Monocyte subsets in blood correlate with obesity related response of macrophages to biomaterials *in vitro*

Biomaterials. 2016;109:32-9.

Geesien S.A. Boersema
Lizette Utomo
Yves Bayon
Nicole Kops
Erwin van der Harst
Johan F. Lange
Yvonne M. Bastiaansen-Jenniskens

ABSTRACT

Macrophages play a key role in the foreign body response. In this study it was investigated whether obesity affects the acute response of macrophages to biomaterials *in vitro* and whether this response is associated with biomarkers in blood. CD14+ monocytes were isolated from blood from obese and age and gender matched lean persons. Monocyte subsets were determined based on CD14 and CD16 on their surface. C-reactive protein (CRP) was measured in peripheral blood. The response of monocyte-derived macrophages to polypropylene (PP), polylactic acid (PLA), polyethylene terephthalate (PET) monofilament, and PET-multifilament (mPET) in culture was based on cytokine production. More IL-6 (for PET), less CCL18 (all materials) and IL-1ra (for PLA) was produced by macrophages from obese patients than lean subjects. Body mass index, serum CRP and to a lesser extend percentages of monocyte subtypes correlated with IL-6, TNFα, CCL18, and IL-1ra production. Taken together, monocyte-derived macrophages of obese patients respond more pro-inflammatory and less anti-inflammatory to biomaterials than macrophages from lean subjects, depending on the material. These results are a step towards personalized medicine for the development of a model or even a blood test to decide which biomaterial might be suitable for each patient.

INTRODUCTION

Biomaterials are often used in several surgical disciplines such as urology, gynaecology and general surgery[165]. The foreign body response to implanted biomaterials is crucial for adapting the material in the human body. Macrophages play a key role in the foreign body reaction to biomaterials[57]. For regenerative biomaterials, an initial pro-inflammatory (M1) response is necessary for recruiting inflammatory cells to encourage the foreign body response, which are necessary events for wound healing including ingrowth. However, a prolonged M1 response results in fibrous capsule formation and extended inflammation. Therefore, a subsequent transition to the anti-inflammatory macrophages (M2), which promotes tissue repair and remodeling, is generally presumed to be the preferred modification[166]. Achieving the desired outcome is individual and biomaterial dependent.

In general, obesity seems to be an important factor for adverse outcomes after surgery. Observed complications are surgical site infections, impairment of cutaneous wound healing, wound failure, anastomotic leakage, and fascia dehiscence[167-169]. These complications are major risk factors to develop incisional hernia or a recurrent incisional hernia after repair[170,171]. Potential factors that increase wound complications by obesity include intrinsic tenuous anatomic properties, poor vascularization, and cellular and molecular alterations. Inflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), leptin, and angiotensin increase simultaneously with increasing mass of adipose tissue and adipocyte size[167]. These factors negatively affect wound healing and are most likely produced by many types of cells than macrophages alone. Obesity is also positively correlated with oxidative stress which can lead to decreased oxygen tension and impaired fibroblast proliferation and collagen synthesis[167].

Due to obesity, macrophages undergo a phenotypic switch from M2 to M1, which leads to a chronic low-grade systemic inflammation[172-176]. Monocytes, the precursors of macrophages, can be divided into subsets, according to their expression of the cell surface antigens CD16 (Fcγ receptor III) and CD14 (a receptor for bacterial lipopolysaccharide (LPS))[177]. The classical monocyte has high CD14 ($CD14^{++}$) cell surface expression and is CD16 negative ($CD16^{-}$). The non-classical monocyte also expresses CD14 at its surface but at an approximately ten times lower level than the classical monocyte ($CD14^{+}$), and is positive for CD16 ($CD16^{++}$). The intermediate monocyte expresses CD14 at a high level ($CD14^{++}$), and CD16 at an approximately ten times lower level than the non-classical monocyte ($CD16^{+}$). In general, monocytes expressing CD16 have a high phagocytic capacity and produce more pro-inflammatory cytokines such as TNFα and IL-6, and are therefore considered pro-inflammatory[178]. The classical $CD14^{++}/CD16^{-}$ subset is the predominant population and accounts for

approximately 90% in healthy persons. It has been suggested that obesity leads to a shift from classical towards intermediate and non-classical monocytes[179,180].

Previous *in vitro* models have shown that culturing macrophages isolated from healthy donors on different biomaterials leads to a biomaterial-specific reaction[43] and that even in a contaminated *in vitro* model, surgical biomaterials still elicit differential reactions in macrophages[116]. These *in vitro* models did not take into account patient specific characteristics, such as age, smoking, diabetes or obesity. Obesity is a growing healthcare issue in the clinics and a subgroup of these patients does receive a biomaterial for several reasons like abdominal wall hernia with an increased risk of unwanted reactions to the biomaterial or delayed wound healing[167,168]. Therefore, the aims of this study were to investigate how obesity affects the acute host response of macrophages to biomaterials *in vitro* and to examine whether this *in vitro* response can be predicted beforehand by determining monocyte subsets in the blood or by measuring the systemic inflammation marker CRP that is a commonly used clinical parameter for inflammation.

MATERIALS AND METHODS

Study population

In total we included 20 obese patients and 20 age and gender matched healthy, lean (BMI 18-25 kg/m^2) volunteers. Obese patients with a BMI >30 kg/m^2 were included at the department of bariatric surgery at the Maasstad Hospital, Rotterdam. Exclusion criteria for both groups were smoking, diabetes mellitus, use of immunosuppressive drugs, autoimmune disease or chronic inflammatory disease, and medical history such as previous surgery or having a prosthesis (e.g., vascular implants, mesh, osteosynthesis material). This study was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam, Netherlands, in accordance with the Dutch law on medical research in humans. Permit number MEC-2014-221, NL47780.078.14. Written informed consent was obtained from all patients.

Biomaterials

Four types of biomaterials were selected for use in all experiments: polypropylene (PP; 0.9 g/cm^3; Ø 0.10 mm monofilament yarn; ETO (ethylene oxide) sterilized), polyethylene terephthalate multifilament (mPET; 1.34 g/cm^3; 50 dTex (10^{-7} kg/m) - 22 filaments per yarn (50/22) multifilament yarn; gamma sterilized), polyethylene terephthalate monofilament (PET; 1.34 g/cm^3; Ø 0.09 mm monofilament yarn; gamma sterilized) and polylactic acid (PLA; 1.25 g/cm^3; Ø 0.15 mm monofilament yarn; ETO sterilized). All materials were provided as yarns braided in the same conformation and with same

volumic density. The length of the yarn was adjusted according to the density (g/cm^3) values of each material (Figure 6.1). The braided materials are created of a mix of micro- and macro-porosity that favors cell attachment, particularly for monofilaments, and even more particularly, for polypropylene monofilaments.

