolism was created (29). In the present study, we investigated the effect of fat depletion on the IS by analyzing T cells with regulatory function and the phenotype of monocyte-derived macrophages (MDMs). We first studied a homogeneous group of LD patients with DUN syndrome, who, when compared to controls revealed lower circulating levels of Tregs and iNKTs and striking change in the population of MDMs, characterized with higher appearance of lipid laden macrophages and depletion of spindle-like cells. Similar results were obtained when we extended the investigation to a larger and more heterogeneous group of LD patients and to OB patients.

Data from animal models showed that Tregs play a crucial role in the suppression of the autoimmune response as indicated by their inhibitory function in the synovial compartment of patients with rheumatoid arthritis and their capacity to inhibit the proliferation of autologous CD4/CD25$^{-}$ effector T cells *in vitro* (30). Decreased levels of Tregs and iNKTs were reported in several diseases, some with a clear autoimmune aetiology including systemic lupus erythematosus (31) and primary Sjögren's syndrome (32, 33). A significantly reduced number of iNKTs was found in patients with autoimmune T1D diabetes (34). It is then somehow expected to find important alterations of these parameters in forms of acquired lipodystrophy with an autoimmune aetiology (35, 36). Less obvious is this finding in genetically determined forms as herein found. In this regard, we could speculate that the altered numbers of T cells with regulatory function might not only be a consequence, but also a determinant of LD by triggering a specific action against adipocytes. Indeed, even in genetically determined partial lipodystrophy, fat depletion is not apparent at birth and progressively appears later in life (37). OB also showed a reduction of T cells with regulatory function; the depletion was mild for Tregs, and more pronounced for iNKTs, this being consistent with what previously documented in animal models and human subjects (10, 29, 38). Interestingly, a recovery to normal levels of iNKT is obtained following weight loss in humans and mice (10).

Far less obvious is the respectively higher and lower representation of lipid-laden (LL-MDMs) and spindle-like cells (Sp-MDMs) among the MDMs from LD and OB. LL-MDMs have been associated with M1 pro inflammatory phenotype while Sp-MDMs with M2 anti-inflammatory macrophages (39).

To further understand the reasons for the different representation of MDM morphotypes in LD, CNT and OB, we can analyse how this relates to other findings of the present study. First, the numbers of Sp-MDMs and LL-MDMs correlated with those of Tregs and iNKTs in a positive and negative way, respectively. Even if no cause-effect relationship can be established in the present study, this result discloses previously uncovered links between these immune cells and may indicate that a common determinant is affecting both MDMs and T cells with regulatory function. Second, correlation analysis between clinical parameters and MDM phenotype gave some indications, albeit indirect, about the nature of such determinant. Indeed, high triglycerides and ox-LDL were positively related to LL-MDMs and negatively related to Sp-MDMs, while the opposite was found for HDL. The MDM phenotype has then the potential of being predictive on the occurrence of dyslipidemia and *vice versa.* Indeed, Tregs and iNKT cells decreased and LL-MDMs increased as the lipid indicators of the metabolic syndrome worsened. The positive correlation between TGs and LL-MDMs is consistent with published data reporting that TGs represent a substantial component of the MDM cytosolic lipid bodies (15). The correlation

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between ox-LDL and LL-MDMs is not surprising (40), given that ox-LDL uptake by macrophages gives rise to foam cells, critical determinants in the formation of atherosclerotic plaques.

Third, higher representation of LL-MDMs in LD patients was associated with a higher expression of the pro-inflammatory M1 markers *CD68* and *CCL20*, and a lower expression of the anti-inflammatory markers *IL10* and *CD163*. The induction of the latters characterizes M2 macrophage polarization (27) and decrease in *IL10* levels are associated with multiple inflammatory conditions and aging (41). Consistent with what herein found it was shown that high ox-LDL is associated to increased *CD68* expression (42), while *IL10* was shown to inhibit ox-LDL induced lipid uptake in macrophages (43). High ox-LDL is a clinically relevant predictor of metabolic syndrome (44). Our findings, in line with the increased ox-LDL found in HIV-related LD (26), point to a condition in which the high levels of this circulating lipoprotein and the expression profile of macrophages concur to the formation of foam cells in both obesity and lipodystrophy.

