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Raman microspectroscopy reveals unsaturation heterogeneity at the lipid droplet level and validates an in vitro model of bone marrow adipocyte subtypes

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Author contribution statement

J.T. planned experiments, analyzed and interpreted the results. G.F. performed Raman acquisitions, interpreted and analyzed the results and compiled the corresponding figures. A.D. and N.B. performed HPLC analyses. J.T., C.B. and N.D.T. performed in vitro culture experiments. V.C. performed co-cultures. G.F. and L.D. performed statistical analyses for Raman data. J.T., G.F., A.D., C.C. and O.N. interpreted the Raman and HPLC data. O.N. and G.P. initiated the project, while C.C. and O.N. co-supervised the project. O.N. and J.T. wrote the manuscript, G.F. and C.C. significantly edited the manuscript.

Keywords

fatty acid, unsaturation (fatty acid), OP9 cell, lipid droplet diameter, Regulated, constitutive, Stroma, Bone marrow stromal cell (BMSC)

Abstract

Word count: 317

Bone marrow adipocytes (BMAds) constitute the most abundant stromal component of adult human BM. Two subtypes of BMAds have been described, the more labile regulated adipocytes (rBMAds) and the more stable constitutive adipocytes (cBMAds), which develop earlier and are more resilient to environmental and metabolic disruptions. In vivo, rBMAds are enriched in saturated fatty acids, contain smaller lipid droplets (LDs) and more readily provide hematopoietic support than their cBMAd counterparts. Mouse models have been used for BMAds research, but isolation of primary BMAds presents many challenges, and thus in vitro models remain the current standard to study nuances of adipocyte differentiation. No in vitro model has been described for the study of rBMAds/cBMAds.

Here, we present an in vitro model of BM adipogenesis with differential rBMAd and cBMAd-like characteristics. We used OP9 BM stromal cells derived from a (C57BL/6xC3H)F2-op/op mouse, which have been extensively characterized as feeder layer in hematopoiesis research. We observed similar canonical adipogenesis transcriptional signatures for spontaneously-differentiated (sOP9) and induced (iOP9) cultures, while fatty acid composition and desaturase expression differed at the population level. To resolve differences at the single adipocyte level we show that Raman microspectroscopy presents a high-resolution method for studying adipogenesis in vitro in a label-free manner, with resolution to individual LDs in this model. We find sOP9 adipocytes have lower unsaturation ratios, smaller LDs and higher hematopoietic support than iOP9 adipocytes, thus functionally resembling rBMAds and cBMAds, respectively. Validation in human samples confirms a higher unsaturation ratio for lipids extracted from stable cBMAds (femoral head upon hip-replacement surgery) versus labile rBMAds (iliac crest after chemotherapy). Moreover, the 16:1/16:0 fatty acid unsaturation ratio, already shown to discriminate cBMAds from rBMAd in rabbit and rat marrow, was validated in both the OP9 model in vitro system and in human samples. We expect our model will be useful for cBMAd and rBMAd studies, particularly where isolation of primary BMAds is a limiting step.

Contribution to the field

Taken together, we conclude that the spontaneous versus induced OP9 in vitro adipogenesis model presented here replicates key differences in lipid droplet size, FA unsaturation ratio and hematopoietic support respectively associated in vivo to regulated versus constitutive BMAd subtypes. We additionally validate the value of the 16:1/16:0 FA unsaturation ratio as a surrogate marker to segregate rBMAd-rich versus cBMAd-rich tissues both in vivo and for our in vitro model, and the feasibility of Raman microspectroscopy to reveal equivalent differences in the unsaturation ratio at the single adipocyte level.

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Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: The studies involving human participants were reviewed and approved by Commission d'étique Canton de Vaud (CER-VD). The patients/participants provided their written informed consent to participate in this study.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Mendeley Data link to original datasets: http://dx.doi.org/10.17632/wdm9gvz3bm.1.



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- 19 constitutive₆, stroma₇, bone marrow stromal cell (BMSC)₈. (Min.5-Max. 8)
- 20

21 Abstract

- 22 Bone marrow adjpocytes (BMAds) constitute the most abundant stromal component of adult human
- 23 bone marrow. Two subtypes of BMAds have been described, the more labile regulated adipocytes
- 24 (rBMAds) and the more stable constitutive adipocytes (cBMAds), which develop earlier and are
- 25 more resilient to environmental and metabolic disruptions. In vivo, rBMAds are enriched in saturated
- 26 fatty acids, contain smaller lipid droplets (LDs) and more readily provide hematopoietic support than
- 27 their cBMAd counterparts. Mouse models have been used for BMAds research, but isolation of
- 28 primary BMAds presents many challenges, and thus *in vitro* models remain the current standard to
- 29 study nuances of adjocyte differentiation. No *in vitro* model has yet been described for the study of
- 30 rBMAds/cBMAds.
- 31 Here, we present an *in vitro* model of BM adipogenesis with differential rBMAd and cBMAd-like
- characteristics. We used OP9 BM stromal cells derived from a (C57BL/6xC3H)F2-op/op mouse, 32
- 33 which have been extensively characterized as feeder layer for hematopoiesis research. We observed
- 34 similar canonical adipogenesis transcriptional signatures for spontaneously-differentiated (sOP9) and

- 35 induced (iOP9) cultures, while fatty acid composition and desaturase expression of *Scd1* and *Fads2*
- 36 differed at the population level. To resolve differences at the single adipocyte level we tested Raman
- 37 microspectroscopy and show it constitutes a high-resolution method for studying adipogenesis *in*
- 38 *vitro* in a label-free manner, with resolution to individual LDs. We found sOP9 adipocytes have
- 39 lower unsaturation ratios, smaller LDs and higher hematopoietic support than iOP9 adipocytes, thus
- 40 functionally resembling rBMAds, while iOP9 more closely resembled cBMAds. Validation in human
- primary samples confirmed a higher unsaturation ratio for lipids extracted from stable cBMAd-rich
 sites (femoral head upon hip-replacement surgery) versus labile rBMAds (iliac crest after
- 42 chemotherapy). As a result, the 16:1/16:0 fatty acid unsaturation ratio, which was already shown to
- 44 discriminate BMAd subtypes in rabbit and rat marrow, was validated to discriminate cBMAds from
- 45 rBMAd in both the OP9 model *in vitro* system and in human samples. We expect our model will be
- 46 useful for cBMAd and rBMAd studies, particularly where isolation of primary BMAds is a limiting
- 47 step.

48 1 Introduction

49 Similar patterns of bone marrow adipocyte (BMAd) formation occur in vertebrate species (including 50 rodents, rabbits, and humans). Specifically, a decreasing rate of BMAd has been observed relative to 51 the decreasing size and lifespan of the animal, with larger skeletons containing more BM adipose tissue 52 (BMAT) which extends farther into the skeleton (1). The distal parts of the skeleton (ie. peripheral 53 bones in human, the distal tibia, paws, and caudal vertebrae in mice) predominantly contain adipocytic 54 marrow interspersed with some hematopoietic cells, comprising the more stable constitutive BMAds 55 (cBMAds) that appear just around birth. The proximal locations of the skeleton (ie. thoracic vertebrae 56 in human and mouse, as well as proximal tibia and other long bones in mouse) contain predominantly 57 hematopoietic marrow interspersed with the more labile regulated BMAds (rBMAds) (1,2, 36). The 58 rBMAds are smaller in size and respond readily to induction of bone marrow adipogenesis through 59 nutritional challenge (high fat diet or caloric restriction) or hematopoietic failure (irradiation or 60 chemotherapy), or BMAd mass reduction in the context of cold exposuror increased hematological demand (e.g. phenylhydrazine). It is argued that cBMAds on the other hand, do not respond readily to 61 62 environmental demands (1,3–6). Stromal cells and BMAds are tightly linked to BM hematopoiesis. 63 Various stromal populations in mouse and human have shown to promote hematopoietic support while 64 more mature BMAds correlate with reduced hematopoietic proliferation ((7,8) and reviewed in (9,10)). 65 Meanwhile Adiponectin expressing cells within the BMAd lineage are beneficial to hematopoietic 66 regeneration in most bones but not the caudal vertebrae of mice (11,12). These studies indicate the 67 probability for population- and location-specific distinctions of BMAd maturation that specifically 68 affect hematopoiesis and respond to hematopoietic demand (reviewed in (13)).

- 69 Isolation of BM stroma for differentiation and analyses of BMAds in vitro to study these processes in
- 70 mouse models is challenging due to the limited amount of BMAd material obtained from each bone
- or bone segment. Moreover, this is compounded by the fact that the defining surface markers that
- 72 would aid in purification of BMAd progenitor populations in homeostatic mice do not discriminate
- 73 between the labile and stable BMAds (7). With such constraints, a promising alternative to the use of
- 74 primary cells for *in vitro* studies are multipotent BM-derived stromal cell lines differentiated *in vitro*,

- especially when studies are possible at the single cell level to address heterogeneity (14). For this
- 76 purpose, we developed a high-throughput image platform to monitor adipocytic differentiation
- though LD accumulation quantified by Digital Holographic Microscopy in live cells, which requires
- 78 minimal handling to avoid perturbation of the culture (15). While not limited by end-point analysis or
- the introduction of handling or preparation biases, this technique does not provide information on the
- 80 molecular composition of the cells. Methods capable of combining manipulation-free, label-free,
- 81 single-cell imaging with analysis of molecular composition are thus of great interest to the field of
- 82 adipogenesis at large. Raman microspectroscopy is a non-invasive and label-free method that does
- not require specific sample preparation, with a sub-cellular resolution on the scale of $\sim 1 \mu$ m that
- provides the molecular composition with spatial data, where standard methods provide bulk
 information (e.g. mass spectroscopic and chromatographic methods) (16). We thus hypothesized th
- information (e.g. mass spectroscopic and chromatographic methods) (16). We thus hypothesized that
 combining lipid profiling by Raman microspectroscopy with the inherently heterogenous *in vitro*
- 87 stromal adipocytic differentiation model would serve as a powerful tool to aid in the understanding of
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- 88 BM adipogenesis.

89 Specifically, LD formation (17) begins with fatty acids (FAs) that enter the cell via fatty acid 90 transport proteins or fatty acid translocase (18,19), or are synthesized through endogenous de novo 91 lipogenesis (DNL). FAs then enter a bioactive pool to form fatty acyl-CoA to be used by glycerolipid 92 synthesis enzymes in the endoplasmic reticulum (ER) to form neutral lipids such as triacylglycerols 93 (TAGs) and eventually coalesce and form LDs (20). If attached to the ER, LDs may grow in size 94 through diffusion of newly synthesized lipids to the LD, and if unattached, through local synthesis or 95 fusion of smaller LDs to larger ones. LDs increase in size during adipocytic differentiation and 96 perilipins (PLINs) bind to promote their stabilization (21). Lipases mobilize neutral lipids in LDs for 97 metabolic energy by fatty acid oxidation, which is triggered through nutritional, hormonal, or 98 inflammatory activation. Adipose triglyceride lipase (ATGL) catalyzes the initial step of intracellular 99 TAG hydrolysis followed by hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) 100 into glycerol moieties (22,23). FAs are necessary for energy production and lipid synthesis for 101 cellular signaling and membrane formation. Despite their importance, increased concentrations of 102 non-esterified FAs can be detrimental contributing to lipotoxicity, and thus TAG hydrolysis and FA 103 cycling are carefully regulated (24,25). As illustrated in Figure S1, the primary product of DNL is 104 palmitic acid (16:0), in the family of saturated FAs that have cytotoxic effects and induce reactive 105 oxygen species (ROS) (26). The monounsaturated FA (MUFA) oleic acid (18:1) may counteract this 106 effect possibly by activating esterification of palmitic acid into TAGs and lipid droplet storage (27). 107 Palmitic acid accumulation is therefore prevented by increased desaturation to palmitoleic acid 108 (16:1n-7) or elongation to stearic acid (18:0) and further desaturation to oleic acid (28). Notably, 109 oleic acid inhibits palmitic-acid dependent osteoclastogenesis and palmitic acid is found to be 110 increased in BM serum of osteoporotic women (29). In fact, BMAT expansion throughout the red marrow with age and disease is associated with bone loss and fracture risk, while cBMAd formation 111 112 is positively associated with bone accrual during early development through correlative and 113 descriptive studies (30).

114 While concrete definitions and BMAd classification markers are being established (30), the oldest 115 standard for classifying the two known types of BMAds is by the composition of their lipid content 116 (31, 32). The first reports by Mehdi Tavassoli showed that the adipocytic-rich yellow marrow -now 117 denominated cBMAd- from the os calcis of rabbits was higher in the proportion of unsaturated FAs (palmitoleic -16:1n-7- and oleic -18:1n-9- acids), whereas fatty acid composition of BMAds from the 118 119 adipocyte-poor red marrow of thoracic vertebrae (rBMAds) was richer in saturated FAs (palmitic -120 16:0- and stearic -18:0- acids) (33). In recent years, primary rat cBMAds from tail vertebrae and distal 121 tibia were likewise shown to contain a higher proportion of unsaturated fatty acids in caudal vertebrae 122 or distal tibia as compared to rat rBMAds isolated from either lumbar vertebrae or femur plus proximal 123 tibia combined (1). Congruently MRI, H-MRS, and gas chromatographic data from human subjects 124 consistently showed that BM adipose tissue (BMAT) from sites of red marrow contained highly 125 saturated lipids, compared to the mostly unsaturated lipids detected at yellow marrow sites (1,33–35). 126 From murine samples, it is considerably more challenging to obtain sufficient BMAT for analysis due 127 to the size of the animal as well as the amount of BMAT present in the bones (32). To our knowledge, 128 the lipid composition of murine BMAds has not been described to date. However, between BMAT of 129 red and yellow marrow there is a conservation of the differences in both the 16:1/16:0 and 18:0/18:1 130 fatty acid unsaturation ratios relative to total lipid content across species (summarized and compiled

131 with our data in Table 1 and Figure S1).