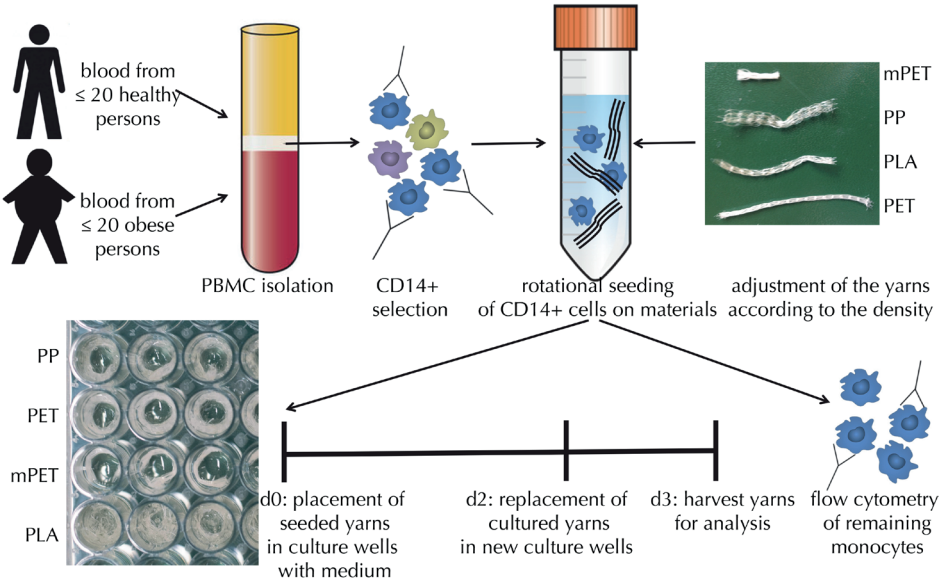


Figure 6.1: Experimental flow of our study, including pictures of the yarns and how the yarns were placed in the culture wells. The length of the yarns was adjusted according to the density of the material. CD14 = cluster of differentiation 14, PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

Monocyte isolation and seeding on biomaterials

Peripheral blood mononuclear cells (PBMC) were isolated from 30 mL blood of obese patients and healthy volunteers by gradient density separation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK). The blood from the obese patients was obtained preoperatively to bariatric surgery. Monocytes were isolated by CD14$^+$ selection. Briefly, the blood was diluted 1:1 with PBS (Gibco; Carlsbad, USA) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, USA), applied on top of a Ficoll layer, and centrifuged at 900 x *g* for 30 minutes to acquire separation of layers. The interphases were collected and washed twice with PBS/0.1% BSA before a 20-minute incubation at 4°C with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Positive selection was performed by Magnetic-acitvated cell sorting (MACS). The isolated CD14$^+$ monocytes were kept in suspension in X-VIVO™

15 medium (Lonza, Verviers, Belgium) containing 20% heat inactivated fetal calf serum (FCS; Lonza), 50 µg/mL gentamicin (Gibco) and 1.5 µg/mL amphotericin B (Fungizone; Gibco), from now on referred to as 'culture medium', until seeding. Prior to seeding, the biomaterials were pre-conditioned in non-heat inactivated FCS for 2 hours at 37°C with agitation. After pre-conditioning, the monocytes were seeded by rotation onto the biomaterials for 2 hours at 37°C at 20 rpm (VWR tube rotator, Radnor, Pennsylvania, USA). The materials were exposed to 425,000 monocytes per yarn at a concentration of 500,000 monocytes/mL. After seeding, the materials were carefully transferred to 96-well plates (Corning Costar, NY, USA) and cultured in 125 µL culture medium per well. Per patient, four different materials in triplicate were cultured. The culture system is shown in Figure 6.1. After 2 days of culture, the materials were transferred to new wells and medium was refreshed to only take into account the biomaterial adherent cells. Twenty-four hours after refreshing, the medium was collected while keeping the three samples separate, centrifuged for 10 minutes at 300 x *g* and stored at -80°C for later cytokine quantification. The macrophages adhering to the biomaterials were lysed in 125 µL PBS/0.1% Triton-X (Sigma-Aldrich) and stored at -20°C before DNA quantification. The remaining $CD14^+$ monocytes that were not used for seeding were stored in 20% dimethyl sulfoxide (DMSO)/FCS in liquid nitrogen for flow cytometric analysis.

Protein adsorption by the biomaterials

To evaluate potential adsorption of the proteins by the materials, the materials were pre-conditioned for 2 hours in non-heat inactivated FCS with agitation, followed by 2 hours incubation in X-VIVO/20% FCS in a tube rotator at 37°C. Next, the materials were transferred to well plates and incubated in X-VIVO/20% FCS for 2 days. After this period, the materials were transferred to new well plates and incubated in medium containing either 1 ng/mL IL-6 (Peprotech), 250 pg/mL CCL18 (R&D Systems), 1.25 ng/mL IL-1RA (R&D Systems), 500 pg/mL TNFα or no cytokine. After an additional incubation day, the media were harvested, centrifuged at 300 x *g* and stored at -80°C until cytokine quantification. The use dosages were based on the detection ranges of the enzyme-linked immunosorbent assays (ELISAs) that were used to determine cytokine concentrations.

Cytokine quantification

Enzyme-linked immunosorbent assays (ELISAs) were performed according to manufacturer's instructions to quantify the concentrations of CCL18, IL-1RA, IL-6, and TNFα (R&D Systems, Minneapolis, MN, USA) released in the cell culture supernatants. These selected cytokines were chosen based on our previous research in which CCL18, IL-1RA, IL-6, and TNFα were the most discriminative for the different macrophages

phenotypes[43,181,182]. All measurements fitted within the standard curve, for every material and cytokine different dilutions had to be made of the culture medium, ranging from a 3 to 100 times dilution. C-Reactive Protein (CRP) levels in the plasma were determined using the standard technique at the hospital's laboratory (Dimension Vista® System, Flex reagent cartridge, Siemens Healthcare Diagnostics Products, Germany) and expressed in mg/L. CRP is a very common used parameter in all hospitals to detect early systemic inflammation, also prior to surgery, especially in obese patients.

DNA quantification

Since cell attachment was different between materials we normalized the protein content in the culture media to the amount of DNA on the biomaterial as an indication for the cell number. By normalizing for DNA, we adjust for variation in cell number allowing to determine the production of cytokines per cell, not influenced by the number of cells that adhered to the material. DNA was quantified with a modified CYQUANT® cell proliferation assay (Invitrogen, Carlsbad, California, USA), in order to normalize the cytokine production for the number of cells. In short, the samples were sonicated for 30 minutes at 48 kHz to completely disintegrate the cells. Next, a solution containing 250 IU heparin (LEO Pharma, Ballerup, Denmark) and 125 µg RNAse (Sigma-Aldrich) were added to the suspensions and incubated for 30 minutes at 37°C. Finally, 0.375 µL CyQUANT GR dye was added to each sample and fluorescence was immediately measured on a SpectraMax Gemini micro plate reader (Molecular Devices, Sunnyvale, USA) at 480 nm excitation and 520 nm emission.