Finally, the morphotype distribution corresponded to a different MDM paracrine action as cells cultivated with MDM conditioned medium from LD or OB exhibited reduced proliferation rate and increased lipid uptake. Importantly, there was a positive correlation between circulating TGs and lipid accumulation induced by MDM-CM, the opposite was true for HDL. These data suggest two, not necessarily alternative, hypotheses. The first, more speculative, is that certain types of macrophages play a role in the ectopic fat deposition that characterizes both LD and morbid obesity. The second, more obvious, is that lipid profile is not only a determinant of MDM phenotype, but also of their function.

Lipids emerge from this study as important determinants of the immune response, both systemic and within tissues, as herein shown *in vitro* by MDMs properties or function. Indeed, patients with LD or OB show similar trends for each aspect of the immune response being considered, but the former often presents with a more pronounced difference, as compared to healthy controls. Of note, high inflammatory markers characterize the obese patients only, supporting the notion that the low grade chronic inflammation, observed in the presence of excess and inflamed WAT (45), do not significantly relate with immune function (at least for the parameters herein analysed), whereas metabolic alterations importantly do.

In conclusion, we established that lipodystrophy and obesity are associated with changes in the immune parameters including: depleted number of T cells with regulatory function (Tregs and iNKTs) and overrepresentation of lipid laden monocytes derived macrophages, associated with enhanced paracrine capacity to induce lipid accumulation. These features correlate with the lipid profile of the patient.

Our findings define a new hallmark characterizing the systemic condition of lipodystrophy and obesity, add a new evidence to the concept that excess or depletion of adipose tissue may result in similar dysfunctions, and finally further strengthen the relationship between metabolism and the immune system.

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CONFLICT OF INTERSEST: the authors have nothing to disclose.

Supplementary Information is available at International Journal of Obesity's website.

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## FIGURE LEGENDS

**Figure 1. Immune parameters in DUN patients.** (A) Representative flow cytometry analysis and gated percentage of circulating Tregs ($CD4^+$/ $CD25^{ih}$) in peripheral blood of Dunnigan patients (DUN, n=6) and lean controls (CNT, n=16). (B) Representative flow cytometry analysis and gated percentage of invariant NKT cells ($CD3^+$/$iNKT^+$) in peripheral blood of Dunnigan patients (DUN, n=6) and lean controls (CNT, n=16). (C) Representative photomicrographs of CNT and DUN MDMs after 7 days in culture, unstained (top panels) or Oil Red O stained (bottom panels). Black and grey arrows indicate a LL-MDM and a Sp-MDM respectively. (D) Number of LL-MDMs and Sp-MDMs, expressed as percentage of total cells, in CNT and DUN. Two-sided Student's t-test (*P<0.05; **P<0.01; ***P<0.001). Data in bar graphs are expressed as mean±SEM.

**Figure 2. T cells with regulatory function in lipodystrophy and in obesity.** Gated percentage of circulating Tregs ($CD4^+$/$CD25^{ih}$) (A) and iNKTs ($CD3^+$/$iNKT^+$) (B) in peripheral blood of healthy controls (CNT), lipodystrophic (LD) and obese (OB) subjects (n=16 per group). (C) Non-parametric correlation analysis between gated percentage of Tregs and HDL cholesterol in CNT, LD (n=13) and OB subjects. CNT, LD and OB were combined in one single population for statistical analysis (Spearman r and P are shown). One-way ANOVA followed by Bonferroni *post-hoc* test (**P<0.01; ***P<0.001). Data in bar graphs are expressed as mean±SEM.

**Figure 3. Characterization of monocyte-derived macrophages (MDMs) in CNT, LD and OB subjects.** (A) Number of LL-MDMs and Sp-MDMs, expressed as percentage of total cells, in CNT (n=14), LD (n=14) and OB (n=16) subjects. (B) Expression of *TNFα*, *CD68*, *MCP1*, *IL1b*, *CCL20*,*IL10*, *CD206* and *CD163* in MDMs from CNT, LD and OB (n=10 per group) as assessed by Real Time PCR, with *GAPDH* used as control for equal loading. (C) Non-parametric correlation analysis between gated percentage of Tregs (left panels) and iNKTs (right panels) and percentage of Sp-MDMs and LL-MDMs. CNT, LD and OB were combined in one single population for statistical analysis (Spearman r and P are shown). One-way ANOVA followed by Bonferroni *post-hoc* test or Kruskal-Wallis followed by Dunn's Multiple Comparison test, as appropriate (*P<0.05; **P<0.01; ***P<0.001). Data in bar graphs are expressed as mean±SEM.