In this study, we harness the inherent adipogenic potential of murine BM-derived OP9 stromal cells to 132 133 further dissect intrinsic properties of BMAds using standard techniques for lipid profiling at the cell 134 population-level, and Raman microspectroscopy for lipid profiling at the single-adipocyte level. We 135 subjected OP9 cells to basal culture conditions or to a standard adipogenic cocktail, allowing the OP9 136 cells to differentiate spontaneously though serum exposure and confluency (sOP9) or through specific 137 induction of the adipogenic differentiation program (iOP9). To test the hematopoietic supportive 138 capacity of OP9 cells from these conditions, we assessed their interaction with a hematopoietic 139 component in short-term co-culture assays with hematopoietic stem and progenitor cells (HSPCs). We 140 show that a full biochemical induction of the adipogenic differentiation program induces iOP9s to more closely resemble cBMAds in their lipid content, lipid droplet size and hematopoietic support than the 141 sOP9 adipocytes that develop upon simple serum induction, which retain rBMAd-like properties. 142 143 Furthermore, we validated the significance of our unsaturation ratio-driven cBMAd versus rBMAd 144 definition. For this, we compared for the first time primary human samples from iliac crest samples 145 drawn after hematological aplasia, which represent the most extreme case of rBMAd remodeling (36), 146 to femoral specimens from hip replacement surgery, which represent one of the best characterized 147 cBMAd depots (37,38).

148 2 Methods

149 **2.1** Cell culture

All stromal cells (MS5, C3H10T1/2, MC3T3-E1-4, MC3T3-E4-24 bone marrow stromal lines,
 AFT024 and BFC012 stromal lines from fetal liver (39) or embryonic stem cell-derived fibroblasts as
 in (40,41) were cultured in complete medium consisting of Minimum Essential Media alpha (MEMα)
 with GlutaMax[™] (Gibco, catalog no. 32561) and 1% Penicillin/Streptomycin (P/S, Gibco, catalog no.

154 15140) supplemented 10% fetal bovine serum (FBS, Gibco, catalog no. 10270-106) at 37°C and 5% 155 CO₂ with media changed every 2-3 days, and differentiated as described below (42). For subsequent analysisOP9 cells were plated in 24-well plates (Falcon) with CaF₂ substrates (Crystran) for Raman 156 157 microspectroscopy and RT-qPCR, in 6-well plates (Falcon) for HPLC analysis, and in flat-bottom 158 tissue culture-treated 96-well plates (Falcon) for co-culture assays. OP9 cells were plated 159 subconfluently at a density of 5,000 cells per cm² (undifferentiated OP9 cells) or plated at confluency 160 of 20,000 cells per cm² in complete medium (spontaneous OP9 cells) for either 7 or 17 days, as 161 specified in figure legends. Both OP9 cells and OP9-EGFP cells (14) were authenticated by the ATCC 162 on June 24, 2021, by short tandem repeat (STR) analysis, indicating an exact match of the ATCC 163 reference OP9 cell line (CRL-2749). OP9 cells are available from the ATCC (CRL-2749), but this

source has not been tested for adipogenesis or hematopoietic support in our laboratory.

165 2.1.1 In vitro differentiation and quantification

166 All stromal lines were plated at 20,000 per cm² and cultured to confluency in 1% gelatin-coated plates 167 (prepared overnight at 4°C), then allowed to spontaneously differentiate over time by confluency or 168 induced toward adipocytic or osteogenic differentiation via a standard differentiation cocktail as in 169 (14,42). The adipogenic induction cocktail consisted of complete medium supplemented $\frac{1\mu M}{\mu}$ 170 dexamethasone (Sigma, catalog no. D4902), 5μ g/ml insulin (Sigma, catalog no. I0516), and 0.5mM isobutyl-methylxanthine (IBMX, Sigma, catalog no. I5879). After four days, the adipogenic induction 171 172 medium was changed to a maintenance medium consisting of complete medium with insulin and 173 dexamethasone only. Media was changed every 3-4 days with aliquots prepared fresh from stock 174 solutions (IBMX in DMSO, insulin in PBS, dexamethasone in ethanol) kept at -20°C in the dark. Short-175 term differentiation experiments were performed at day seven of adipocytic differentiation with 176 minimal culture manipulation as in (15), and long-term experiments were carried out on day 17. Neutral lipids were stained at day 17 of culture with Oil Red O for quantification of adipogenesis in 96-well 177 178 plates. First, cells were gently washed with phosphate buffered saline (PBS, Gibco, catalog no. 10010015) then fixed for 10min at room temperature with 4% paraformaldehyde (PFA) diluted in PBS 179 180 from 32% stock solution, and gently washed three times with PBS. 100µl filtered Oil Red O solution 181 freshly prepared from stock solution (Sigma, catalog no.01391-250ML) diluted 3:2 with distilled water 182 was added to the wells. Cells were incubated with Oil Red O at room temperature for 45min on a 183 shaker, then washed gently with PBS three times and light transmission micrographs were obtained. 100μ l isopropanol was then added to the wells and incubated at room temperature. After 10min, 70 μ l 184 185 of the solution was transferred to a new 96 well plate and OD measurements read at 520nm with 186 isopropanol as background. Osteogenic differentiation was performed by addition of dexamethasone 187 1μ M, 2-phospho-L-ascorbic acid 50μ g/ml (1000x stock in PBS), glycerophosphate 10mM (100x stock 188 in PBS) and 1,25-hydroxyvitamine D3 0.01µM (stock 1000x in ethanol) for 28 days. Every 3-4 days differentiation media was changed. All media changes were made with fresh stock aliquots. Efficiency 189 190 of osteoblastic differentiation was determined with Alizarin Red stain (Sigma) in stromal cells fixed as 191 described above for O Red Oil stains. Alizarin Red staining solution (alizarin red 2% in distilled water, 192 filtered through $45-\mu$ m pore, pH adjusted to 4.0-4.3 with NH4OH and filtered again) was then added 193 for 10-15 minutes until precipitates were visible. Alkaline Phosphatase development kits were 194 purchased from Promega (\$3771), and OP9 stains were performed according to manufacturer's

instructions in lightly fixed cells (4%PFA for 1 minute at room temperature) by dilution of NBT in AP
 staining buffer (100mM Tris HCl pH9.5, 50mM MgCl2, 100mM NaCl and 0.1% Tween-20). Pictures

- 197 were taken immediately after staining.
- 198 2.2 Raman microspectroscopy

Fixed 24-well plates were kept in PBS at 4°C and shipped to the University of Lille. Raman acquisitions 199 200 were done on a LabRAM HR800 equipped with an immersion objective (Nikon, obj x100, numerical 201 aperture = 1, Japan) and a diode laser λ =785 nm. The laser power at the sample was 30mW. The lateral 202 resolution was $1-2\mu$ m. Spectral acquisition was made in the 400–1800cm⁻¹range and spectral resolution 203 was 4cm⁻¹. The acquisition time was set at 60s per spectrum. Raman spectra were processed using 204 Labspec software (HORIBA, Jobin-Yvon, France). The water immersion objective focused the laser 205 on the center of individual lipid droplets where one spectrum corresponds to one adipocyte lipid 206 droplet. In total 2944 spectra were measured over 120 sOP9-adipocytes (on average 24 spectra per 207 adipocyte) and 2971 spectra over 138 iOP9-adipocytes (with an average of 21 spectra per adipocyte). 208 The number of spectra per well was between 60 to 110. The unsaturation ratio was calculated as ratio 209 of area under the curve of bands 1654cm¹ / 1441cm¹. The optical image of the adipocyte was saved for each acquisition. The diameter of LDs was evaluated from the optical image by using the software FIJI 210 211 (43).

The instrument is also equipped with a XYZ motorized stage which allows the acquisition of Raman images. Raman images were acquired using the point-by-point imaging mode (Pt-Img). The laser beam was focused perpendicular to the sample surface. Acquisition time was set to 3sec (×2) for each spectrum. The laser beam was stepped in two dimensions (x and y), and a spectrum was recorded at each position (x,y). The step was set to 1 μ m between 2 positions. The Pt-Img mode generated x×y spectra.

218 2.3 High-performance liquid chromatography

219 Cells were fixed with PFA as described for the adipocytic quantification above. After washing, 500µl 220 of PBS was left in the wells, plates were sealed with parafilm and stored at 4°C until processing for 221 HPLC analysis. Cells were detached from the plate with 1mL (2 x 0.5ml) of PBS and lipids extracted 222 with chloroform/methanol (2:1; v/v) under agitation for 30min at room temperature. The resulting lipid 223 extract was then subjected to a saponification, followed by a fatty acid derivatization into naphthacyl 224 esters as described previously (44). Fatty acid derivatives were applied into the HPLC Alliance system 225 (2695 Separations Module, Waters, Saint-Quentin-en-Yvelines, France) equipped of an autosampler 226 (200ml-loop sample), a photodiode array detector (model 2998), and the Empower software for data 227 analyses. Fatty acid derivatives were eluted on a reverse phase YMC PRO C18 column (3mm, 228 4.6x150mm, 120Å) by using two solvent systems: a) methanol/acetonitrile/water (64:24:12; v/v/v) and, 229 b) methanol/dichloromethane/water (65:28:7; v/v/v) under identical conditions reported previously 230 (44). Fatty acid derivatives were detected at 246 nm and quantified by an external standard curve 231 realized with the naphthacyl 19:0 derivative. Note that 19:0 was also used as internal standard to

evaluate FA recoveries. Lipidomics LC-HRMS standards and solvents are provided in Table S1.

233 2.4 Real-time quantitative PCR

234 At day seven or 17 of adipocytic differentiation, RNA was extracted with Trizol and cDNA was synthesized using Taq DNA polymerase (catalog no. 10342020, Life Technologies) according to 235 236 manufacturer's instructions. Quantitative PCR was performed in technical triplicates using the Fast 237 SYBR Green qPCR Mastermix with 250 µM of primer concentration (Applied Biosystems) on 238 QuantStudio 6 (Life Technologies). Primers were pre-validated for high efficiency and internally tested 239 not to vary upon OP9 differentiation. The geometric mean of housekeeping genes (RPL13 and YWHAZ) 240 was used as reference to calculate the fold expression of each gene relative to the samples of 241 undifferentiated OP9 cells as described for the delta-delta normalization method (45,46). Primer 242 sequences obtained from Microsynth are listed in Supplementary Table S2.

243 **2.5** Bone marrow extraction and HSPC sorting

244 Total bone marrow was extracted from eight-week-old B6 ACTb-EGFP females housed in 12-hour 245 day-night light cycles and provided ad-libitum sterile food and water as described previously, in 246 accordance to Swiss law and with the approval of cantonal authorities (Service Veterinaire de l'Etat de 247 Vaud) and complying with ARRIVE guidelines. Total bone marrow cells were extracted from femur, 248 tibia and pelvis by crushing using a mortar and pestle in ice-cold PBS supplemented 1mM 249 ethylenediaminetetraacetic acid (EDTA, catalog no. 15575020, Thermo Fisher Scientific). The 250 samples were dissociated and filtered through a 70µM cell strainer (catalog no. 352350, Falcon), lysed 251 for 30sec at room temperature in red blood cell lysis buffer (Biolegend, catalog no. 420301), washed 252 with ice-cold PBS-EDTA and centrifuged at 1300rpm for 10min at 4°C. For HSPC sorting prior to co-253 culture with OP9 stroma, the cell pellet was stained with 50μ l biotinylated 'lineage' antibody cocktail 254 (BD, catalog no. 558451) in 1ml PBS-EDTA per six bones for 15min on ice. The sample was washed 255 with PBS-EDTA and stained with 50µl magnetic beads of the same kit in PBS-EDTA for 10min on 256 ice. The samples were then washed and filtered through a 70μ m cell strainer (catalog no. 352350, 257 Falcon) prior to lineage depletion using the AutoMACS Pro (Miltenyi Biotec, USA). After depletion 258 the negative fraction was resuspended in an antibody mix containing antibodies against Streptavidin-259 TxRed (1:200), cKit-PECy7 (1:200), Sca1-APC (1:100) and PI (1:1000). Cell sorting was performed 260 on a FACSAria Fusion (Becton Dickinson, USA) cell sorter.

261 2.6 In vitro HSPC co-culture

262 OP9 cell seeding as detailed in Section 2.1, was followed by a short-term in vitro adipocyte 263 differentiation of OP9 cells, and then a gently wash with PBS and pre-warmed Iscove's Modified 264 Dulbecco's Medium (IMDM, catalog no. 12440053, Gibco) supplemented 10 % FBS and 1% 265 Pen/Strep (P/S) was added. The washing was done using the Caliper Sciclone ALH 3000 (Caliper Life 266 Sciences, USA) and consisted of four cycles of removing 140μ l media and adding 140μ l fresh IMDM 267 media, to dilute the previous media so as to keep mature adipocyte detachment to a minimum. The wells were left with 100µ1 IMDM 10% FBS and 1% P/S. HSPCs extracted from B6 ACTb-EGFP mice 268 269 were plated at a ratio of 1:10 initial OP9 cells (in volume of 100μ l IMDM, totaling 200 μ l volume per 270 well).

- 271 After seven days of co-culture, the 96-well plates were removed from the incubator and placed on ice.
- To count non-adherent cells, the cells in suspension were retrieved through repeated washes and stained
- with CD45-PacBlue at final concentration of 1:200, PI at 1:1000 and 5μ l CountBright beads (catalog
- no. C36950, Invitrogen) directly added to the media. Without any further manipulation, the plates were analyzed using the High Throughput Sampler (HTS) module of a LSRII (Becton Dickinson, USA)
- flow cytometer. For accuracy, the settings were programmed to mix the wells thoroughly before sample
- 277 uptake. To count adherent cells, a separate set of duplicate plates were taken from the incubator, the
- media manually removed, and wells washed once with 200μ l PBS. 40μ l of Trypsin EDTA (0.5 %,
- 279 catalog no. 25300-054, Gibco) was added to each well and incubated for five minutes at 37°C. 160µ1
- of ice-cold PBS containing FBS (to neutralize the trypsin), CD45-APCCy7 (1:200), PI (1:1000) and 5μ l CountBright beads were then added to the plates. The plates were stained on ice and directly
- analyzed with flow cytomet.