Flow cytometric analysis

Monocytes were thawed from -180°C and re-suspended at 500,000 cells/mL in FACSFlow solution (BD Biosciences) and stained for 30 minutes at 4°C with antibodies against human CD14 conjugated with APC-H7 and CD16 conjugated with PE (both BD Biosciences), according to the manufacturer's guidelines. Unstained cells were used as negative control. Flow cytometric analysis was performed using the FACSJazz™ (BD Biosciences), and data were analyzed using FlowJo (FlowJo v7.6.4/v10; Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM Inc., Chicago, USA). Basic characteristics are presented as mean ± standard deviation (SD) and data related to cytokines are presented as mean and standard of mean (S.E.M.). An independent T-test was used for the age and BMI due to normal distribution of these parameters. Mann Whitney U analysis was used for statin use. To compare cytokine levels between macrophages obtained from lean and obese subjects and compare cytokine levels

between the different materials within the obese and control group, a Kruskal-Wallis analysis followed by a post-hoc Mann Whitney U analysis. An M1/M2-index per material was calculated based on the cytokine production of pro-inflammatory (M1) cytokines IL-6 and TNFα and anti-inflammatory (M2) cytokines CCL18 and IL-1RA. To prevent that the calculation is skewed to a certain cytokine that is produced in a higher quantity, the production per cytokine is first normalized to the average production of that cytokine. The mean of the normalized M1 cytokine levels per patient to the overall M1 cytokine levels of all patients, was divided over the mean of the normalized M2 cytokine levels per patient to the overall M2 cytokine production of all patients, as shown in the following formula.

$$M1/M2\text{-}index = \frac{\frac{(\text{IL-6}_{\text{per patient}} + \text{TNF}\alpha_{\text{per patient}})}{(\overline{\text{IL-6}_{\text{all patients}}} + \overline{\text{TNF}\alpha_{\text{all patients}}})}}{\frac{(\text{CCL18}_{\text{per patient}} + \text{IL1ra}_{\text{per patient}})}{(\overline{\text{CCL18}_{\text{all patients}}} + \overline{\text{IL1ra}_{\text{all patients}}})}}$$

To determine correlations, a non-parametric Spearman test was performed. All reported *p*-values were two-sided; a p-value < 0.05 was considered to indicate statistical significance. Since the analyses were exploratory and the groups sizes small, no adjustment for multiple testing was performed.

RESULTS

As a result of our inclusion criteria, BMI was significantly different between the included lean and obese subjects. Age, gender and the use of statins were not significantly different between the two groups (Table 6.1).

Table 6.1: Patient characteristics lean group vs obese patients.

	lean (n=20)	obese (n=20)	p-value
BMI (kg/m^2)	22.9 ± 2.6	43.8 ± 6.5	<0.001
gender (male/ female)	2/18	2/18	1.0
age (years)	41.8 ± 13.1	41.3 ± 13.5	0.916
use of statins	0/20	2/20	0.154

Values are means (SD), p-value was estimated by using independent sample T-test

Obesity influenced cytokine production by macrophages on biomaterials

The production of IL-6 and TNFα as indicators for a pro-inflammatory response and CCL18 and IL-1ra as indicators for an anti-inflammatory response were measured.

Macrophages from obese patients produced significantly more IL-6 than macrophages from lean subjects when cultured on PET (144.0 pg IL-6/ng DNA vs 102.0 pg IL-6/ng DNA, $p = 0.022$). No significant differences were seen for the other materials regarding IL-6 production (Table 6.2 and Supplementary Figure S6.2A). TNFα production was not significantly different between the groups for any of the tested materials (Table 6.2 and Supplementary Figure S6.2B). CCL18 production was significantly higher for all materials in the lean group than in the obese group (Table 6.2 and Supplementary Figure S6.2C). IL-1ra production was higher in the lean group than in the obese group when cultured on PLA (34.6 pg IL-1RA/ng DNA vs 15.5 pg IL-1RA/ng DNA, $p = 0.026$) but not on the other materials (Table 6.2 and Supplementary Figure S6.2D).

No IL-6, TNFα, CCL18, and IL-1RA were detectable in medium with serum alone and thus also no difference was seen after incubation of the material in medium with serum but without adherent cells. When the proteins of interest were spiked in the culture medium, adsorption of these proteins was seen to the materials, with the most adsorption of all four proteins to PP, and PLA in the case of IL-6 (Figure 6.2).

Table 6.2: The average production of IL-6, TNFα, CCL18, and IL-1RA corrected for DNA by macrophages on the different materials.

		cytokine production by macrophages (pg protein/ng DNA)		
cytokine	material	lean (mean ± SD)	obese (mean ± SD)	*p* value
IL-6	PP	116.6 ± 97.2	172.4 ± 114.1	0.106
	PLA	109.2 ± 67.1	157.4 ± 146.8	0.247
	PET	102.0 ± 73.9	144.0 ± 58.4	**0.022**
	mPET	39.2 ± 23.9	68.7 ± 64.0	0.140
TNFα	PP	7.9 ± 8.3	5.9 ± 4.9	0.300
	PLA	5.0 ± 4.4	3.3 ± 3.1	0.119
	PET	3.6 ± 3.8	3.3 ± 1.5	0.417
	mPET	1.0 ± 0.9	0.7 ± 0.5	0.421
CCL18	PP	0.8 ± 0.7	0.2 ± 0.3	**< 0.001**
	PLA	1.4 ± 0.9	0.4 ± 0.4	**0.002**
	PET	1.6 ± 1.3	0.5 ± 0.6	**0.002**
	mPET	0.7 ± 0.6	0.3 ± 0.3	**0.007**
IL-1ra	PP	49.4 ± 52.2	24.4 ± 16.8	0.128
	PLA	34.6 ± 28.5	15.5 ± 9.0	**0.026**
	PET	32.3 ± 22.6	20.2 ± 13.6	0.071
	mPET	18.0 ± 18.4	10.0 ± 12.3	0.057