**Figure 4. Relationship between lipid profile and MDM morphotype.** (A) Non-parametric correlation analysis between Triglycerides (left), ox-LDL (center), HDL (right) and Sp-MDMs in CNT, LD and OB subjects. (B) Non-parametric correlation analysis between Triglycerides (left), ox-LDL (center), HDL (right) and LL-MDMs in CNT, LD and OB subjects. CNT, LD and OB were combined in one single population for correlation analysis (Spearman r and P are shown).

**Figure 5. Effect of conditioned medium (CM) from CNT, LD and OB MDMs on SGBS cells.** Undifferentiated SGBS cells were incubated for 96 hrs with standard medium (DMEM) or MDM-CM from CNT, LD and OB (n=16 per group). They were then assessed for proliferation (A) and lipid accumulation by ORO staining (B): in A data are expressed as percentage of the proliferation in standard culture conditions (dashed line). In B bars indicate mean area of ORO staining. (C) Representative photomicrographs of the ORO-stained SGBS cells after 96 hours with standard DMEM medium or MDM-CM from CNT, LD and OB groups (obj. 20X). (D) Non-parametric

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correlation analysis between SGBS lipid accumulation and plasma triglycerides (left), ox-LDL (center), and HDL (right) in CNT, LD and OB subjects. CNT, LD and OB were combined in one single population for statistical analysis (Spearman r and P are shown). Kruskal-Wallis followed by Dunn's Multiple Comparison test (*P<0.05; **P<0.01; ***P<0.001). Data in bar graphs are expressed as mean±SEM.

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**Table 1. Clinical characteristics**

| | CNT (N=9-16) | LD (N=11-16) | OB (N=13-16) | P value | Result of the *post-hoc* test |
|---|---|---|---|---|---|
| Age (years) | 33.2±2.6 | 38.6±3.9 | 39.3±3.4 | P=0.397 | ns |
| BMI ($Kg/m^2$) | 20.9±0.4 | 21.9±1.5 | 47.8±2.8 | P<0.0001 | a ns; b ***; c *** |
| Waist/Hip | 0.79±0.02 | 0.97±0.02 | 1.00±0.03 | P<0.0001 | a ***; b ***; c ns |
| Leptin (ng/ml) | 11.2±2.1 | 8.35±2.0 | 44.4±3.8 | P<0.0001 | a ns; b ***; c *** |
| Glucose (mg/dl) | 83.2±2.6 | 112.9±9.0 | 88.2±4.5 | P=0.0041 | a **; b ns; c * |
| HbA1c (%) | 5.4±0.03 | 7.2±0.49 | 6.0±0.22 | P=0.0114 | a *; b ns; c ns |
| Triglycerides (mg/dl) | 71.4±6.9 | 191.1±36.5 | 134±22.2 | P=0.0062 | a **; b ns; c ns |
| Total Cholesterol (mg/dl) | 184.5±20.8 | 200.5±16.1 | 174.4±11.7 | P=0.3543 | ns |
| HDL (mg/dl) | 69.2±4.9 | 40.6±3.9 | 43.5±4.2 | P<0.0001 | a ***; b ***; c ns |
| LDL (mg/dl) | 105.4±10.9 | 134±13.8 | 108.8±10.0 | P=0.0975 | ns |
| oxLDL (mU/ml) | 69.23±4.8 | 93.12±5.8 | 87.79±5.4 | P=0.028 | a *; b ns; c ns |
| C-reactive protein (mg/dl) | 0.05±0.02 | 0.178±0.05 | 1.03±0.23 | P<0.0001 | a ns; b ***; c ** |
| Hp (mg/dl) | 85±9.0 | 111.7±13.9 | 166.6±15.1 | P<0.0002 | a ns; b ***; c * |

One-Way ANOVA followed by Bonferroni post-hoc test or Kruskal-Wallis test followed by Dunns multiple comparison test, as appropriate; a = CNT *vs* LD; b = CNT *vs* OB; c = OB *vs* LD. Data are presented as Mean±SEM.