283 **2.6.1** In vitro conditioned media preparation and culture

284 Conditioned media was prepared for four different conditions as follows. For the undifferentiated 285 condition, undifferentiated OP9 cells were seeded in six-well plates at confluency (20,000 OP9 286 cells/cm²) in IMDM (catalog no. 12440053, Gibco) supplemented 10% FBS and 1% P/S (basal IMDM) 287 and conditioned medium was harvested after two days in culture and stored at -20°C. For the spontaneous differentiation conditions, confluent OP9 cells were grown in MEMa as described for the 288 short-term differentiation assays except that at day five media was changed to IMDM following three 289 290 washes; conditioned medium was then harvested after two days and stored at -20°C. For the induced 291 adipocytic conditions, confluent OP9 cells underwent the short (5 day) or long (17-day) DMI 292 adipogenic induction protocols before changing media to basal IMDM for two additional days of 293 culture; conditioned medium was then harvested and stored at -20°C. Subsequently 2000 live-sorted 294 HSPCs were plated per well in 96-well round-bottom plates in the absence of stroma. Different ratios 295 of conditioned-to-basal IMDM media were added. After two-day culture the cells were stained with 296 Annexin V, Propidium Iodine (PI) and CD45 to select for live CD45 hematopoietic cells via flow 297 cytometric analysis with an LSRII cytometer (Becton Dickinson, USA). CountBright beads were added 298 to count absolute cell numbers as described above.

299 **2.6.2** Cobblestone formation assay

300 OP9 cells were plated at 20,000 cells/cm² in gelatin-pre-coated 6-well plates and cultured with α-MEM 301 media supplemented with 10%FBS and 1%P/S. The cells were induced to differentiate using the 302 standard differentiation cocktail DMI, which was changed two times per week. After seven days of 303 differentiation, the media was washed three times with PBS and changed to basal IMDM. On the same 304 day, 200 sorted KLS cells were added into each well. After 7 days of co-culture the hematopoietic colonies were manually counted (47) and scored the following way: (i) shiny colonies (defined by 305 306 majority of round and bright cells, as they float above the OP9 stromal layer, with no more than 10 307 cobblestone cells visible per colony; (ii) cobblestone colonies (defined as darker 'cobblestone'-like 308 cells, because they are below the OP9 stromal layer) should not contain more than 20% of shiny cells; 309 and (iii) mixed colonies that contain both shiny and cobblestone cells(48–50).

310 2.7 Clinical samples

- 311 This study complied with the Declaration of Helsinki and the local ethical authorities (CER-VD). All
- 312 patients signed a specific consent for the reuse of biological samples in the context of our study. For
- 313 HPLC, iliac crest BM aspirates from female and male patients undergoing treatment for acute myeloid
- leukemia (between ages 48 and 73, n=5) or surgical debris from patients undergoing hip replacement
- 315 surgery (between ages 56 and 80, n=4) were received for analysis from the Lausanne University
- 316 Hospital (Centre Hospitalier Universitaire Vaudois, CHUV). Floating adipose tissue, serum, and oil
- 317 fractions were isolated and processed for lipid extraction and processed as described for OP9
- 318 lipidomics.

319 2.8 Statistical Analysis

320 Values are shown as mean plus or minus the standard deviation or standard error of the mean as

- indicated. Student's t-test was performed for all experiments when comparing two conditions only, or
 a Two-Way ANOVA when comparing multiple conditions, with P-values indicated for statistical
- 323 significance.

324 Raman spectra were processed by using the PLS Toolbox and the MIA toolbox (v8.7 eigenvector 325 Research, Inc., USA). The spectra were baseline corrected, normalized and mean centered. Two processes were done on Raman spectra. First, cluster analysis (CA) was performed using Ward's 326 method. CA clustered individual Raman spectra into 3 categories (saturated-rich lipids, unsaturated-327 328 rich and mixture of saturated and unsaturated lipids). Second, principal component analysis (PCA) was 329 used which highlighted the difference in spectral features between the spontaneous and induced 330 conditions. All data are reported as mean ± standard deviation. All statistical analyses were performed 331 using GraphPad Prism (version 8.0.0, GraphPad Software). Mean values were compared by Mann and 332 Whitney test, or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. 333 Statistical significance was accepted for P<0.05, and reported.

334

335 **3 Results**

336 3.1 Differential adipocyte lipidation and similar transcriptional signature upon adipogenic 337 induction by confluency (spontaneous) versus DMI cocktail (induced).

338

We chose murine BM-derived OP9 stromal cells as a model for this study because of their propensity to differentiate into adipocytes while displaying both hematopoietic support and dual adipogenic and osteogenic differentiation capacities as compared to other commonly used murine stromal lines including BM-derived MS5 and C3H10T1/2 lines or MC3T3 subclones (Figure S2A-C and (42)). Note retention of alkaline phosphatase activity in confluent and fully mature, lipidated OP9-derived O Red Oil positive adipocytes (Figure S2D), reminiscent of properties assigned to hematopoietic supportive AP+ reticular adventitial cells in the bone marrow (51) and possibly of AdipoCARs (52).

The non-clonal OP9 stromal cell line (53,54) was originally derived from the calvaria of newborn osteopetrotic mice ((C57BL/6xC3H) F_2 -op/op) deficient in macrophage colony-stimulating factor (M-

348 CSF), and were shown optimal for studies of hematopoietic cell development and differentiation 349 sensitive to M-CSF (55,56). OP9 cells have been described as preadipocytes with multilineage differentiation capacity which readily generate mature adipocytes, and express the transcriptional 350 351 activators CCAAT/enhancer binding proteins (C/EBP) α and β , together with the master regulator of 352 adipocyte differentiation peroxisome proliferator activated receptor-y (PPARg), and perilipin (PLIN), 353 a phosphoprotein associated with the surface of lipid droplets (LDs) (21,54). Thanks to their 354 multipotency, hematopoietic support, and adipocytic differentiation capacities, OP9 cells are thus ideal 355 for in vitro studies on BM adipogenesis. Due to their non-clonal nature and inherent heterogeneity, 356 they serve as a BM model for studying BMAd differentiation at the single cell level.

357 In vitro, the OP9 cell line spontaneously differentiates into adipocytes upon confluency in the presence of serum (spontaneous OP9 adipocytes, sOP9), a phenomenon that can be more robustly promoted by 358 359 exposure to a classical adipocytic differentiation cocktail consisting of insulin, dexamethasone, and 3-360 isobutyl-1-methylxanthine (induced OP9 adipocytes, iOP9) (21,53). After 17 days in culture, both sOP9 and iOP9 culture conditions contained numerous differentiated OP9-adipocytes (Figure 1) with 361 362 a significantly greater prevalence of Oil Red O staining of lipids in the induced condition (iOP9) 363 (p<0.01) as compared to control baseline undifferentiated OP9 cells plated at sub-confluency (uOP9) (Figure 1G). Spontaneous OP9-adipocytes formed under the minimal confluency condition (sOP9) and 364 365 stained with Oil Red O at intermediate levels between undifferentiated uOP9 cells and induced iOP9 366 cultures (Figure 1G). No significant amount of mature adipocytes could be visualized in the uOP9 367 condition (Figure 1A,D). Adipocytes were defined as previously described, where cells containing at 368 least four identifiable lipid droplets were accounted as mature adipocytes (57). Morphologically, both 369 sOP9 and iOP9 adipocytes stained with Oil Red O at day 17, but iOP9 adipocytes constituted the 370 majority of the cultures and often contained large lipid droplets, while sOP9 adipocytes were sparser 371 and their lipid droplets seemed smaller.

Given the intermediate phenotype of sOP9 adipocytic cultures, containing a mixture of mature adipocytes and non lipidated stromal cells likely including preadipocytes, we wished to determine whether the transcriptional profile and lipid composition of these cultures more resembled the undifferentiated uOP9 cultures or the highly lipidated iOP9 adipocytic cultures. We thus compared transcriptional adipocyte differentiation markers by RT-qPCR and the global fatty acid (FA) composition by high performance liquid chromatography (HPLC) for the three conditions.

378 RNA transcripts for the canonical adipocyte differentiation and maturation program (Fabp4, Adipoq, 379 Cebpa, Pparg, Lpl, Pnpla2 -also known as Atgl-) in the sOP9 condition most resembled iOP9 380 adipocytic cultures, with average gene expression more than 10 times higher for the sOP9 and iOP9 381 conditions than for the control uOP9 condition (Figure 2A). These results were further validated in the 382 OP9-EGFP cell line used in previous studies (Figure S3A-B, (14)). Further analysis of the desaturation 383 and elongation pathway for fatty acid synthesis showed a similar pattern (Figure 2B), with overall higher expression for the sOP9 and iOP9 adipocytic conditions as compared to the uOP9 384 385 undifferentiated condition. Of note RNA transcripts for Sds1 and Fads2 were however significantly 386 between sOP9 and iOP9 cultures. We thus conclude that, at the population level, expression of genes 387 related to the adipocyte differentiation and fatty acid desaturation programs is similar for both sOP9 388 and iOP9 adipocytic conditions after 7 days of culture, suggesting that the transcriptional program is

triggered in sOP9 cells prior to lipid droplet accumulation.

390 Conversely, total FA content and profiles were similar for control uOP9 cells and the sOP9 adipocytic 391 condition as measured by HPLC analysis. Classical adipogenic induction of iOP9 cells resulted in a 392 16-fold increase of total FA content as compared with sOP9 cells or uOP9 cells 393 $(2.4x10^{4}\pm 2.2x10^{4}pmol/cm^{2})$ versus $1.5 x 10^{4} \pm 7.0 x 10^{3} pmol/cm^{2}$ and 17.4x10⁴±3.7x10⁴pmol/cm² 394 respectively, Figure 2C). FA profiles revealed a preferential enrichment of MUFAs in iOP9 cells (47% 395 MUFAs versus 38% in uOP9s and 36% in sOP9 cells), mostly to the detriment of PUFAs (only 2% in 396 iOP9 cells versus 6% PUFAs in uOP9s and 10% in sOP9 cells, p<0.0001) (Figure 2D, G). In terms of 397 individual FAs, the most striking finding was the marked high ratio of palmitoleic acid (16:1) in iOP9 398 cells, representing 29% of total FA (versus 9% in uOP9 or sOP9 cells, p=0.007) (Figure 2D, E). This 399 result was associated to a reduction of 18 carbon FAs in iOP9 cells and a trend towards a consistent 400 reciprocal enrichment of 18 carbon FAs in sOP9 cells: stearic acid (18:0, sOP9 11% vs. iOP9 4%, 401 p=0.02), oleic acid (18:1, sOP9 26% vs. iOP9 16%, p=0.07) and linoleic acid (18:2 n-6, sOP9 4% vs. 402 iOP9 1%, p=0.007). The most abundant PUFA species was arachidonic acid (20:4 n-6). In relative 403 terms, linoleic (18:2 n-6) and arachidonic acid (20:4 n-6) were significantly enriched in the sOP9-404 adipocytes at 5% of total FA while making up only 1% of total FA in iOP9-adipocytes. Notably, the 405 ratio of palmitoleic acid (16:1) versus its saturated form (stearic acid, 16:0) in iOP9 adipocytes was 406 three times higher than in sOP9 adipocyte cultures or uOP9 control cells (Figure 2E). The ratio of oleic 407 acid (18:1) versus its saturated form (stearic acid, 18:0) showed a similar but non-significant trend 408 (Figure 2F). These results are very similar to those obtained on rats' tibiae when comparing lipid 409 profiles from constitutive adipocytes versus regulated adipocytes (1). Our data is also congruent with 410 the results shown for rabbit BMAds when comparing FA composition of rabbit BMAs from rBMAd-411 rich versus cBMAd-rich sites ((33) and summarized in Table 1).

412 Overall, these results indicate that, at the population level, sOP9 adipocytic cultures share 413 transcriptional similarities in the adipocyte differentiation program with iOP9 cells, but are less 414 lipidated and present lower abundance of unsaturated FAs. This constellation would be consistent with 415 our hypothesis that sOP9 adipocytic cultures present similarities with regulated BMAds, and thus an 416 enrichment in saturated FA, as compared to cBMAd-like iOP9 adipocytic cultures. Given the 417 heterogeneous lipidation of sOP9 adipocytic cultures and the inevitably bulk nature of our RT-PCR 418 and HPLC-based analysis, we set out to validate our hypothesis through a single cell analysis pipeline 419 that could directly compare sOP9 and iOP9 adipocytes on their relative FA acid composition at the 420 single cell level.

421

422 3.2 Raman microspectroscopy detects unsaturated spectra at the single lipid droplet level in 423 spontaneous and induced OP9-derived adipocytes.

Raman microspectroscopy represents a powerful yet non-invasive and label-free method to assess the lipid composition of adipocytes *in vitro*. The unsaturation ratio is recognized as a measure of the 426 proportion of unsaturated FA obtained by the ratio of the peak area assigned to unsaturated bonds 427 (C=C) divided by the peak area assigned to saturated bonds (CH₂) (Equation 1) (58–61).