Bold values denote statistical significance

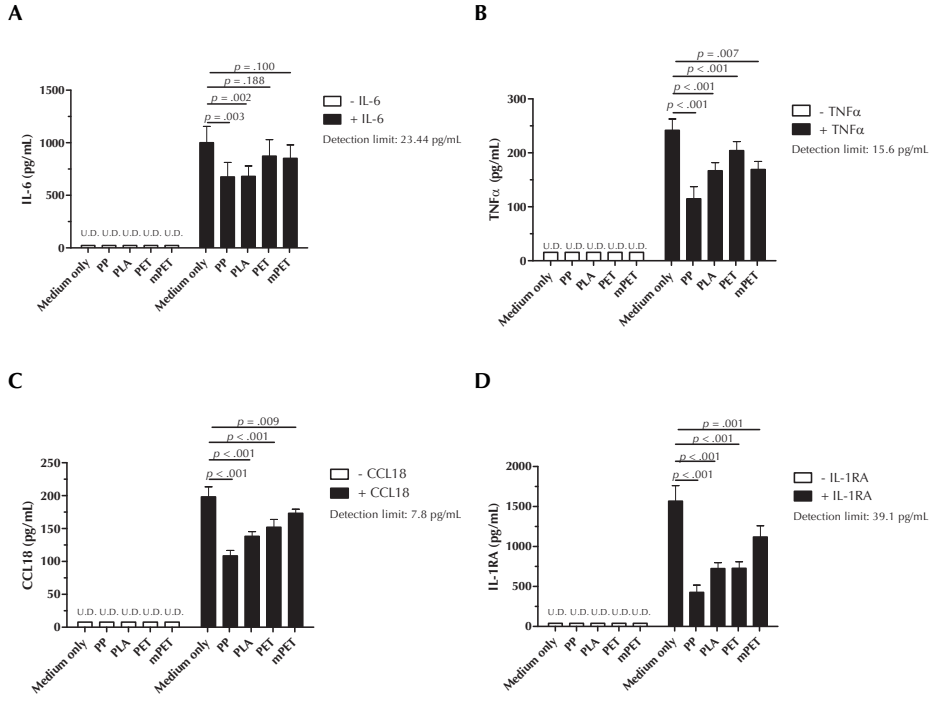


Figure 6.2: Measurements of **(A)** IL-6, **(B)** TNFα, **(C)** CCL18, **(D)** and IL-1RA in the culture medium with and without the incubation of the biomaterials and with and without spiking of the protein of interest. White bar indicates measurements in medium with or without incubation of the materials. Black bars indicate measurements in medium alone or after incubation with the material in the presence of the spiked proteins. Bars represent n= 6 + SD for every bar.U.D.: undetectable.

The DNA concentration as an indication for the number of attached macrophages to the biomaterials, was not significantly different between the lean and obese patients in all biomaterials (Supplementary Figure S6.3A). Absolute protein production per individual is shown in Supplementary Figure S6.2B-D. When comparing the effect of the materials on macrophages within the obese and lean group and per material, PP induced higher levels of IL-6, TNFα, and IL-1RA than the other materials, especially when compared to mPET. Less clear differences between materials were seen for CCL18 production (Figure 6.3).

To compare overall response of the different materials in lean and obese subjects, an M1/M2 index was calculated for each condition. The M1/M2 index was significantly higher of the obese group than for the lean subjects for PP ($p < 0.001$), PET ($p = 0.001$), and mPET ($p = 0.003$) but not for PLA. No differences regarding the M1/M2 index were seen between materials for the lean subjects. In obese patients, PLA resulted in the lowest M1/M2 index, and mPET the highest (Figure 6.4).

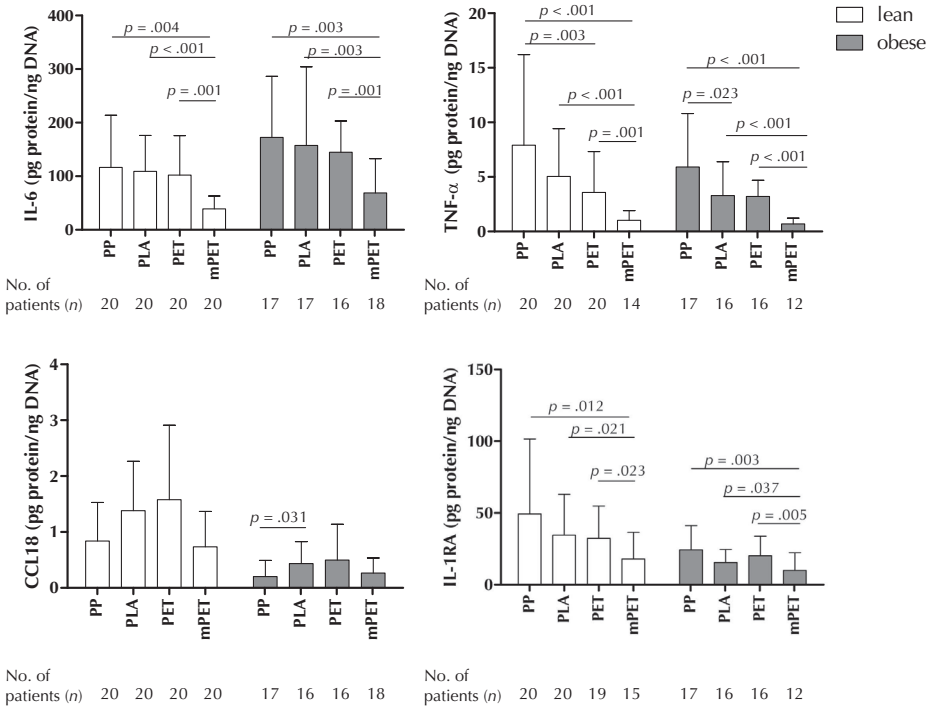


Figure 6.3 Cytokine production corrected for DNA compared per material, in lean subjects or in obese subjects. Number of patients per cytokine and per material are indicated in the bars or just above the error bar.

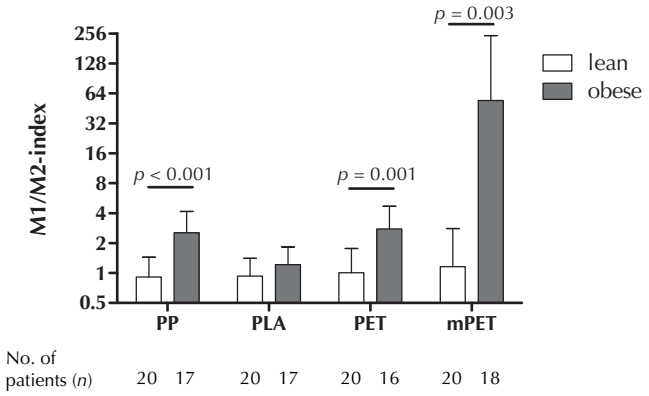


Figure 6.4: M1/M2 index between materials. Bars represent the mean, error bars the SD, *p*-values indicate significant differences. A base 2-log scale is used for the Y-axis. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate. The number of patients included per material, per group are indicated in the bars.