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**Table 2. Presence of diabetes and medications**

| | Controls (N=16) | LD (N=16) | Obese (N=16) |
|---|---|---|---|
| Presence of Diabetes | 0/16 | 9/16 (56.2%) | 4/16 (25%) |
| Steroids | 0 | 1/16 (6.25%) | 0 |
| Hypolipidemic drugs | 0 | 9/16 (56.25%) | 1/16 (6.25%) |
| Oestrogen/progestin drugs | 0 | 0 | 0 |
| Antidiabetic drugs | 0 | 12/16 (75%) | 8/16 (50%) |

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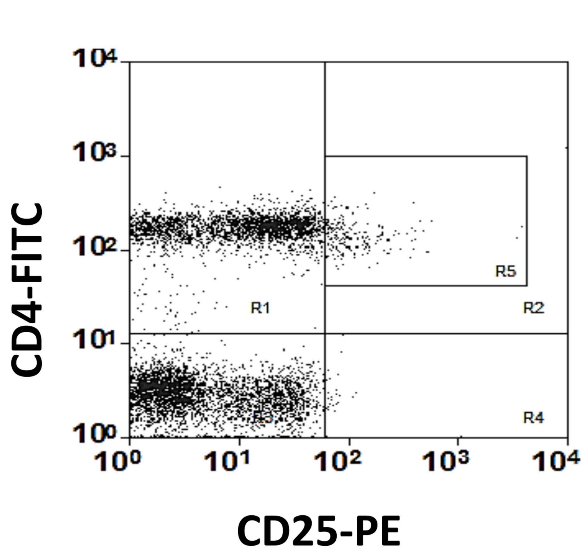


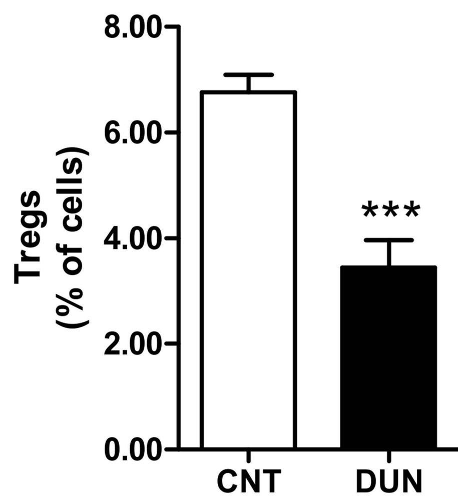


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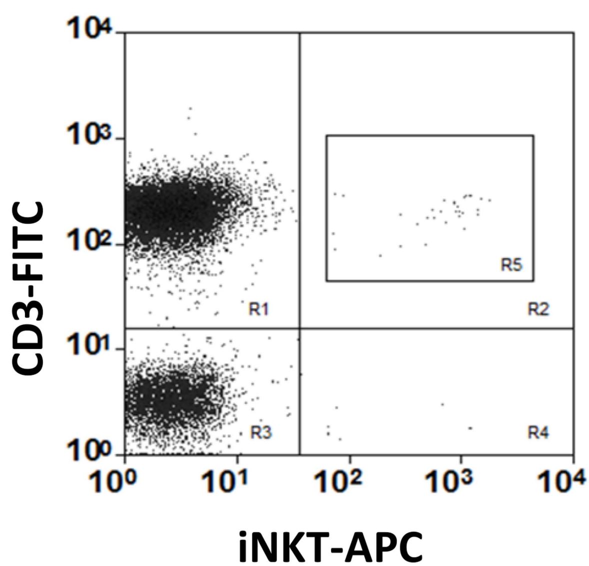