428

429
$$Unsaturation \ ratio = \frac{A(vC=C)}{A(vCH_2)}$$
(1)

430 Equation 1: Lipid unsaturation ratio measured by Raman microspectroscopy.

431 Our feasibility test with Raman microspectroscopy imaging showed that it was possible to acquire 432 Raman spectra at single lipid droplet (LD) resolution in our model (Figure 3). After 17 days of culture, 433 Raman spectra, representative of the molecular composition of individual LDs, were acquired for sOP9 434 and iOP9 adipocytes through high resolution Raman imaging. No analysis was performed for the 435 undifferentiated (uOP9) condition as it presented with very few, if any, LDs. Raman images of individual adipocytes were processed by PCA. The PCA shows that a mixture of spectra exists between 436 437 the adipocytes but also within the LDs themselves, as illustrated by the Raman images (Figures 3B, 438 3E). In Figure 3C, the PC1 scores (96.68%) show a representative spectrum rich in unsaturated lipids 439 for the iOP9 adipocyte shown in Figure 3A-B. In Figure 3F, the PC1 scores (95.69%) show a spectrum 440 representative of a saturated rich lipids for the sOP9 adipocyte shown in Figure 3D-E. Figure S4 shows 441 the additional variation captured by PC2 and PC3 (<5%) for the same two adipocytes shown in Figure 442 3. We conclude that LDs composed of predominantly unsaturated-rich or saturated-rich lipids can be 443 identified through Raman microspectroscopy within OP9-derived adipocytes with single LD 444 resolution.

3.3 Raman microspectroscopy reveals differential predominance of unsaturated spectra in large lipid droplets from induced adipocytes

447 In order to determine if significant differences exist in the unsaturation ratio of FAs accumulated within 448 sOP9 versus iOP9 adipocytes, we thus proceeded to the acquisition of Raman spectra from individual LD in both conditions and throughout 3 separate experimental campaigns (iOP9: n = 138 adipocytes 449 and 2971 spectra; sOP9: n = 120 adipocytes and 2944 spectra; lateral resolution $1-2\mu m$), and combined 450 451 all data for aggregated principal component (PC) analysis in both conditions. Figure S5 shows the 452 classification of spectra using unsupervised hierarchical cluster analysis, which predicted three main 453 categories: saturated-rich, unsaturated-rich and mixture. The representative spectrum of each category 454 is shown in Figure 4A. PCA was then performed on the averaged spectra per adipocyte. The PC1 455 loading plot contains characteristic spectra where the positive peaks correspond to saturated lipids (1061, 1129, and 1296 cm⁻¹, represented in red for Figures 4A-B) and the negative peaks correspond to 456 457 unsaturated lipids (1080, 1267, and 1655 cm⁻¹, represented in blue for Figures 4A-B) (58). PC1 captures 458 78.20% of the variability of the overall dataset, and separates the Raman spectra based on the saturated-459 rich and unsaturated-rich profiles (Figure 4B). On the PCA score plot, each point represents an 460 averaged spectrum per adipocyte (Figure 4C). Most of the spectra from iOP9-adipocytes (81%) form 461 a cluster in the negative score along PC1, while the spectra of sOP9-adipocytes are scattered along 462 PC1. This indicates that the molecular composition of LDs in sOP9-adipocytes is heterogeneous 463 compared to iOP9-adipocytes, and that iOP9-adipocytes are enriched in unsaturated-rich lipids. Score

464 plots in Figure 4C and 4D are equivalent, but the points in Figure 4C are colored according to PC1 465 scores, and the points in Figure 4D are colored according to the culture condition. Specifically, the 466 points in dark red and dark blue in Figure 4C correspond to Raman spectra of saturated-rich and 467 unsaturated-rich lipids, while intermediate colors point to Raman spectra of a rather balanced mixture of saturated and unsaturated lipids. PCA analysis shows that LDs in iOP9-adipocytes are mainly rich 468 469 in unsaturated lipids, while LDs in sOP9-adipocytes are composed of a rather balanced mixture of 470 unsaturated and saturated lipids (Figure 4D). However, when comparing the averaged raw unsaturation 471 ratio (based on averaged spectra per adipocyte) we found no significant difference between iOP9 472 adipocytes and sOP9 adipocytes (Figure 4E). At the adipocyte level, both conditions have a similar 473 averaged unsaturation ratio. This result is explained by PCA analysis which shows that LDs of both conditions are not solely composed of either uniformly saturated or unsaturated lipids, but rather by 474 475 different mixtures thereof. We thus took advantage of the 1-2µ resolution of our setup and performed 476 the analysis at the single LD level.

477 First, we hypothesized that the smaller diameter of LDs in sOP9 adipocytes may be related to their 478 lower unsaturation ratio, owing to the higher mobilization rate of unsaturated FAs (62). We thus plotted 479 LD diameter versus unsaturation ratio (Figure 5A-B). The LD diameter did not correlate with the 480 unsaturation ratio. Specifically, correlation analysis for the LD diameter to unsaturation ratio shows 481 that the parameters are not linearly correlated in either condition (Figure 5A-B, R²_{sout}=0.0013; 482 $R_{ind}^2=0.0107$). Even when the spectra are separated according the 3 types of lipids (saturated-rich, 483 unsaturated-rich and mixture) the LD diameter and the unsaturation ratio are not linearly correlated. 484 All the R² are inferior to 0.05 (Figure S6). Since, no correlation was observed, the LD diameter and the 485 unsaturation ratio were explored separately.

486 The averaged LD diameter was significantly lower (p<0.0001) in the sOP9-adipocytes ($5.24\pm1.85\mu$ m) 487 than the iOP9-adipocytes (7.87 \pm 3.83 μ m) (Figure 5C). When we further separated LDs by type of 488 lipids, we found that saturated-rich, unsaturated-rich or mixture LDs are not different in their average 489 composition when originating from iOP9 or sOP9 adipocytes, but unsaturated LDs are more frequent 490 in iOP9s while saturated LDs are more frequent in the sOP9 condition (Figure 5D-E, Figure S5B). 491 Unsaturated-rich LDs are overall bigger in size than saturated-rich LDs, with a predominance in iOP9 492 adipocytes, particularly above the diameter of 10 μ m. In iOP9 adipocytes, the number of unsaturated-493 rich LDs is higher compared to sOP9 adipocytes for LD diameter superior to 10 μ m.

494 Then, the averaged unsaturation ratio per condition were obtained from individual spectra of LDs. The 495 unsaturation ratio of iOP9 condition (0.17 ± 0.05) is significantly higher than sOP9 condition 496 (0.14±0.05, p=0.0014). Also on average, LDs of iOP9 condition have more unsaturated-rich lipids 497 compared to the LDs in sOP9 condition. Since differences are observed between sOP9 and iOP9 around 498 a LD diameter of 10 μ m (Figure 5E), the unsaturation ratio of LDs was investigated as function of the threshold of $10 \,\mu m$ (Figure 5G). The larger LDs (>10 μm) have the highest unsaturation ratio compared 499 500 to smaller LDs. Smaller LDs ($<10\mu$ m) from iOP9 adipocytes have a higher unsaturation ratio compared 501 to sOP9-adipocyte. Then, we investigated the unsaturation ratio as a function of the 3 types of LDs, 502 namely saturated-rich, mixture or unsaturated-rich. Although we found consistent differences between 503 the three classes of LDs, there were no significant differences between LDs of the same class in sOP9

adipocytes versus iOP9-adipocytes (Figure 5H). Not surprisingly, mixture LDs showed the greatest heterogeneity in their unsaturation ratio (Figure 5H). Overall, we conclude from this data that iOP9 adipocytes contain significantly bigger LDs and more frequently unsaturated LDs than sOP9 adipocytes. *In vivo*, both higher LD diameter and higher FA unsaturation are characteristics of cBMAds, while smaller LD diameter and lower unsaturation ratio are characteristics of rBMAds. We could not find a consistent relationship between LD diameter and unsaturation ratio.

510

511 **3.4** Hematopoietic support capacity of sOP9-adipocytes is superior to that of iOP9-adipocytes

512 Another *in vivo* property of rBMAds as compared to cBMAds is their differential capacity to serve as 513 a supportive niche to hematopoietic progenitor cells. Indeed, cBMAds are associated to the 514 hematopoietic poor -yellow- regions of the marrow, while rBMAds are associated to the hematopoietic-515 rich -red- regions of the marrow. To determine whether sOP9 versus iOP9 cultures behave different in 516 terms of hematopoietic support, we co-cultured murine hematopoietic stem and progenitor cells 517 (HSPCs) isolated as cKit⁺LinScal⁺ (KLS) cells for seven days with the differentiated OP9 cells at a 518 ratio of 1 HSPC to 10 OP9 cells. As in previous work (63), this high ratio of KLS to OP9 cells serves 519 to avoid dispersion due to HSPC heterogeneity and avoids downstream stochastic, clone effects on analysis secondary to differences in the content of the most primitive and thus proliferative HSPCs 520 521 across wells, which although small, would be exponentially exaggerated after co-culture. At the end of 522 the seven-day co-culture period, flow cytometric analysis revealed that total hematopoietic expansion 523 of 2,000 seeded HSPCs was 4.5-fold lower (p<0.0001) when iOP9 cultures were used as feeder as 524 compared to the sOP9 condition (Figure 6A). Of note, total hematopoietic cells in suspension were 525 significantly lower in the iOP9 versus sOP9 condition (p<0.05) whereas the more immature progenitor cells that are typically in close contact and adherent to the feeder cells were proportionally lower in the 526 527 sOP9 condition. This suggests that iOP9 cultures may preferentially favor support of the more primitive 528 hematopoietic progenitors, which is congruent with previous data from purified human BMAd as 529 compared to undifferentiated stromal cells (8). The more hematopoietic progenitor supportive function 530 of the sOP9 condition is also illustrated by the quantification of colonies formed by day 7 of co-culture 531 (Figure 6B). The total number of hematopoietic colonies per initial number of HSPCs seeded, was 532 significantly higher in the sOP9 coculture (0.13 colonies per initial KLS seeded) than in the iOP9 533 coculture (0.01 colonies per initial KLS seeded). All three colony categories, from the shiny consisting 534 of more mature hematopoietic cells to the mixed and cobblestone colonies which contain the most 535 immature hematopoietic progenitors were significantly increased (P<0.05) in the sOP9 condition. To 536 understand to which extent secreted factors from either condition contributed to hematopoietic 537 expansion, HSPCs were cultured for two days in the absence of feeder cells but in the presence of 538 different concentrations of 48-hour conditioned media from either uOP9, sOP9 or iOP9 cultures. For 539 the iOP9 condition we tested both a short (5+2 days) and a long (17+2 days) DMI adipogenesis induction protocol (Figure 7C). Hematopoietic expansion was greatest for HSPCs cultured in the 540 541 highest concentration of undifferentiated-conditioned medium. HSPCs cultured in conditioned 542 medium from iOP9-adipocytes did not show a significant expansion after two days in culture, 543 independently of the length of the iOP9 DMI induction protocol. However, the conditioned medium

- 544 from sOP9 cultures was substantially more supportive to HSPC maintenance than iOP9, although less
- 545 than those cultured in conditioned media from undifferentiated OP9 cells. This effect increased with
- 546 higher ratios of conditioned-to-basal medium. We therefore conclude that the *in vitro* hematopoietic
- 547 progenitor support capacity of sOP9 cultures is superior to that of iOP9 cultures, aligning with the *in*
- 548 *vivo* functionality of regulated versus constitutive BMAds.

549 3.5 HPLC fatty acid quantification in primary human samples confirms a higher 550 unsaturation ratio in constitutive BMAd-rich marrow (femoral head) versus regulated 551 BMAd-rich marrow (iliac crest post-chemotherapy).

- 552 In order to contextualize and validate the value of the FA unsaturation ratio used throughout this
- work as a surrogate marker for regulated versus constitutive BMAds, we set to test whether we could
- 554 find differences in the unsaturation ratio of lipids extracted from one of the most extreme BM
- remodeling scenarios. Patients suffering from acute leukemias receive intensive aplastic
- 556 chemotherapy, a regime that eliminates leukemic cells but also virtually all short-term hematopoietic
- 557 progenitors in the BM, leading to a rapid adipocytic conversion of the marrow. BMAds formed in
- this context should represent a stereotypical example of rBMAd, and we thus set out to compare the
- unsaturation ratio of lipids obtained from iliac crest samples from these patients as compared to the
- 560 lipid fraction obtained from surgical debris of adult patients undergoing elective hip replacement
- surgery, which is thought to represent a cBMAd-rich site. Analysis of these primary human BM lipid
- samples reflected a preferential enrichment of MUFAs in the post-chemotherapy iliac crest BM
- samples (regulated BMAd site) versus the femoral head BM samples (constitutive BMAd site) from
- hip replacement surgery (39.6% versus 51.2%, p=0.02) to the detriment of PUFAs (18.6% versus
- 565 17.0% p=0.80) and SFAs (41.8% and 36.6%, p=0.04) (Figure 7D). While rapid conversion to
- adipocytic marrow is induced by chemotherapy, and thus iliac crest samples are interpreted as
- 567 rBMAd-rich, the differences in fatty acid profiles displayed could also be an inherent function of the
- 568 anatomical site. The largest difference between FA species in the regulated versus constitutive
- 569 marrow sites was seen in the 18:1 to 18:0 unsaturation ratio (4.40 versus 7.91, p=0.007), and the 16:1
- 570 versus 16:0 unsaturation ratio (0.11 versus 0.19, p=0.031) (Figure 7B-C). We thus conclude that
- 571 human BM samples from a rBMAd-rich site in the context of BM remodeling driven by
- hematopoietic demand present a lower unsaturation ratio, as measured by 16:1/16:0 and 18:1/18:0-
- 573 FA content, than samples obtained from a cBMAd-rich site, which complements previously reported
- results for rabbit and rats marrow at steady-state (1,33–35) as summarized in Table 1.