Serum CRP and BMI correlate with cytokine production by macrophages

The average C-reactive protein level in lean subjects was 1.3 ± 1.8 mg/L versus 15.6 ± 17.1 mg/L in obese patients, $p = 0.004$ (Supplementary Figure S6.4). CRP concentration positively correlated with BMI (Table 6.3). A positive correlation was also seen between CRP and IL-6 production in response to the material for all materials, but only significant for PP and mPET. A significant negative correlation was seen between serum CRP concentration and CCL18 production by macrophages in response to PP, PLA, and mPET and between BMI and CCL18 production by macrophages in response to PP, PLA, and mPET. CRP also negatively correlated with IL-1RA production in response to PP, PLA, and mPET. CRP or BMI did not correlate with TNFα production (Table 6.3).

Table 6.3: Correlations between CRP concentration in peripheral blood, BMI of all subjects, and cytokine production by the macrophages.

	material	CRP		BMI	
		r	*p*- value	r	*p*-value
CRP	-	-	-	0.64	**< 0.001**
IL-6	PP	0.37	**0.046**	0.27	0.111
	PLA	0.20	0.310	0.22	0.198
	PET	0.40	0.035	0.27	0.146
	mPET	0.53	**0.003**	0.22	0.186
CCL18	PP	-0.45	**0.012**	-0.44	**0.006**
	PLA	-0.56	**0.002**	-0.37	**0.028**
	PET	-0.36	0.063	-0.39	**0.017**
	mPET	-0.54	**0.002**	-0.30	0.068
IL-1ra	PP	-0.36	**0.05**	-0.15	0.391
	PLA	-0.45	**0.015**	-0.20	0.245
	PET	-0.35	0.075	-0.22	0.211
	mPET	-0.54	**0.013**	-0.22	0.267
TNFα	PP	-0.17	0.382	-0.18	0.295
	PLA	-0.15	0.438	-0.24	0.16
	PET	0.14	0.492	0.02	0.903
	mPET	-0.17	0.476	-0.30	0.143

Bold values denote statistically significant *p*-values

Table 6.4 Percentages of peripheral blood monocytes subsets in lean (n=9) and obese (n=8) subjects. Values are mean +/- SD.

% of monocyte	lean	obese	*p*-value
classical ($CD14^{++}CD16^{-}$)	90.9 ± 5.3	77.4 ± 22.0	0.290
intermediate ($CD14^{++}CD16^{+}$)	2.2 ± 3.4	7.9 ± 13.4	0.336
non-classical ($CD14^{+}CD16^{++}$)	4.0 ± 3.8	12.9 ± 13.7	0.211

Table 6.5A Spearman correlation between percentages of $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate), or $CD14^{+}CD16^{++}$ (non-classical) monocyte subsets and production of cytokines by cultured macrophages on the four different materials. The Spearman correlation coefficients (r) define the relationship between monocyte subsets from peripheral blood and the production of IL-6, CCL18, IL-1ra, and TNFα) by macrophages cultured on PP, PLA, PET, and mPET. Table **6.5B** shows the correlation between the percentages of monocyte subsets with the M1/M2 index for the four different materials. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

A	material	$CD14^{++}CD16^{-}$		$CD14^{++}CD16^{+}$		$CD14^{+}CD16^{++}$	
		r	*p*- value	r	*p*-value	r	*p*-value
CRP	-	-0.42	0.120	0.35	0.198	0.35	0.203
BMI		-0.16	0.535	0.12	0.636	0.31	0.231
IL-6	PP	-0.17	0.541	0.27	0.334	0.26	0.355
	PLA	-0.43	0.086	0.53	**0.028**	0.41	0.103
	PET	-0.54	**0.038**	0.51	0.052	0.47	0.079
	mPET	-0.36	0.158	0.38	0.133	0.26	0.323
CCL18	PP	0.28	0.321	-0.13	0.639	-0.23	0.405
	PLA	0.16	0.549	-0.32	0.107	-0.18	0.370
	PET	0.21	0.451	-0.40	**0.045**	-0.36	0.073
	mPET	0.24	0.353	-0.50	**0.007**	-0.39	**0.039**
IL-1ra	PP	0.12	0.676	-0.43	0.108	0.18	0.516
	PLA	0.16	0.529	-0.29	0.252	0.04	0.889
	PET	0.28	0.334	-0.65	**0.011**	-0.03	0.911
	mPET	0.13	0.658	-0.53	0.052	0.16	0.594
TNFα	PP	-0.37	0.173	0.17	0.550	0.42	0.121
	PLA	-0.35	0.171	0.37	0.141	0.21	0.428
	PET	-0.46	0.084	0.30	0.283	0.30	0.296
	mPET	-0.41	0.167	0.01	0.986	0.42	0.152

B Spearman correlation between percentages of monocyte subsets and M1/M2 index

		$CD14^{++}CD16^{-}$		$CD14^{++}CD16^{+}$		$CD14^{+}CD16^{++}$	
	material	r	*p*-value	r	*p*-value	r	*p*-value
M1/M2	PP	-0.59	**0.020**	0.36	0.182	0.48	0.069
	PLA	-0.40	0.112	0.37	0.144	0.35	0.174
	PET	-0.45	0.092	0.26	0.341	0.27	0.328
	mPET	-0.51	**0.038**	0.58	**0.016**	0.41	0.098

Bold values denote statistically significant *p*-values

Differences in monocyte subsets between lean and obese patients

Monocytes changed in shape and granularity based on the forward scatter and sideward scatter as a result of freezing and thawing (Supplementary Figure S6.1A and S6.1C). Percentages of monocytes subsets remained however comparable and not affected by freezing and thawing (Supplementary Figure S6.1B and S6.1D). The percentages of classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), or non-classical monocytes ($CD14^{+}CD16^{++}$) in peripheral blood were not statistically significantly different between lean and obese subjects (Table 6.4). However, the percentages of intermediate monocytes correlated positively with IL-6 for PLA and negatively with the CCL18 protein production for PET and mPET, and with IL-1RA for mPET. The percentages of non-classical monocytes correlated negatively with CCL18 production when macrophages were cultured on mPET. No statistically significant correlations were seen between percentages of monocyte subsets and TNFα production by the macrophages cultured on any of the biomaterials (Table 6.5A). For PP and mPET the M1/M2 index significantly correlated with the percentage of classical monocytes. Intermediate monocytes significantly correlated negative with the M1/M2 index for mPET.

DISCUSSION

The use of biomaterials has become common in regenerative medicine. The reaction of primary human macrophages to biomaterials has been shown *in vitro* to be biomaterial specific, even when an inflammatory situation is simulated[43,116]. However, the person-dependent foreign body response has not been taken into account in these models. In the current explorative study, we investigated the effect of obesity, a growing problem in the Western world, on the response of macrophages to biomaterials and found that on average macrophages from obese patients respond more pro-inflammatory to biomaterials as indicated by higher IL-6 and lower CCL18 and IL-1RA production than in macrophages from lean persons that were cultured on the same materials. In addition, we found that BMI, serum CRP and percentages of monocyte subsets in the peripheral blood correlate with the response of the macrophages to the biomaterials *in vitro*, and that these correlations were biomaterial specific. In addition, we showed that macrophages derived from monocytes from obese patients still respond pro-inflammatory, even when they are not in an obese environment anymore. To our knowledge, this is the first study that investigated the differences in macrophage response to biomaterials between lean and obese patients.