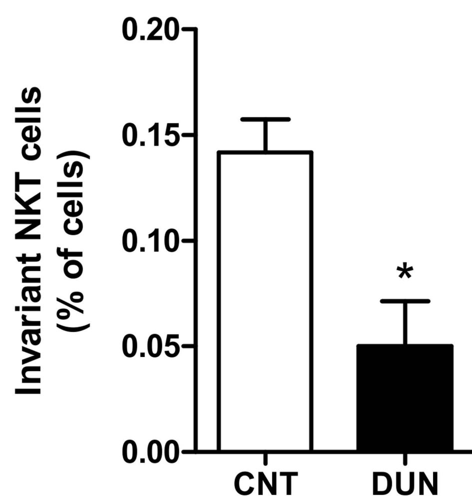


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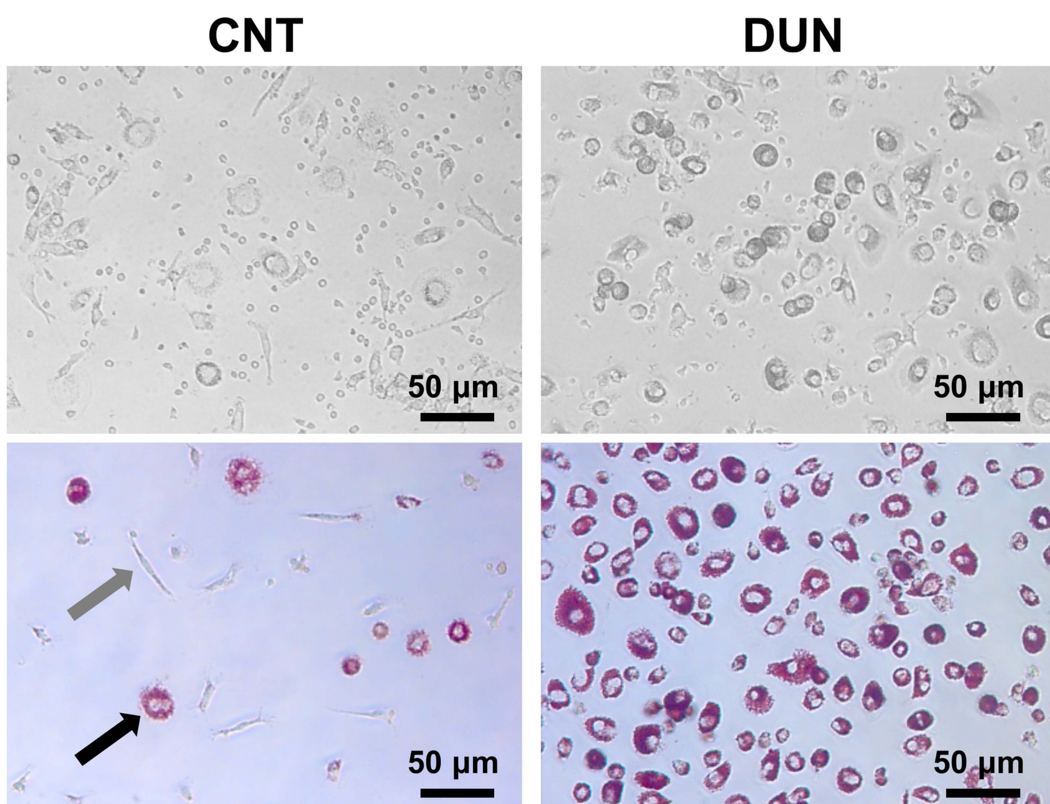


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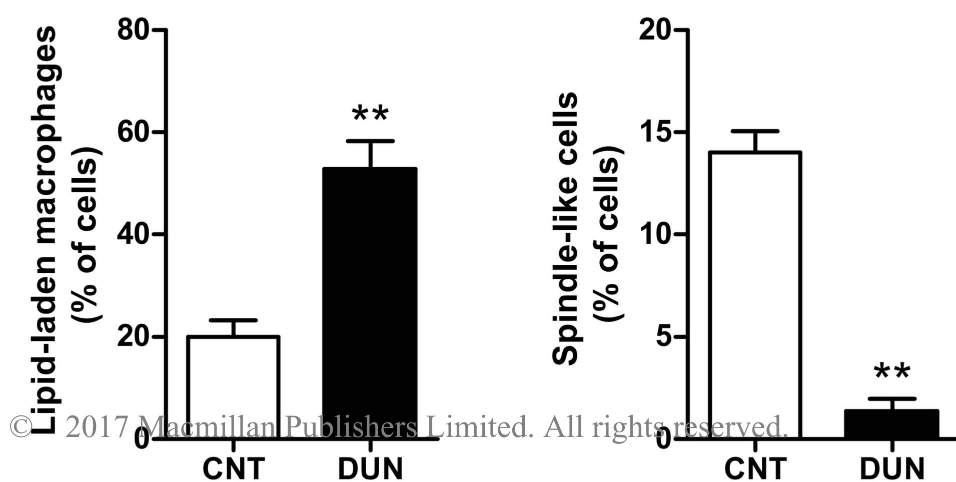