575 Table 1: Fatty acid unsaturation ratios in rat, rabbit, human, and OP9 cells from red and yellow

576 **BMAds**. The 16:1/16:0 and 18:1/18:0 unsaturation ratios were calculated from the proportion of total fatty acids. Rat BM data from Scheller et al. 2015 Supplementary Data 1 (regulated: femur and proximal tibia,

- 578 constitutive: distal tibia). Rabbit BM data from Tavassoli 1977 Table 1 (regulated: vertebrae, constitutive: os
- 579 calcis). Human original data from Figure 7 (regulated: iliac crest post-chemotherapy, constitutive: femoral head)
- and OP9 cell original data from Figure 6 (regulated: spontaneous, constitutive: induced) from HPLC analysis.
- 581 Additional measurements in human BM have been made by MRI spectroscopy but cannot be compiled with
- these data due to the different methodology (35,64). c: constitutive, r: regulated.

Running Title

Origin	BMAd type	16:1/16:0	c/r	18:1/18:0	c/r	Reference
Rat	constitutive regulated	0.25 0.09	2.8	1.18 0.81	1.5	Scheller et al. 2015
Rabbit	constitutive regulated	0.53 0.16	3.3	7.52 2.68	2.8	Tavassoli et al. 1977
Human	constitutive regulated	0.19 0.11	1.7	7.91 4.40	1.8	original data
OP9 cells	induced spontaneous	0.84 0.24	1.6	4.74 2.33	2.0	original data

Taken together, we conclude that the spontaneous versus induced OP9 *in vitro* adipogenesis model presented here replicates key differences in lipid droplet size, FA unsaturation ratio and hematopoietic support respectively associated *in vivo* to regulated versus constitutive BMAd subtypes. We additionally validate the value of the 16:1/16:0 FA unsaturation ratio as a surrogate marker to segregate rBMAd-rich versus cBMAd-rich tissues both *in vivo* and for our *in vitro* model, and the feasibility of Raman microspectroscopy to reveal equivalent differences in the unsaturation ratio at the single adipocyte level.

590

591 4 Discussion

592 BMAds constitute a distinct fat depot with low responsiveness to cold and insulin, overall relative 593 resistance to lipolysis, and high expression of adiponectin as compared to subcutaneous fat depots 594 (37,65,66). Two BMAd subtypes have been described based on their differential response to 595 physiological and metabolic stimuli: the more stable so-called constitutive (cBMAd) depots, 596 preferentially located in the distal parts of the skeleton, and the more labile so-called regulated 597 (rBMAd) adipocytes, which are interspersed within the hematopoietic marrow in more proximal 598 skeletal sites. Due to their fragility and location within the bone cavity, access to live BMAd cells for 599 functional and cell trajectory analysis is not trivial, especially for murine BMAd depots where BM 600 adipose tissue volume is very limiting. Although possibly limited on the extrapolation of specific metabolic behaviors (37), in vitro models of adipogenesis from undifferentiated stromal progenitors 601 602 have been proposed as a complementary tool to study BMAd at the cellular level, so as to facilitate 603 both gene function studies and cell behavior analysis (reviewed in (32,67)). To our knowledge, no in 604 vitro model has been described to recapitulate some or all of the differential characteristics of the in 605 vivo defined rBMAd versus cBMAd depots. Here we present a model of BM adipogenesis in the form 606 of a differential in vitro culture system for BM-derived OP9 stromal cells. When classically induced 607 by an adipogenic cocktail (iOP9s) OP9-derived adipocytes resemble cBMAds and, when allowed to

608 spontaneously differentiate by confluency in serum-containing conditions (sOP9s), they resemble 609 rBMAds in lipid droplet size, overall lipid saturation, 16:1/16:0 FA unsaturation ratio and 610 hematopoietic supportive properties. Indeed, we validated the value of the 16:1/16:0 FA unsaturation 611 ratio, already described as a discriminating factor for cBMAd and rBMAd depots in rabbit and rat 612 homeostatic tissues, in both human BM and as a marker of cBMAd-like (iOP9) versus rBMAd-like 613 (sOP9) in vitro cultures. Furthermore, we have extended the reproducibility of the 16:1/16:0 FA 614 unsaturation ratio as a discriminating factor to highly remodeled rBMAd depots in the context of 615 patients undergoing intensive ablative chemotherapy. It is worth noting that the robustness of 616 differences in the 16:1/16:0 FA unsaturation ratio in our model (summarized in Table 1; (1,68), as 617 opposed to other proposed FA pairs (e.g. 18:1/18:0), is somehow surprising and may be associated to 618 the low mobilization rate for palmitate (16:0) and palmitoleate (16:1) (69,70). Further studies should 619 mechanistically address the relative contribution of de novo lipogenesis and FA re/uptake in lipid 620 composition differences underlying the 16:1/16:0 FA unsaturation ratio. Indeed, at the RNA level, 621 sOP9 adipocytes had significantly higher expression of Lpl, which mediates fatty acid uptake for 622 storage, while iOP9 adjocytes had increased expression of *Atgl*, the key enzyme that initiates 623 hydrolysis of TAGs.

After HPLC-based lipid composition analysis of OP9 cultures at the cell population level, and given 624 625 the relative heterogeneity of in vitro adipogenic cultures (67), we implemented a novel application of 626 Raman microspectroscopy to measure lipid unsaturation heterogeneity at the single cell level, with 627 individual LD resolution and in a label-free manner. This approach allowed us to focus lipid 628 composition analysis exclusively on mature adipocytes and not on the remaining unlipidated stromal 629 component. Previous studies have shown the application of Coherent anti-Stokes Raman 630 microspectroscopy to embryonic stem cells undergoing adipogenic differentiation, showing a measure 631 of chemical lipid saturation composition and an increase in LD accumulation with differentiation 632 (71,72). While, very interestingly, individual LDs within one adipocyte appear to have an 633 inhomogeneity in their chemical composition, this was not further analyzed. Raman microspectroscopy has allowed for the characterization of LD lipid composition in animal and in vitro models. Lipid 634 635 droplets have been measured directly in the endothelial layer of murine aortic sections by, pointing to smaller LDs induced by TNFalpha preferentially containing more unsaturated lipids whereas larger 636 637 LDs were more rich in saturated lipids (73). Another Raman-based study showed interesting 638 differences in the pattern of distribution between saturated FA species (palmitic acid -16:0- and stearic 639 acid -18:0), which were evenly distributed in broad areas of the cell, and unsaturated FA species (oleic 640 acid -18:1- and linoleic acid -18:2-), which were concentrated in LDs, after exposure and uptake in 641 macrophages (74). The effect of the high fat diet was evaluated on the lipidic composition of 642 perivascular adipocytes in a mouse model. The size of the LDs was increased as an adaptive response 643 to the excessive lipid income. In an *in-vitro* study, the effect of TNF- α on endothelial HMEC-1 cells 644 during 24h was monitored by Raman microspectroscopy. The composition and the distribution of 645 cellular lipids was modified upon exposure to TNF- α , and the increase in LD size was concomitant 646 with the formation LDs composed of unsaturated rich lipids (75,76). However, none of these studies 647 provide supporting statistical analysis for an univocal association between LDs size and molecular 648 composition. In all, we found Raman microspectroscopy to be robust for the measurement of the 649 unsaturation ratio at the lipid droplet level. Congruently to the HPLC-derived data, we could thus 650 confirm at the single adipocyte level the higher unsaturation ratio of iOP9 lipids as compared to the 651 sOP9 condition. It is worth noting that the overall differences in unsaturation ratio were more 652 pronounced when measured with HPLC analysis (1.02±0.27 for iOP9-cultures versus 0.19±0.11 sOP9cultures, p=0.0004, Figure 2E), than with Raman (0.17±0.05 for iOP9-cultures versus 0.14±0.05 sOP9-653 654 cultures, p=0.014, Figure 5F). This difference could be related to the sample bias, as HPLC analysis 655 was done in lipid extracts from the bulk culture while Raman microspectroscopy acquired spectra from 656 mature adipocytes only, or to the method itself. For the HPLC analysis, the unsaturation ratio is 657 calculated from the contribution of isolated and purified lipids. On the other hand, Raman analysis is 658 done inside the LD where lipids are mixed. The unsaturation ratio is calculated from the contribution 659 of the mixture of lipids which hamper the signal. Indeed, the small differences we report in the 660 unsaturation ratio are in the range of previous Raman studies cited above, although differences are 661 more pronounced if only the more defined fractions are considered (unsaturation-rich versus saturated-662 rich, Figure S5).

663 Diseases of lipodystrophy may provide insights into the formation of BMAds and the selective 664 accumulation of FAs that we are struggling to understand (1,77). Congenital generalized lipodystrophy 665 (CGL) is a family of diseases that include a complete or partial loss of adipose depots, which reveal 666 important insights into the consequences of genetic deficiencies involved in adipocytic maturation and 667 function (reviewed in (77)) and may point us in the direction of the underlying nature of BMAd formation. One of the best understood CGL mouse models within the context of BMAd research are 668 669 the *Ptrf*⁴ deficient mice, which contain BMAT only in the tail vertebrae and very distal tibia with a loss 670 of BMAds at the tibia-fibula junction and proximal thereof (1,78-80). This is accompanied by a small 671 increase in cortical bone mineral content in adult male mice and a large increase in trabecular number 672 and cortical bone mineral content in females (1). The differential effect on BMAT upon PTRF loss of 673 function, points toward a differential regulation of LD formation in regulated versus constitutive 674 BMAds. Alternatively, differences in unsaturated lipid ratios could be involved in the specific 675 sensitivity of BMAds to such a mutation. More recently, diphtheria-toxin mediated ablation of Adipoq-676 Cre expressing cells also revealed differences in BMAd plasticity throughout the long bones in "fatfree" mice. In particular BMAds were increasingly present in the mid-diaphysis with age (81,82). 677 678 Furthermore, specific depletion of BMAds through a double conditional Osterix and Adipoqdependent strategy caused severe reduction in rBMAds with a milder reduction in the cBMAd mass, 679 680 while ATGL (*Pnpla2* gene) loss in BMAd through the same strategy caused massive rBMAd expansion 681 in the mid diaphysis (83,84). To what extent these discrepancies are due to divergent transcriptional 682 programs or part of a stabilization of the adipocytic differentiation program remains to be determined. 683 Moreover, seipin-deficient lymphoblastoid cell-lines derived from patients suffering congenital 684 generalized lipodystrophy type 2 were shown to have an increased proportion of SFAs with a decrease 685 in MUFAs, which are the principle products of Scd1, indicating decreased Scd1 activity. LD number 686 and size were increased compared to control (85). Impaired Δ 9-desaturase activity in nematodes also 687 led to decreased TAG accumulation and LD size that could not be rescued completely by supplemented 688 dietary oleate (86). Therefore, it appears that LD size is at least partially driven by the ability of Scd1 689 to synthesize unsaturated FAs. Although our results did not show a strong correlation of LD size with 690 lipid unsaturation, we did observe that the largest LDs were always predominantly unsaturated. Taken 691 together, this points to tissue-specific regulation of lipid composition independently of nutrient and metabolite availability, and possibly to sequential stages of LD (and adipocyte) maturation in the sOP9

693 versus the iOP9 conditions. Overall, it is tempting to suggest a continuum with smaller LD being

mostly saturated, an intermediate stage of LD maturation that are of largely mixed composition, and

695 the most mature LDs that are large and unsaturated. Follow-up studies should address this hypothesis

696 through Raman microspectroscopy in the context of inhibition versus activation of lipolysis and

697 reuptake with relation to lipid droplet and adipocyte size. Extrapolation to the *in vivo* scenario should

698 be done with caution, as the primary mechanism of lipid remodeling in primary human BMAd has

699 been shown to be independent of ATGL-mediated lipolysis (37).

700 Finally, we found that sOP9-adipocytes are more supportive to hematopoiesis than iOP9-adipocytes, 701 as seen by their capacity to form hematopoietic colonies and to support hematopoietic expansion. This agrees with reports of adiponectin expressing BM pre/adipocytes -including so-called Adipo-CAR 702 703 cells- being necessary for hematopoietic expansion (12,87-88), whereas mature BMAds rather 704 inducing HSCs quiescence or reduced proliferation (7,8,11,89). Of note, depletion of recently 705 described marrow adipocyte lineage precursors (MALPs) did not affect hematopoiesis, at least in 706 homeostasis (90). The reduced proliferation of hematopoietic progenitors in iOP9 adipocytic 707 cocultures was replicated in a dose-dependent manner through condition media experiments (Figure 708 6C), pointing to availability of soluble factors as one of the implicated mechanisms. Indeed, previous 709 data revealed a higher expression of the hematopoietic supportive factors C-X-C Motif Chemokine 710 Ligand 12 (Cxcl12) and c-Kit Ligand (KitL) in undifferentiated OP9 cultures versus OP9-derived 711 adipocytes analogous to iOP9s (42), and the regulation of BM stromal cell differentiation precisely via 712 *Cxcl12* (91). Note however that the undifferentiated OP9 condition was shown even more supportive 713 of hematopoietic proliferation than sOP9s (Figure 6). In congruence with previous *in vivo* data (11), 714 this observation in the OP9 system suggests that hematopoiesis is favored when mature marrow 715 adipocytes are absent. We therefore suggest an optimum of maturation along the adipocytic 716 differentiation axis with regard to hematopoietic support, in particular through at least limited 717 expression of Angpt1, SCF, and Cxcl12 that are attenuated in terminally differentiated adipocytes (92). 718 Taken together, our results indicate that, at the population level, sOP9 adipocytic cultures share 719 similarities in terms of hematopoietic support with the labile rBMAds of the red marrow, while iOP9 720 adipocytic cultures impair rapid hematopoietic progenitor proliferation as described for the stable 721 cBMAds of the yellow marrow.