Obese patients included in this trial had no insulin resistance and therefore, according to the WHO criteria, no metabolic syndrome[183]. Because of the strict

inclusion and exclusion criteria, such as no smoking, no implants, and the absence of diabetes mellitus we assume that the different responses to the biomaterials between lean and obese patients is the result of obesity only and not because of a difference in the presence of diabetes or previous operations in which biomaterials were used. However, certain risk factors that are unknown at the moment might have influenced our measurements and have resulted in the large variation that is sometimes observed in the cytokine measurements. Although these patients do not have a metabolic syndrome, 50% of them had a CRP level >10 mg/L, indicating systemic low-grade inflammation. CRP levels in the serum correlated positively with IL-6 production by the macrophages on PP and mPET and negatively with CCL18 and IL-1RA levels on PP, PLA and mPET *in vitro*, showing that CRP has a pro-inflammatory effect on macrophages. This was supported by an *in vitro* study, where it was shown that CRP polarizes human macrophages to an M1 phenotype[184]. A shift from classic monocytes in the peripheral blood to intermediate or non-classic monocytes has been seen before as a result of obesity[174,185,186], of which the latter two subsets are regarded as the pro-inflammatory subsets with increasing CD16 positivity[174,185,186]. We did not observe a statistically significant shift when comparing the presence of these subsets between lean persons and obese patients. This can be due to the fact that the inclusion criteria were strict and only obese patients without a metabolic syndrome were included. In addition, the numbers of patients from whom we were able to obtain a sufficient number of monocytes to perform additional flow cytometric analysis next to culture with biomaterials were low and thus resulting in a low power. Interestingly however, when comparing percentages of monocyte subsets in the peripheral blood with the cytokine production of monocyte-derived macrophages on biomaterials *in vitro*, CCL18 and IL-1RA production by macrophages on mPET and PET *in vitro* were correlated with the percentages of the different monocyte subsets in the peripheral blood. The percentages of classical monocytes correlated positively with CCL18 and IL-1ra levels produced by macrophages in culture, the percentages of the more pro-inflammatory intermediate and non-classical subsets correlated negatively with CCL18 production in culture. CCL18 is a chemokine that is predominantly made by anti-inflammatory macrophages [43,69], indicating that the initial presence of classical monocytes is associated with the differentiation towards anti-inflammatory macrophages. As could be seen from the individual levels of IL-6, CCL18 and IL-1RA, not all obese patients had macrophages producing high levels of IL-6 and low levels of CCL18 or IL-1RA. No corrections for baseline production of the cytokines of interest were made however, because in our opinion, this best represents the *in vivo* situation. Even though no corrections were made, differences were still seen between the effects of different biomaterials on cells of the same patient. This underlines potential patient specific responses even when obesity already changed the metabolic status of the patient and these responses can be

explained by serum CRP levels and percentages of monocyte subsets in the blood. The production of TNFα in our culture system was not influenced by obesity, this might be explained by the short time detection range of TNFα[187]. Based on our data, it seems that PLA followed by PP and PET, are more preferable for obese patients and that all tested materials can be more or less equivalently be used for lean for lean patients, assuming that a pre-dominant anti-inflammatory reaction is preferred. Although the choice of material may be better guided by the inflammatory reaction at the individual patient level rather than at the comorbidity category such as obesity. As shown in previous clinical studies, no enormous undesirable behavior of multifilament PET mesh (e.g., Parietex™ Composite mesh) for hernia repair in obese patients has been reported till now, therefore the clinical impact might be moderate[188,189]. Nevertheless, the patient's outcome can always improve with careful and personalized selection of meshes.

The polymers used in this study are commonly used for materials for soft tissue repair. The host-response to materials is not only material dependent but also the porosity, topography, and surface of the material influence the biocompatibility[166,190-192]. The many different properties of the material influence the polarization of the macrophages[166]. In the current study, the materials were braided in the same way, but because of different diameters of the individual fibers between the materials, the topography was not exactly the same. Therefore, the length of the knitted yarn was adjusted to the diameter to achieve the best possible comparable material appearance. Interestingly, PET and mPET resulted in different M1/M2 indexes, especially when macrophages of obese patients were cultured on the materials. This demonstrates that indeed not only the polymer but also the architecture of the material is important for elicited responses. In this study, PP did not elicit an anti-inflammatory effect based on the cytokines measured. This underlines again that not only the polymer itself is important for the reaction the material elicits, but also the architecture of the material since in our earlier studies we have used meshes instead of yarns[43,116,193-195]. To optimize the macrophage-biomaterial contact, necessary for the low numbers of patient cells available for this study, braided yarns were chosen in the current study. After spiking of IL-6, TNFα, CCL18, and IL-1RA in the culture medium, adsorption was seen, which varied between the materials. Since PP had the most adsorption of our proteins of interest, the values for PP (and for PLA in the case of IL-6) are most likely an underestimation. However, most of the associations and comparisons are made within a biomaterial and a cytokine, not comparing two different cytokines or materials with each other. These comparisons and associations are therefore unaffected in our opinion by the adsorption of the protein of interest. However, the difference in adsorption to each material, and especially the high adsorption to PP, might overshadow the differences in reactions elicited by the materials.