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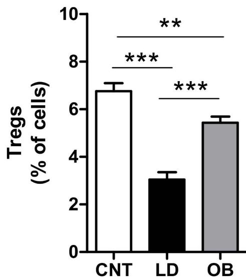


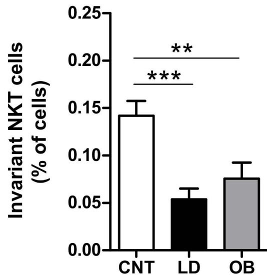


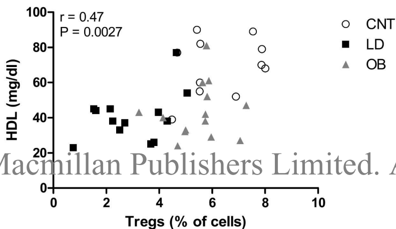


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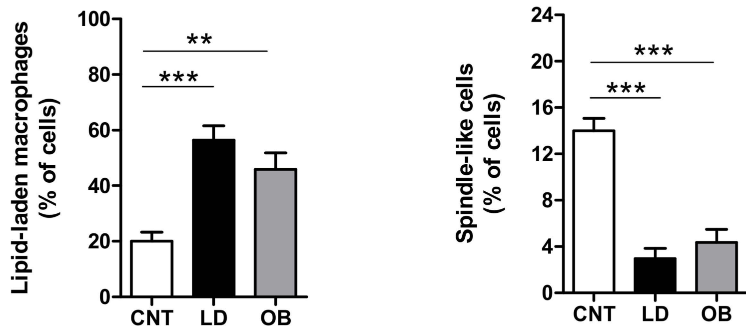


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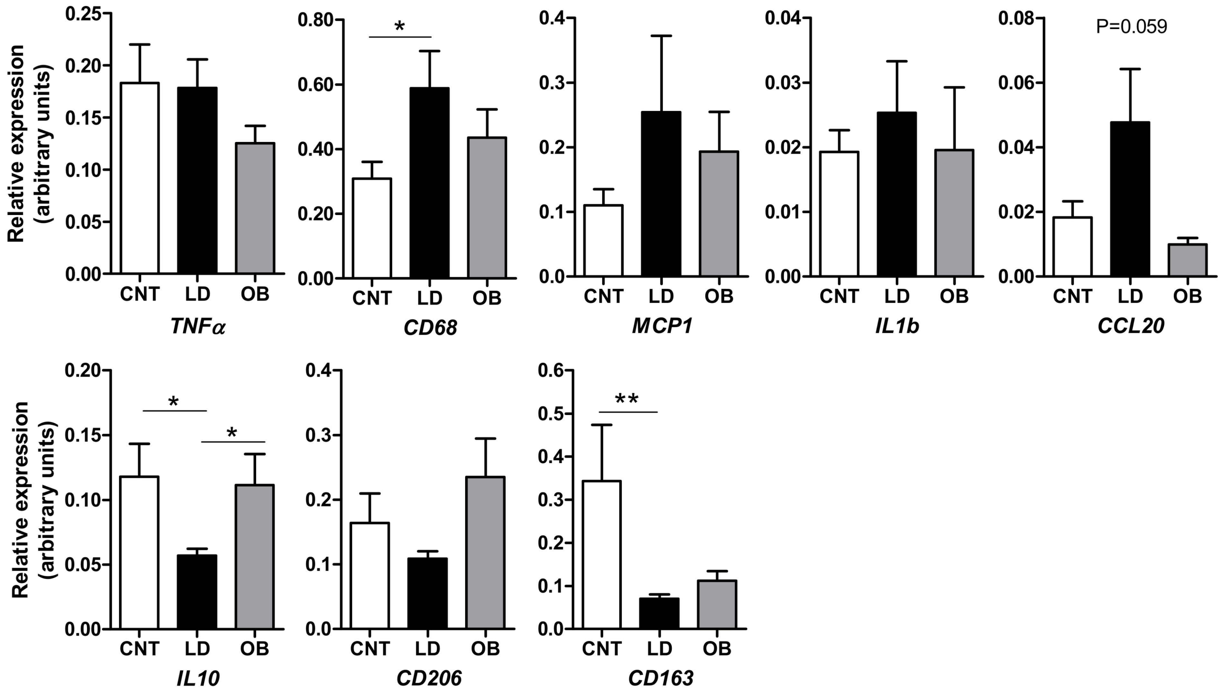