722 The mechanisms for differential hematopoietic support in vivo are still poorly understood, but 723 extrapolation from the collective findings of the field suggests that cBMAd-dense regions of the distal 724 skeleton maintain HSCs, while rBMAd-containing red marrow would contribute to expansion of their 725 progenitors, possibly through increased FA transfer (83). Most HSCs have low mitochondrial potential and rely mainly on anaerobic glycolysis, while downstream progenitors rely on high energy production 726 727 through mitochondrial oxidative phosphorylation (93-95). Previous studies have also revealed that 728 HSCs and leukemic cells can rely on PPAR-mediated FA oxidation (96), with activation of oxidative 729 metabolism being a predictive factor for leukemic stem cell behavior (97). Indeed, we have shown that 730 BM stromal cells with lower overall lipid content (sOP9 cultures) associate to increased hematopoietic 731 expansion capacity compared with more mature BMAds that are high in lipid content (iOP9 cultures). 732 Specific differences in FA of BMAds may contribute to the nuances of differential hematopoietic support. For example, palmitic acid was shown to have a lipotoxic effect on hematopoietic-supportive

- osteoblasts, and high levels are present in aged and osteoporotic bone associated with decreased hematopoiesis (98,99). This may in part explain the overt myeloproliferation that is seen on the aged
- remain opolesis (98,99). This may in part explain the overt invelopion relation that is seen on the aged
 skeleton. In the current study, the stromal OP9 cell line was selected for its adipocytic differentiation
- potential. The inherent M-CSF deficiency may influence outcomes with regard to the hematopoietic
- supportive function. Previous studies have shown lymphoid and myeloid support of bone marrow
- adipocytes, although the extent of hematopoietic support as a function of the extent of adipocytic
- 740 differentiation has not been addressed. In future studies, a comparison to marrow stromal cell lines
- 741 without cytokine expression defects is warranted to provide a more representative understanding of the
- 742 marrow hematopoietic and adipocytic lineages.
- Future studies to understand the functional metabolic differences of rBMAd-like sOP9 versus cBMAd-

144 like iOP9 adipocytes should focus on *in vitro* lipase- and desaturase- inhibition, complemented by the

- study of their role in BMAd-deficient animal models (such as the Atgl-KO or Scd1-KO) to better
- understand the role of rBMAd vs cBMAd FA mobilization on hematopoiesis. Altogether, our results
- open avenues for future work on the OP9-adipocyte system to understand the mechanisms underlying
- 748 BMAd heterogeneity and its impact on both hematopoiesis and bone health.
- 749

750 **5 Conflict of Interest**

- 751 The authors declare no conflict of interest at this time.
- The authors declare that the research was conducted in the absence of any commercial or financial
 relationships that could be construed as a potential conflict of interest.

754 **6** Author Contributions

- J.T. planned experiments, analyzed and interpreted the results. G.F. performed Raman acquisitions,
- interpreted and analyzed the results and compiled the corresponding figures. A.D. and N.B. performed
- 757 HPLC analyses. J.T., C.B. and N.D.T. performed *in vitro* culture experiments. V.C. performed co-
- cultures. G.F. and L.D. performed statistical analyses for Raman data. J.T., G.F., A.D., C.C. and O.N.
 interpreted the Raman and HPLC data. G.Q.D. and O.N. designed and optimized OP9 differentiation
- 759 interpreted the Kaman and TFLC data. 0.0.D. and 0.N. designed and optimized OF 9 differentiation 760 protocols and associated hematopoietic support assays. O.N. and G.P. initiated the project, while C.C.
- and O.N. co-supervised the project. O.N. and J.T. wrote the manuscript, G.F. and C.C. significantly
- redited the manuscript.

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779 <mark>9 Data Availability</mark>

780 Original datasets are available in Mendeley Data publicly accessible repository: 781 [http://dx.doi.org/10.17632/wdm9gyz3bm.1].

782 10 References

- Scheller EL, Doucette CR, Learman BS, Cawthorn WP, Khandaker S, Schell B, et al. Region specific variation in the properties of skeletal adipocytes reveals regulated and constitutive
 marrow adipose tissues. Nature Communications. 2015 Nov;6(1).
- Kricun ME. Red-yellow marrow conversion: Its effect on the location of some solitary bone lesions. Skeletal Radiology. 1985 Jun;14(1):10–9.
- Doucette CR, Horowitz MC, Berry R, MacDougald OA, Anunciado-Koza R, Koza RA, et al. A
 High Fat Diet Increases Bone Marrow Adipose Tissue (MAT) But Does Not Alter Trabecular or
 Cortical Bone Mass in C57BL/6J Mice. J Cell Physiol. 2015 Sep;230(9):2032–7.
- Scheller EL, Khandaker S, Learman BS, Cawthorn WP, Anderson LM, Pham HA, et al. Bone marrow adipocytes resist lipolysis and remodeling in response to β-adrenergic stimulation.
 Bone. 2019 Jan;118:32–41.
- 794 5. Craft CS, Scheller EL. Evolution of the Marrow Adipose Tissue Microenvironment. Calcified
 795 Tissue International. 2017 May;100(5):461–75.
- Horowitz MC, Berry R, Holtrup B, Sebo Z, Nelson T, Fretz JA, et al. Bone marrow adipocytes.
 Adipocyte [Internet]. 2017;3945. Available from: https://doi.org/10.1080/21623945.2017.1367881
- 7. Ambrosi TH, Scialdone A, Graja A, Gohlke S, Jank AM, Bocian C, et al. Adipocyte
 800 Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based
 801 Hematopoietic and Bone Regeneration. Cell Stem Cell. 2017 Jun;20(6):771-784.e6.
- Mattiucci D, Maurizi G, Izzi V, Cenci L, Ciarlantini M, Mancini S, et al. Bone marrow
 adipocytes support hematopoietic stem cell survival. J Cell Physiol. 2018;233(2):1500–11.

- 804 9. Mattiucci D, Naveiras O, Poloni A. Bone Marrow "Yellow" and "Red" Adipocytes: Good or
 805 Bad Cells ? 2018;117–22.
- Wei Q, Frenette PS. Niches for Hematopoietic Stem Cells and Their Progeny. Immunity. 2018
 Apr;48(4):632–48.
- Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes
 as negative regulators of the haematopoietic microenvironment. Nature. 2009
 Jul;460(7252):259–63.
- I2. Zhou BO, Yu H, Yue R, Zhao Z, Rios JJ, Naveiras O, et al. Bone marrow adipocytes promote
 the regeneration of stem cells and haematopoiesis by secreting SCF. Nature Cell Biology. 2017
 Jul 17;19(8):891–903.
- Tratwal J, Rojas-Sutterlin S, Bataclan C, Blum S, Naveiras O. Bone marrow adiposity and the
 hematopoietic niche: A historical perspective of reciprocity, heterogeneity, and lineage
 commitment. Best Practice & Research Clinical Endocrinology & Metabolism. 2021
 Jul;35(4):101564.
- 818 14. Ghali O, Broux O, Falgayrac G, Haren N, van Leeuwen J, Penel G, et al. Dexamethasone in
 819 osteogenic medium strongly induces adipocyte differentiation of mouse bone marrow stromal
 820 cells and increases osteoblast differentiation. BMC Cell Biology. 2015;16(1):9.
- 15. Campos V, Rappaz B, Kuttler F, Turcatti G, Naveiras O. High-throughput, nonperturbing
 quantification of lipid droplets with digital holographic microscopy. Journal of Lipid Research.
 2018 Jul;59(7):1301–10.
- 824 16. Stiebing C, Schmölz L, Wallert M, Matthäus C, Lorkowski S, Popp J. Raman imaging of
 825 macrophages incubated with triglyceride-enriched oxLDL visualizes translocation of lipids
 826 between endocytic vesicles and lipid droplets. Journal of Lipid Research. 2017 May;58(5):876–
 827 83.
- 828 17. Guo Y, Cordes KR, Farese RV, Walther TC. Lipid droplets at a glance. Journal of Cell Science.
 829 2009 Mar 15;122(6):749–52.
- 18. Ehehalt R, Füllekrug J, Pohl J, Ring A, Herrmann T, Stremmel W. Translocation of long chain
 fatty acids across the plasma membrane lipid rafts and fatty acid transport proteins. Mol Cell
 Biochem. 2006 May 12;284(1–2):135–40.
- 833 19. Schaffer JE, Lodish HF. Expression cloning and characterization of a novel adipocyte long
 834 chain fatty acid transport protein. Cell. 1994 Nov 4;79(3):427–36.
- 835 20. Thiam AR, Beller M. The why, when and how of lipid droplet diversity. Journal of Cell
 836 Science. 2017 Jan 15;130(2):315–24.
- Kimmel AR, Sztalryd C. The Perilipins: Major Cytosolic Lipid Droplet–Associated Proteins
 and Their Roles in Cellular Lipid Storage, Mobilization, and Systemic Homeostasis. Annu Rev
 Nutr. 2016 Jul 17;36(1):471–509.

- 22. Zimmermann R. Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride
 Lipase. Science. 2004 Nov 19;306(5700):1383–6.
- Zechner R, Zimmermann R, Eichmann TO, Kohlwein SD, Haemmerle G, Lass A, et al. FAT
 SIGNALS Lipases and Lipolysis in Lipid Metabolism and Signaling. Cell Metabolism. 2012
 Mar;15(3):279–91.
- 845 24. Unger RH, Clark GO, Scherer PE, Orci L. Lipid homeostasis, lipotoxicity and the metabolic
 846 syndrome. Biochimica et Biophysica Acta (BBA) Molecular and Cell Biology of Lipids. 2010
 847 Mar;1801(3):209–14.
- 848 25. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat
 849 Rev Immunol. 2011 Feb;11(2):85–97.
- 26. Carta G, Murru E, Banni S, Manca C. Palmitic Acid: Physiological Role, Metabolism and
 Nutritional Implications. Frontiers in Physiology. 2017 Nov 8;8.
- Ly LD, Xu S, Choi SK, Ha CM, Thoudam T, Cha SK, Wiederkehr A, Wollheim CB, Lee IK,
 Park KS. Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. Exp Mol
 Med. 2017 Feb 3;49(2):e291.
- 855 28. Gillet C, Spruyt D, Rigutto S, Dalla Valle A, Berlier J, Louis C, et al. Oleate abrogates
 856 palmitate-induced lipotoxicity and proinflammatory response in human bone marrow-derived
 857 mesenchymal stem cells and osteoblastic cells. Endocrinology. 2015;156:4081–93.
- 858 29. Miranda M, Pino AM, Fuenzalida K, Rosen CJ, Seitz G, Rodríguez JP. Characterization of
 859 Fatty Acid Composition in Bone Marrow Fluid From Postmenopausal Women: Modification
 860 After Hip Fracture. Journal of Cellular Biochemistry. 2016 Oct;117(10):2370–6.
- 30. Tencerova, Michaela, Michaela Ferencakova, and Moustapha Kassem. Bone marrow adipose
 tissue: Role in bone remodeling and energy metabolism. Best Practice & Research Clinical
 Endocrinology & Metabolism 35.4 (2021): 101545.
- Bravenboer N, Bredella MA, Chauveau C, Corsi A, Douni E, Ferris WF, Riminucci M, Robey
 PG, Rojas-Sutterlin S, Rosen C, Schulz TJ, Cawthorn WP. Standardised Nomenclature,
 Abbreviations, and Units for the Study of Bone Marrow Adiposity: Report of the Nomenclature
 Working Group of the International Bone Marrow Adiposity Society. Front Endocrinol
 (Lausanne). 2020 Jan 24;10:923.n
- 869 31. Craft CS, Li Z, MacDougald OA, Scheller EL. Molecular Differences Between Subtypes of
 870 Bone Marrow Adipocytes. Current Molecular Biology Reports. 2018 Mar;4(1):16–23.
- Tratwal J, Labella R, Bravenboer N, Kerckhofs G, Douni E, Scheller EL, et al. Reporting
 Guidelines, Review of Methodological Standards, and Challenges Toward Harmonization in
 Bone Marrow Adiposity Research. Report of the Methodologies Working Group of the
 International Bone Marrow Adiposity Society. Frontiers in Endocrinology. 2020 Feb 28;11.
- 33. Tavassoli M, Houchin DN, Jacobs P. Fatty Acid Composition of Adipose Cells in Red and
 Yellow Marrow: A Possible Determinant of Haematopoietic Potential. Scandinavian Journal of
 Haematology. 1977;18(1):47–53.

878 34. Ren J, Dimitrov I, Sherry AD, Malloy CR. Composition of adipose tissue and marrow fat in 879 humans by 1H-NMR at 7 Tesla. Journal of Lipid Research. 2008 Sep:49(9):2055–62. 880 35. Beekman KM, Regenboog M, Nederveen AJ, Bravenboer N, Akkerman EM, Maas M. Gender-881 and Age-Associated Differences in Bone Marrow Adipose Tissue and Bone Marrow Fat 882 Unsaturation Throughout the Skeleton, Quantified Using Chemical Shift Encoding-Based 883 Water-Fat MRI. Frontiers in Endocrinology. 2022;13:10. 884 Tratwal J, Bekri D, Boussema C, Sarkis R, Kunz N, Koligi T, et al. MarrowQuant Across Aging 36. 885 and Aplasia: A Digital Pathology Workflow for Quantification of Bone Marrow Compartments 886 in Histological Sections. Front Endocrinol. 2020 Sep 24;11:480. 887 37. Attané C, Estève D, Chaoui K, Iacovoni JS, Corre J, Moutahir M, et al. Human Bone Marrow Is 888 Comprised of Adipocytes with Specific Lipid Metabolism. Cell Reports. 2020 Jan;30(4):949-889 958.e6. 890 38. Lucas S, Tencerova M, von der Weid B, Andersen TL, Attané C, Behler-Janbeck F, et al. 891 Guidelines for Biobanking of Bone Marrow Adipose Tissue and Related Cell Types: Report of 892 the Biobanking Working Group of the International Bone Marrow Adiposity Society. Front 893 Endocrinol. 2021 Sep 27;12:744527. 894 39. Moore KA, Ema H, Lemischka IR. In Vitro Maintenance of Highly Purified, Transplantable 895 Hematopoietic Stem Cells. Blood. 1997 Jun 15;89(12):4337-47. 896 40. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced 897 pluripotent stem cells. Nature. 2010 Sep;467(7313):285-90. 898 41. McKinney-Freeman SL, Naveiras O, Daley GO, Isolation of Hematopoietic Stem Cells from 899 Mouse Embryonic Stem Cells. Current Protocols in Stem Cell Biology. 2008;4(1):1F.3.1-900 1F.3.10. 901 42. Naveiras O. Novel determinants of the hematopoietic microenvironment in development and 902 homeostasis. Harvard University: 2008. 903 43. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, et al. ImageJ2: 904 ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017 905 Dec;18(1):529. 906 During A. Lipid determination in bone marrow and mineralized bone tissue: From sample 44. 907 preparation to improved high-performance thin-layer and liquid chromatographic approaches. 908 Journal of Chromatography A. 2017 Sep;1515:232-44. 909 45 Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The Ultimate qPCR 910 Experiment: Producing Publication Quality, Reproducible Data the First Time. Trends in Biotechnology. 2019 Jul;37(7):761-74. 911 912 46. Vandesompele J, Preter KD, Roy NV, Paepe AD. Accurate normalization of real-time 913 quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome 914 Biology. 2002 Jun 18;3(7):12.