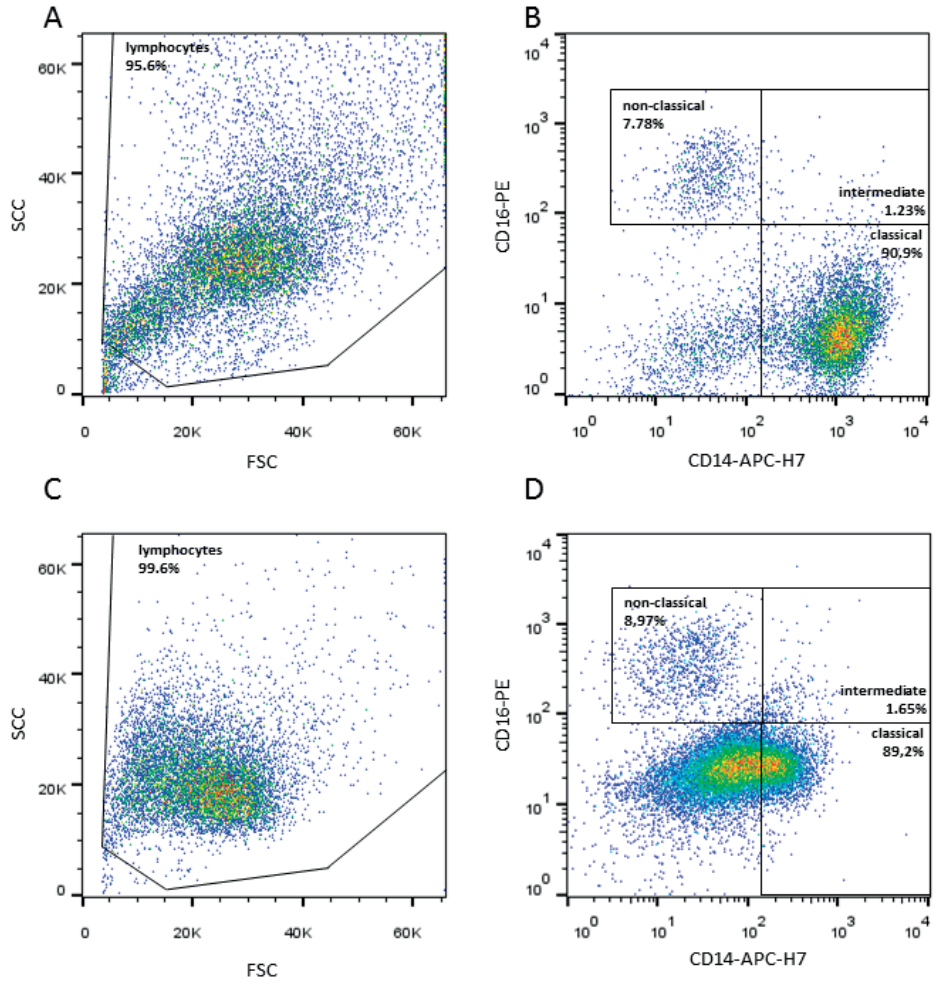
After implantation, the biomaterial eventually will be in contact with macrophages, but it will also be surrounded by non-adherent macrophages and extracellular matrix. We however specifically chose not to include non-adherent macrophages in our experimental set-up. The biomaterials were cultured on tissue culture plates made of tissue culture polystyrene (TCPS), also a polymer. By transferring the materials with their adherent cells to new wells, the medium contained mainly the cytokines from the macrophages adhering to the yarns. TCPS most likely will have a totally different effect than the extracellular matrix that normally surrounds an implanted biomaterial[196,197]. In fact, we have seen that collagen indeed exerts different effects on macrophages than polymers[43,116]. Therefore, we believed that including cytokine production from macrophages adhering to the TCPS would make the system even more artificial, especially since the TCPS has a completely different architecture than the used yarns

The proteins IL-6, TNFα, CCL18, and IL-1RA were selected as indicators of pro-inflammatory and anti-inflammatory responses. We are aware that these cytokines do not represent the full spectrum of mediators produced during the foreign body response. However previously, we have seen that these mediators are most discriminative between pro-inflammatory and anti-inflammatory macrophages[43,69]. Studies to determine the actual *in vivo* response to the biomaterial and correlating this with the parameters in the peripheral blood are necessary to draw firmer conclusions about the predictive value of monocyte subset percentages and serum CRP levels for the reaction biomaterials elicit in a certain patient.

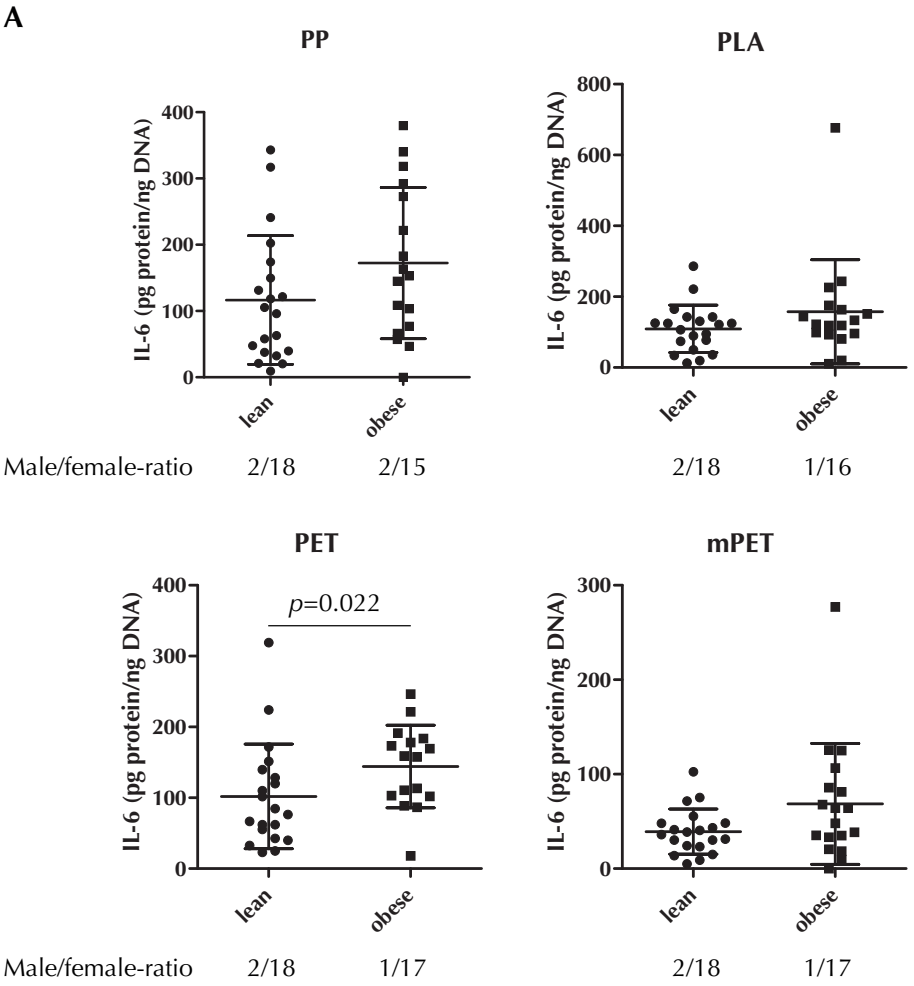
CONCLUSION

Monocyte-derived macrophages of obese patients respond more pro-inflammatory and less anti-inflammatory to biomaterials than macrophages from lean subjects and this response depends on the type of biomaterial. This variation in cytokine production by the macrophages was associated by the percentages of monocyte subsets in the peripheral blood, serum CRP levels, or BMI of the patient. The results of this *in vitro* study offer possibilities and could stimulate future research towards personalized medicine, eventually leading to a model that can be used to test biomaterials for tissue repair and tissue engineering using patient's own cells prior to implantation of a biomaterial. In addition, our results offer the prospect that monocyte subsets in the blood or serum CRP might be measured prior to surgery to predict which biomaterial might be suitable for each patient. Studies indeed examining the clinical outcome after implantation of a biomaterial in relation to serum CRP, BMI, and monocyte subsets are however needed to confirm this.