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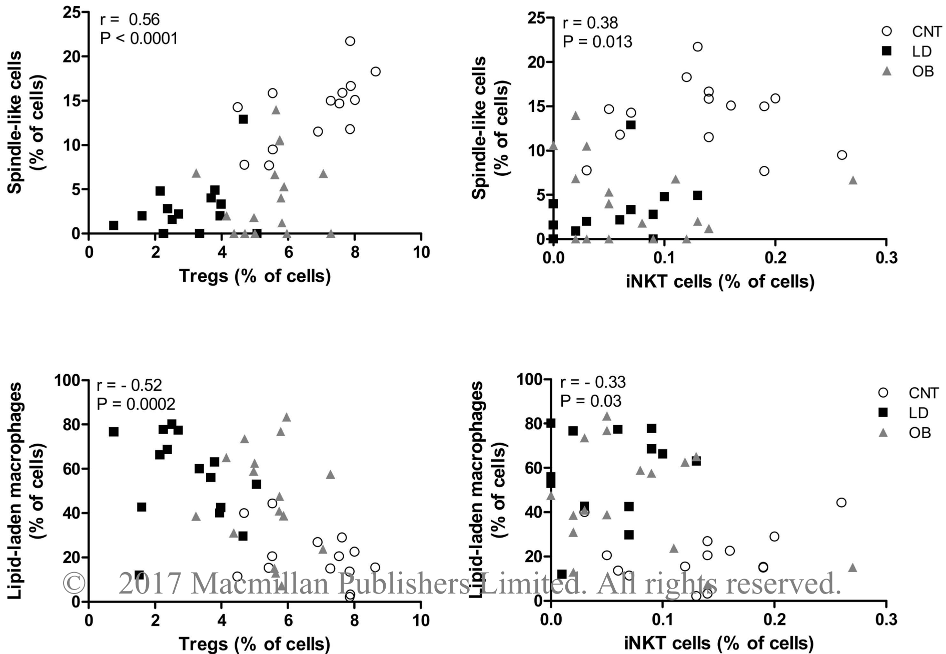


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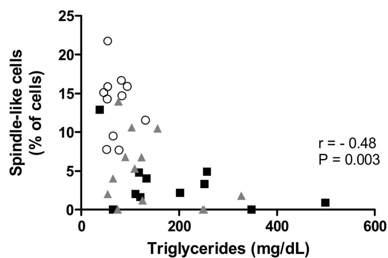


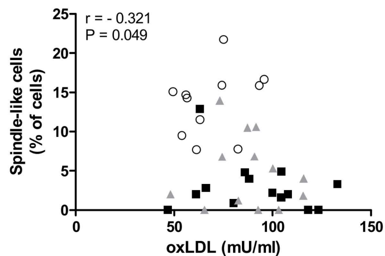


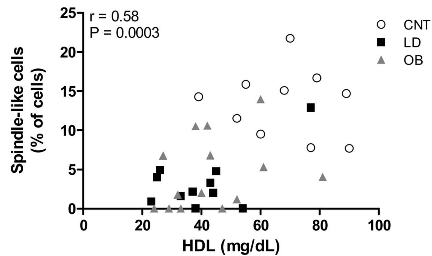


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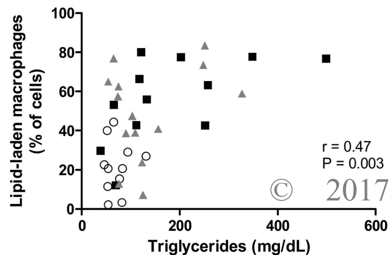