915 916 917	47.	Feugier P, Li N, Jo DY, Shieh JH, MacKenzie KL, Lesesve JF, et al. Osteopetrotic Mouse Stroma with Thrombopoietin, c-kit Ligand, and flk-2 Ligand Supports Long-Term Mobilized CD34 ⁺ Hematopoiesis In Vitro. Stem Cells and Development. 2005 Oct;14(5):505–16.
918 919	48.	Dexter TM, Allen TD, Lajtha L. Conditions controlling the proliferation of haemopoietic stem cells in vitro. Journal of cellular physiology. 1977;91(3):335–44.
920 921	49.	Dexter T, Lajtha L. Proliferation of haemopoietic stem cells in vitro. British journal of Haematology. 1974;28(4):525–30.
922 923	50.	Dexter T, Testa N. Differentiation and proliferation of hemopoietic cells in culture. In: Methods in cell biology. Elsevier; 1976. p. 387–405.
924 925 926	51.	Westen H, Bainton DF. Association of alkaline-phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. Journal of Experimental Medicine. 1979 Oct 1;150(4):919–37.
927 928 929	52.	Baccin C, Al-Sabah J, Velten L, Helbling PM, Grünschläger F, Hernández-Malmierca P, et al. Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. Nat Cell Biol. 2020 Jan;22(1):38–48.
930 931	53.	Gao J, Yan XL, Li R, Liu Y, He W, Sun S, et al. Characterization of OP9 as authentic mesenchymal stem cell line. Journal of Genetics and Genomics. 2010 Jul;37(7):475–82.
932 933 934	54.	Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Park C, Choi K, et al. OP9 mouse stromal cells rapidly differentiate into adipocytes: characterization of a useful new model of adipogenesis. Journal of Lipid Research. 2006 Feb;47(2):450–60.
935 936 937	55.	Hisahiro Y, Hayashi SI, Takahiro K, Ogawa M, Nishikawa S, Okamnura H, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature. 1990 May 31;345.
938 939	56.	Nakano T, Kodama H, Honjo T. Generation of Lymphohematopoietic Cells from Embryonic Stem Cells in Culture. Science, New Series. 1994;265(5175):1098–101.
940 941 942	57.	Gubelmann C, Schwalie PC, Raghav SK, Roder E, Delessa T, Kiehlmann E, et al. Identification of the transcription factor ZEB1 as a central component of the adipogenic gene regulatory network. Elife. 2014;3:e03346.
943 944	58.	Czamara K, Majzner K, Pacia MZ, Kochan K, Kaczor A, Baranska M. Raman spectroscopy of lipids: a review. Journal of Raman Spectroscopy. 2015;46(1):4–20.
945 946 947	59.	Kochan K, Maslak E, Krafft C, Kostogrys R, Chlopicki S, Baranska M. Raman spectroscopy analysis of lipid droplets content, distribution and saturation level in Non-Alcoholic Fatty Liver Disease in mice. Journal of Biophotonics. 2015;8(7):597–609.
948 949	60.	Le TT, Duren HM, Slipchenko MN, Hu CD, Cheng JX. Label-free quantitative analysis of lipid metabolism in living Caenorhabditis elegans. Journal of Lipid Research. 2010 Mar;51(3):672–7.

- 950 61. Wu H, Volponi JV, Oliver AE, Parikh AN, Simmons BA, Singh S. In vivo lipidomics using
 951 single-cell Raman spectroscopy. Proc Natl Acad Sci USA. 2011 Mar;108(9):3809–14.
- Raclot T, Groscolas R. Differential mobilization of white adipose tissue fatty acids according to
 chain length, unsaturation, and positional isomerism. Journal of Lipid Research. 1993
 Sep;34(9):1515–26.
- 63. Tavakol DN, Tratwal J, Bonini F, Genta M, Campos V, Burch P, et al. Injectable, scalable 3D
 tissue-engineered model of marrow hematopoiesis. Biomaterials. 2020 Feb 1;232:119665.
- Maciel JG, de Araújo IM, Carvalho AL, Simão MN, Bastos CM, Troncon LEA, et al. Marrow
 Fat Quality Differences by Sex in Healthy Adults. Journal of Clinical Densitometry. 2017
 Jan;20(1):106–13.
- Suchacki KJ, Tavares AAS, Mattiucci D, Scheller EL, Papanastasiou G, Gray C, et al. Bone marrow adipose tissue is a unique adipose subtype with distinct roles in glucose homeostasis.
 Nat Commun. 2020 Dec;11(1):3097.
- 66. Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, et al. Bone Marrow
 Adipose Tissue Is an Endocrine Organ that Contributes to Increased Circulating Adiponectin
 during Caloric Restriction. Cell Metabolism. 2014 Aug;20(2):368–75.
- 966 67. Dufau J, Shen JX, Couchet M, De Castro Barbosa T, Mejhert N, Massier L, et al. In vitro and ex vivo models of adipocytes. American Journal of Physiology-Cell Physiology. 2021 May 1;320(5):C822–41.
- 68. Tavassoli M. Marrow adipose cells. Histochemical identification of labile and stable
 components. Arch Pathol Lab Med. 1976 Jan;100(1):16–8.
- 871 69. Raclot T. Differential mobilization of white adipose tissue fatty acids according to chain length,
 972 unsaturation, and positional isomerism. :12.
- 70. Raclot T, Oudart H. Selectivity of fatty acids on lipid metabolism and gene expression. Proc
 Nutr Soc. 1999 Aug;58(3):633–46.
- 975 71. Masia F, Glen A, Stephens P, Borri P, Langbein W. Quantitative Chemical Imaging and
 976 Unsupervised Analysis Using Hyperspectral Coherent Anti-Stokes Raman Scattering
 977 Microscopy. Anal Chem. 2013 Nov 19;85(22):10820–8.
- Masia F, Glen A, Stephens P, Langbein W, Borri P. Label-free quantitative chemical imaging
 and classification analysis of adipogenesis using mouse embryonic stem cells. J Biophotonics.
 2018 Jul;11(7):e201700219.
- 981 73. Pacia MZ, Sternak M, Mateuszuk L, Stojak M, Kaczor A, Chlopicki S. Heterogeneity of
 982 chemical composition of lipid droplets in endothelial inflammation and apoptosis. Biochimica et
 983 Biophysica Acta (BBA) Molecular Cell Research. 2020 Jun;1867(6):118681.
- Sugiyama T, Hobro AJ, Pavillon N, Umakoshi T, Verma P, Smith N. Label-free Raman mapping of saturated and unsaturated fatty acid uptake, storage, and return toward baseline levels in macrophages. Analyst. 2021 Feb 22;146(4):1268–80.

- 987 75. Majka Z, Czamara K, Janus J, Kępczyński M, Kaczor A. Prominent hypertrophy of perivascular
 988 adipocytes due to short-term high fat diet. Biochimica et Biophysica Acta (BBA) Molecular
 989 Basis of Disease. 2022 Feb 1;1868(2):166315.
- 76. Czamara K, Majzner K, Selmi A, Baranska M, Ozaki Y, Kaczor A. Unsaturated lipid bodies as
 a hallmark of inflammation studied by Raman 2D and 3D microscopy. Sci Rep. 2017
 Feb;7(1):40889.
- 993 77. Scheller EL, Rosen CJ. What's the matter with MAT? Marrow adipose tissue, metabolism, and
 994 skeletal health: Marrow adipose tissue and skeletal health. Annals of the New York Academy of
 995 Sciences. 2014 Apr;1311(1):14–30.
- Hayashi YK, Matsuda C, Ogawa M, Goto K, Tominaga K, Mitsuhashi S, et al. Human PTRF
 mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with
 generalized lipodystrophy. Journal of Clinical Investigation. 2009 Sep 1;119(9):2623–33.
- P100
 P100
 P100
 P100
 Cavin/PTRF Causes Global Loss of Caveolae, Dyslipidemia, and Glucose Intolerance. Cell Metabolism. 2008 Oct;8(4):310–7.
- 1002 80. Ding SY, Lee MJ, Summer R, Liu L, Fried SK, Pilch PF. Pleiotropic Effects of Cavin-1
 1003 Deficiency on Lipid Metabolism. Journal of Biological Chemistry. 2014 Mar 21;289(12):8473–
 1004 83.
- 1005 81. Zou W, Rohatgi N, Brestoff JR, Zhang Y, Scheller EL, Craft CS, et al. Congenital
 1006 lipodystrophy induces severe osteosclerosis. Baron R, editor. PLoS Genet. 2019 Jun
 1007 24;15(6):e1008244.
- 1008 82. Zhang X, Robles H, Magee KL, Lorenz MR, Wang Z, Harris CA, et al. A bone-specific
 1009 adipogenesis pathway in fat-free mice defines key origins and adaptations of bone marrow
 1010 adipocytes with age and disease. eLife. 2021 Aug 11;10:e66275.
- 1011 83. Li Z, Bowers E, Zhu J, Yu H, Hardij J, Bagchi DP, et al. Lipolysis of bone marrow adipocytes
 1012 is required to fuel bone and the marrow niche during energy deficits. Zaidi M, Cawthorn WP,
 1013 editors. eLife. 2022 Jun 22;11:e78496.
- 1014 84. Li Z, Bagchi DP, Zhu J, Bowers E, Yu H, Hardij J, et al. Constitutive bone marrow adipocytes
 1015 suppress local bone formation. JCI Insight [Internet]. 2022 Sep 1 [cited 2022 Sep 27]; Available
 1016 from: http://insight.jci.org/articles/view/160915
- 1017 85. Boutet E, El Mourabit H, Prot M, Nemani M, Khallouf E, Colard O, et al. Seipin deficiency
 1018 alters fatty acid Δ9 desaturation and lipid droplet formation in Berardinelli-Seip congenital
 1019 lipodystrophy. Biochimie. 2009 Jun;91(6):796–803.
- 1020 86. Shi X, Li J, Zou X, Greggain J, Rødkær SV, Færgeman NJ, et al. Regulation of lipid droplet
 1021 size and phospholipid composition by stearoyl-CoA desaturase. Journal of Lipid Research. 2013
 1022 Sep;54(9):2504–14.

- 1023 87. Aoki K, Kurashige M, Ichii M, Higaki K, Sugiyama T, Kaito T, et al. Identification of
 1024 CXCL12-abundant reticular cells in human adult bone marrow. British Journal of Haematology.
 1025 2021;193(3):659–68.
- 1026 88. Zhang Z, Huang Z, Ong B, Sahu C, Zeng H, Ruan HB. Bone marrow adipose tissue-derived
 1027 stem cell factor mediates metabolic regulation of hematopoiesis. Haematologica. 2019
 1028 Sep;104(9):1731-1743.
- 1029 89. Spindler TJ, Tseng AW, Zhou X, Adams GB. Adipocytic Cells Augment the Support of
 1030 Primitive Hematopoietic Cells In Vitro But Have No Effect in the Bone Marrow Niche Under
 1031 Homeostatic Conditions. Stem Cells and Development. 2014 Feb 15;23(4):434–41.
- 2 90. Zhong L, Yao L, Tower RJ, Wei Y, Miao Z, Park J, et al. Single cell transcriptomics identifies a
 unique adipose lineage cell population that regulates bone marrow environment. Elife. 2020 Apr
 1034 14;9:e54695.
- 1035 91. Tzeng YS, Chung NC, Chen YR, Huang HY, Chuang WP, Lai DM. Imbalanced Osteogenesis
 1036 and Adipogenesis in Mice Deficient in the Chemokine Cxcl12/Sdf1 in the Bone Mesenchymal
 1037 Stem/Progenitor Cells. Journal of Bone and Mineral Research. 2018 Apr;33(4):679–90.
- 1038 92. Oh I, Ozaki K, Miyazato A, Sato K, Meguro A, Muroi K, et al. Screening of genes responsible
 1039 for differentiation of mouse mesenchymal stromal cells by DNA micro-array analysis of
 1040 C3H10T1/2 and C3H10T1/2-derived cell lines. Cytotherapy. 2007;9(1):80–90.
- 1041 93. Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, et al.
 1042 Regulation of Glycolysis by Pdk Functions as a Metabolic Checkpoint for Cell Cycle
 1043 Quiescence in Hematopoietic Stem Cells. Cell Stem Cell. 2013 Jan;12(1):49–61.
- 1044 94. Yu WM, Liu X, Shen J, Jovanovic O, Pohl EE, Gerson SL, et al. Metabolic Regulation by the
 1045 Mitochondrial Phosphatase PTPMT1 Is Required for Hematopoietic Stem Cell Differentiation.
 1046 Cell Stem Cell. 2013 Jan;12(1):62–74.
- 1047 95. Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the
 1048 hypoxic niche. Cell Stem Cell. 2011 Oct 4;9(4):298-310.
- 1049 96. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Schafer ZT, et al. A PML-PPARδ pathway for
 1050 fatty acid oxidation regulates haematopoietic stem cell maintenance. 2013;20.
- 1051 97. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, et al. Chemotherapy-Resistant
 1052 Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require
 1053 Oxidative Metabolism. Cancer Discovery. 2017 Jul 5;7(7):716–35.
- 1054 98. Al Saedi A, Bermeo S, Plotkin L, Myers DE, Duque G. Mechanisms of palmitate-induced
 1055 lipotoxicity in osteocytes. Bone. 2019 Oct;127:353–9.
- 1056 99. Singh L, Tyagi S, Myers D, Duque G. Good, Bad, or Ugly: the Biological Roles of Bone
 1057 Marrow Fat. Current Osteoporosis Reports. 2018 Apr;16(2):130–7.

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1059 11 Supplementary Material

Supplementary Figures have been uploaded separately on submission. Please provide Frontiers linkfor access here.

1062

1063 Figure 1: Heterogeneity in the formation of lipidated adipocytes upon induction of adipogenesis 1064 by confluency and serum exposure (spontaneous) versus DMI cocktail (induced). OP9 cells were 1065 (A, D) passaged subconfluently and remained undifferentiated on day 17 of culture, (B, E, H) 1066 differentiated spontaneously upon confluency in the presence of serum for 17 days, or (E, F, I) induced 1067 with a classical adipogenic differentiation cocktail in the same serum-containing conditions after 1068 confluent plating and 17 days in culture. (G) Oil red O measurements show the highest adipogenic differentiation in the induced condition at day 17 in culture. **P<0.01 by student's t-test. Error bars 1069 1070 represent mean \pm s.d. (n=3 biological replicates). DMI: 1µM dexamethasone, 0.5mM isobutyl-1071 methylxanthine, 5µg/ml insulin.

1072

1073 Figure 2: Population level differences in lipid composition are more marked than differences in 1074 the canonical adipocytic transcriptional signature for spontaneous versus induced cultures. (A) 1075 RT-qPCR markers of adipogenesis are increased in induced OP9-adipocytes. (B) Desaturase 1076 expression is highest in day 17 induced OP9-adipocytes while one of the elongases in fatty acid de novo synthesis is decreased. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 by Bonferroni's multiple 1077 1078 comparisons test. Error bars represent mean \pm s.d. (n=3 independent experiments). (C-G) HPLC 1079 analysis of spontaneously differentiated or induced OP9 adipocytes revealed (C) nuances of the most 1080 abundant saturated, mono-, and polyunsaturated lipid species, with (**D**) overall total higher unsaturation 1081 content in induced versus spontaneous conditions. (E) Percentage of individual fatty acid species 1082 reveals (F) a greater 16:1/16:0 unsaturation ratio in the induced condition. (G) Unsaturation ratios 18:1/18:0 were not significantly different. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 by 1083 1084 ANOVA test with Bonferroni's multiple comparisons adjustment. Error bars represent mean \pm s.d. 1085 (n=3 independent experiments). HKGs: housekeeping genes.

1086

1087 Figure 3: Raman microspectroscopy detects unsaturated spectra at the single lipid droplet level 1088 in spontaneous and induced adipocytes. (A) An optical image of an adipocyte in the induced 1089 condition (iOP9) analyzed by Raman imaging. (B) Raman image of the scores of PC1 in induced 1090 condition. Pixels in black represent a score of PC1 equal to zero, which corresponds to the absence of 1091 PC1. Pixels in shades of blue correspond to the contribution of PC1. (C) Loading PC1 is characteristic 1092 of a spectrum of unsaturated lipid-rich and captures more than 95% of the variation. (**D**) An optical 1093 image of an adipocyte in spontaneous condition (sOP9) analyzed by Raman imaging. (E) Raman image 1094 of the scores of PC1 in spontaneous condition. Pixels in black represent a score of PC1 equal to zero, 1095 which corresponds to the absence of PC1. Pixels in shades of red correspond to the contribution of 1096 PC1. (F) Loading PC1 is characteristic of a spectrum of saturated lipid-rich. The Raman images of the 1097 scores and the loadings of PC2 and PC3 capture less than 2% and are presented in supplementary files 1098 (Figure S4).

1099

Figure 4: Raman microspectroscopy reveals predominance of unsaturated spectra in induced adipocytes, with heterogeneity in the unsaturation ratio for the spontaneous condition. (A) Representative Raman spectrum of saturated-rich, unsaturated-rich lipids and mixture identified by unguided hierarchical cluster analysis. (B) Loading plot PC1 indicates the discriminant peaks for saturated versus unsaturated designation, the color is associated to the attribution of specific Raman bands: saturated in blue, unsaturated in red (n=3 independent experiments, pooled for PCA analysis).

- 1106 (C) PCA score plot of Raman spectra labelled according to spontaneous (orange) and induced (green)
- 1107 OP9-adipocytes. (**D**) The same PCA score plot as (**C**) labelled according to the assignment of Raman
- 1108 spectra as saturated (red) and unsaturated (blue) lipids based on the PC1 loading determined by B. For
- 1109 panels C and D, each point is a single adipocyte with all its lipid droplet data points averaged. n
- 1110 (induced) = 138 adipocytes, 2971 spectra; n (spontaneous) = 120 adipocytes, 2944 spectra. (E)
- 1111 Unsaturation ratios of spontaneous and induced at adipocyte level. The unsaturation ratio of sOP9
- adipocytes is not different from iOP9 adipocytes (P=0.0855 by Mann-Whitney test).
- 1113

1114 Figure 5: Induced adipocytes are composed of larger lipid droplets than spontaneous adipocytes, but there is no overall correlation between lipid droplet diameter and unsaturation ratio. (A-B) 1115 1116 Unsaturation ratio as a function of the diameter of the LDs for sOP9 versus iOP9 adipocytes (n=3)independent experiments, pooled for LD analysis). The low R2 for both conditions indicates that the 1117 1118 diameter of the LDs is not correlated with the unsaturation ratio. Dashed lines represent the correlation 1119 line. The Y axis is colored as function of the score of PC1. The circles in dark blue corresponds to LDs 1120 identified as unsaturated rich. The circles in dark red correspond to LDs identified as saturated-rich. 1121 The circles in yellow and light blue correspond to LDs identified as mixture. (C) Histogram for 1122 frequency of LDs as a function of the diameter for the spontaneous and induced condition. Vertical 1123 lines show the mean diameter of the LD for each condition. The mean diameter of LDs is 5.24µm 1124 (±1.85µm) and 7.87 µm (±3.83µm) for sOP9 adipocytes and iOP9 adipocytes, respectively. The LDs mean diameter of sOP9-adipocytes is significantly lower compared to iOP9-adipocytes (P<0.0001, 1125 1126 Mann-Whitney test). (D, E) When separated according to LD type a trend toward larger unsaturated 1127 lipid droplets in the induced condition is observed. (F) Unsaturation ratios of sOP9 versus iOP9 at LD 1128 level. LDs from iOP9 adipocytes have a higher mean unsaturation ratio compared to LDs from sOP9 1129 adipocytes. (P<0.0014, Mann-Whitney test). (G) At single droplet resolution, larger droplets (>10µm) 1130 have a higher unsaturation ratio as compared to smaller lipid droplets, while smaller droplets (<10µm) 1131 in induced OP9-adipocytes (n=1973) have a higher unsaturation ratio than those of spontaneous OP9-1132 adipocytes (n=2579). Highest unsaturation ratio overall is seen in large droplets from spontaneous 1133 OP9-adipocytes (n=41). (F) Unsaturation ratio as a function of the condition (spontaneous and 1134 induced) and the nature of the lipid (saturated-rich, unsaturated-rich and mixture). *P<0.05, 1135 ****P<0.001 by Kruskal-Wallis test. The lipid droplet of spontaneous unsaturated-rich have the higher 1136 unsaturation ratio compared to induced unsaturated-rich.

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1138 Figure 6: Short-term co-culture of OP9-adipocytes with hematopoietic stem and progenitor cells 1139 favours hematopoietic expansion in the spontaneous condition. (A) Total hematopoietic cell expansion of 2000 initial HSPCs after seven days in co-culture (including cells in adherent and cells 1140 1141 in suspension) is significantly higher (p<0.0001) when hematopoietic stem and progenitor cells, HSPCs, (cKit⁺Lin⁻Sca1⁺, KLS) are co-seeded with spontaneous- compared to induced OP9 adipocytes. 1142 1143 (B) The colony forming capacity of HSPCs seeded with spontaneous OP9 adipocytes is significantly 1144 higher (P<0.05) than when cultured with induced OP9 adipocytes. (C), Total hematopoietic expansion (in the absence of stroma) after two-day co-culture of 2000 HSPCs in conditioned medium from OP9 1145 cultures comprising increasing ratios of conditioned-to-fresh media. ****P<0.0001, **P<0.01, 1146 *P<0.05 by Bonferroni's multiple comparisons test. Error bars represent mean \pm s.d. (n=3 biological 1147 1148 replicates).

1150 Figure 7: HPLC fatty acid quantification of primary human BM samples confirms a higher 1151 unsaturation ratio in constitutive BMAd-rich marrow (femoral head) versus regulated BMAd-1152 rich marrow (ileac crest post-chemotherapy). (A-C) HPLC analysis of lipids isolated from human 1153 iliac crest bone marrow aspirates after induction chemotherapy (regulated site, labile marrow) or from 1154 the bone marrow of femoral specimens collected upon hip replacement surgery (constitutive site, stable 1155 marrow). (A) Higher unsaturation content in stable versus labile marrow oil reveal nuances of 1156 individual saturated, mono-, and polyunsaturated lipid species. (B-C) Greater unsaturation ratios in the human marrow from the constitutive site versus the regulated siten. (D) **P < 0.01, *P < 0.05 by 1157 student's t-test. Error bars represent mean \pm s.d. (n=4 experiments from independent donors). 1158 1159 Regulated site (iliac crest): 3 females, 1 male (age = 55 ± 12 years). Constitutive site (hip replacement): 1160 3 males, 1 female (age = 68 ± 10 years).

1161

Table 2: List of general abbreviations

Abbreviation	Definition
ACTb	Actin beta
AdipoQ	Adiponectin
Angpt1	Angiopoietin 1
ANOVA	Analysis of variance
ATGL	Adipose triglyceride lipase
Ap2	Activating protein 2
BM	Bone marrow
BMAd	Bone marrow adipocyte
BMAT	Bone marrow adipose tissue
BMSC	Bone marrow stromal cell
CA	Cluster of analysis
CAR cell	CXCL12 abundant reticular cell
cBMAd	Constitutive bone marrow adipocyte
cDNA	Complementary deoxyribonucleic acid
CEBPa	CCAAT Enhancer Binding Protein Alpha
CFU	Colony-forming unit
CGL	Congenital generalized lipodystrophy
cKit	Kit proto-oncogene, receptor tyrosine kinase
CXCL12	C-X-C motif chemokine ligand receptor 12
DMI	Cocktail for Dexamethasone, IBMX and insulin
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FA	Fatty acid
FABP4	Fatty acid binding protein 4
FACS	Fluorescence-activated cell sorting
FADS	Fatty acid desaturase
FBS	Fetal bovine serum
HKGs	Housekeeping genes
¹ H-MRS	proton magnetic resonance spectroscopy
HPLC	High performance liquid chromatography
HSC	Hematopoietic stem cell
HSL	Hormone sensitive lipoase
HSPC	Hematopoietic stem and progenitor cell
IBMX	isobutylmethylxanthin
IMDM	Iscove's Modified Dulbecco's Medium
iOP9	induced OP9 cell
KitL	cKit ligand
KLS	cKit ⁺ Lineage ⁻ Sca1 ⁺

LD	Lipid droplet
LPL	lipoprotein lipase
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acid
M-CSF	Macrophage colony-stimulating factor
ΜΕΜα	Minimum Essential Medium alpha
ORO	Oil red O
PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PLIN	Perilipin
PPARγ	Peroxisome proliferator-activated receptor gamma
PTRF	Polymerase I and transcript-release factor
P/S	Penicillin/Streptomycin
PUFA	Polyunsaturated fatty acid
rBMAd	Regulated bone marrow adipocyte
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
Sca1	Stem cell antigen 1
Scd	Stearyl-CoenzymeA desaturase
SCF	Stem cell factor
SD	Standard deviation
SFA	Saturated fatty acid
sOP9	spontaneous OP9 cell
STR	Short tandem repeats
TAG	Triacylglycerols
uOP9	Undifferentiated OP9 cell

Table 3: List of fatty acid abbreviations

Abbreviation	Definition	Formula	IUPAC Name
14:1	Myristoleic acid	$C_{14}H_{26}O_2$	(Z)-tetradec-9-enoic acid
16:0	Palmitic acid	$C_{16}H_{32}O_2$	hexadecanoic acid
16:1n-7	Palmitoleic acid	$C_{16}H_{30}O_2$	(Z)-hexadec-9-enoic acid
16:1n-10	Sapienic acid	$C_{16}H_{30}O_2$	(Z)-hexadec-6-enoic acid
18:0	Stearic acid	$C_{18}H_{36}O_2$	octadecanoic acid
18:1n-9	Oleic acid	$C_{18}H_{34}O_2$	(Z)-octadec-9-enoic acid
18:2n-6	Linoleic acid	$C_{18}H_{32}O_2$	(9Z,12Z)-octadeca-9,12-dienoic acid
20:4n-6	Arachidonic acid	$C_{20}H_{32}O_2$	(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid



Δ

Undifferentiated

Spontaneous









10x objective















Figure 6.JPEG