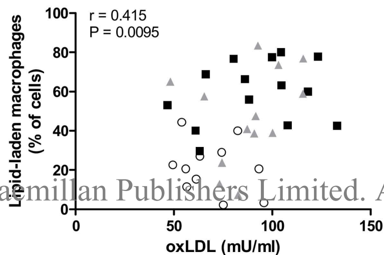


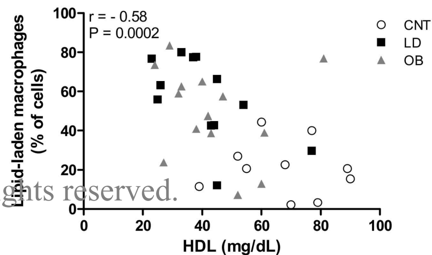


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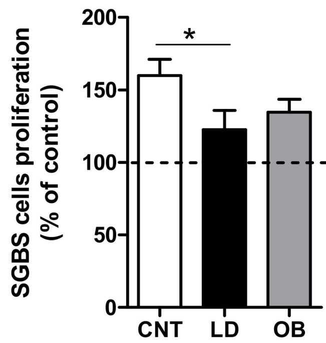


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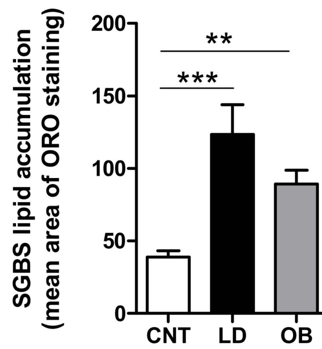


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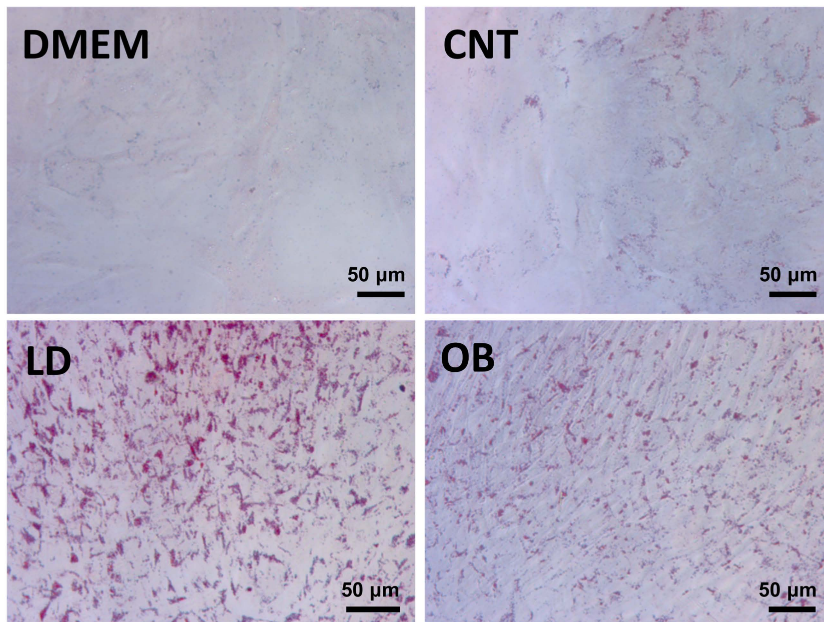


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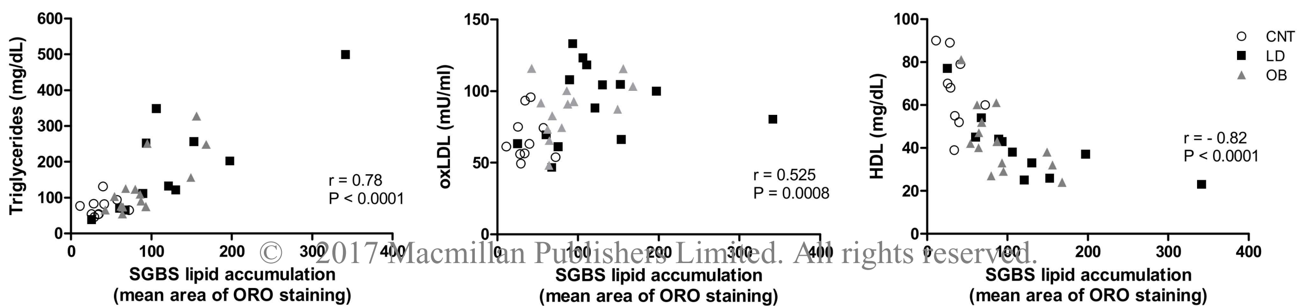


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