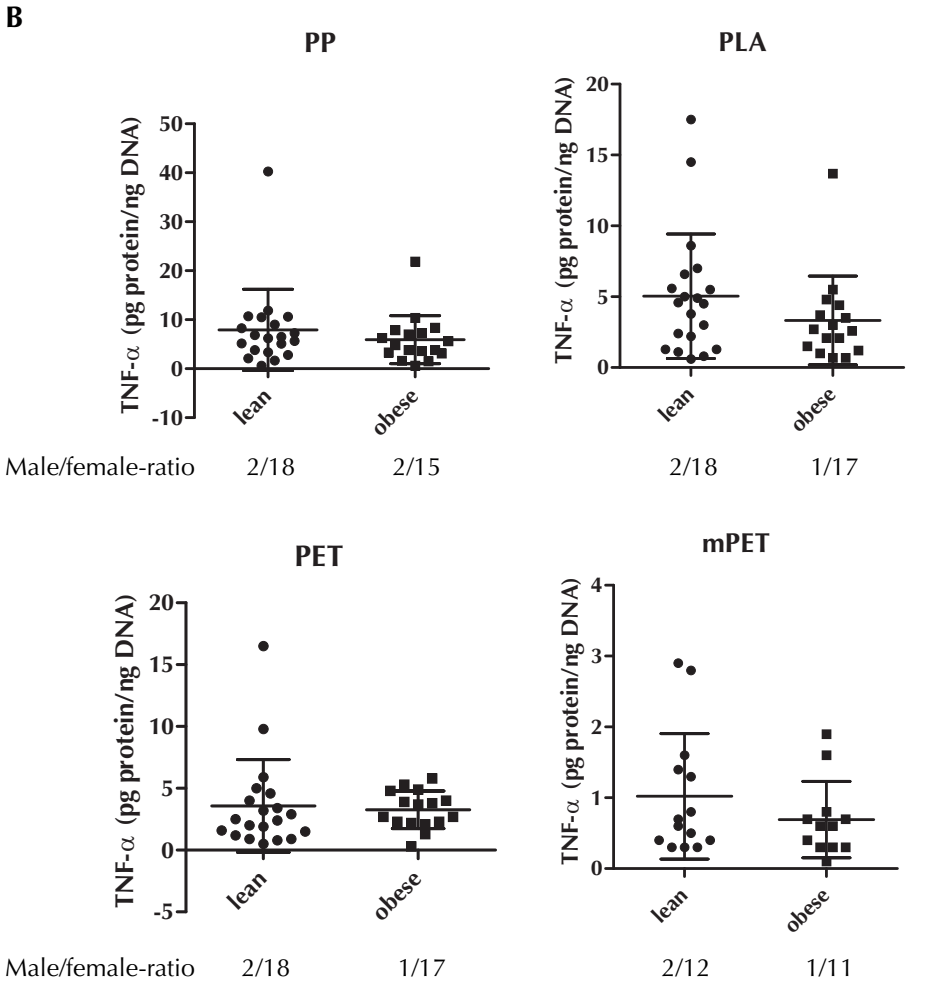
SUPPLEMENTARY DATA



Supplementary Figure S6.1: flow cytometric analysis of fresh (**A, B**), and frozen monocytes (**C, D**). Forward scatter (FSC) and sideward scatter (SSC) show size and granularity of the cells (**A, C**) and monocyte subsets were determined based on the presence of cluster of differentiation 14 (CD14) and CD16 (**B, D**).

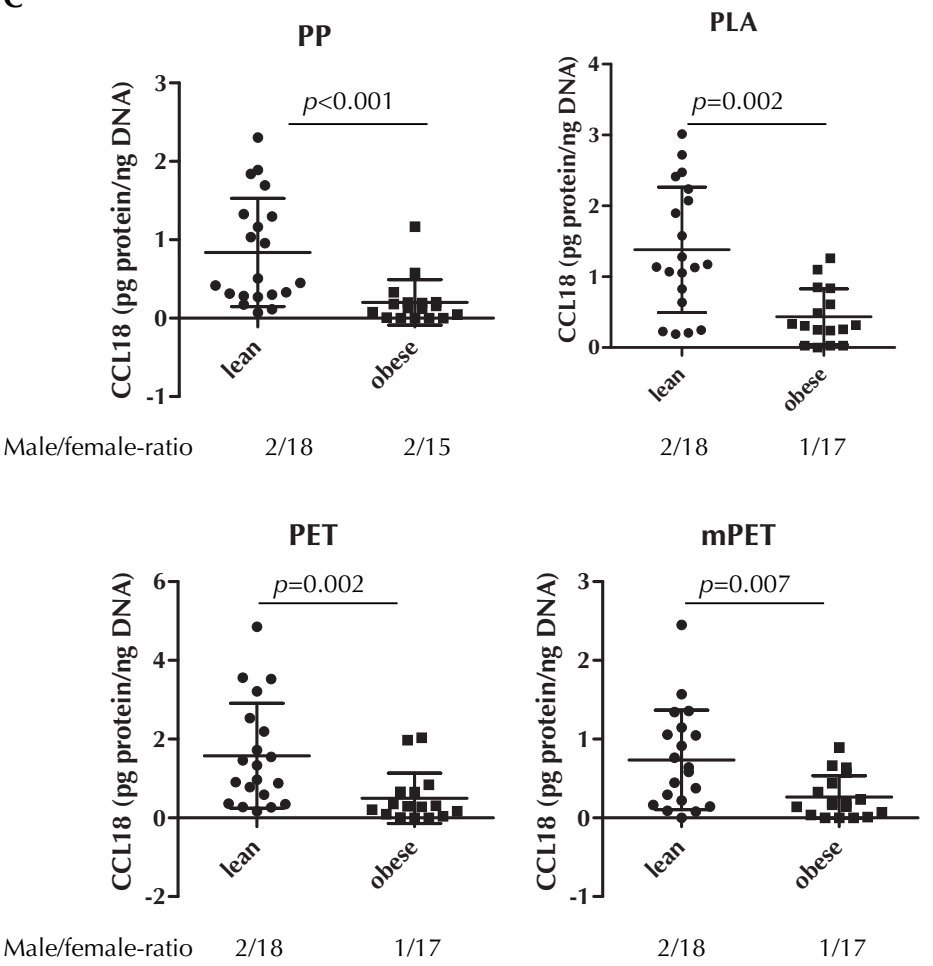


Supplementary Figure S6.2: (A) IL-6, **(B)** TNFα, **(C)** CCL18, and **(D)** IL-1RA production by macrophages seeded on different materials corrected for DNA, lean vs. obese groups shown per material. Every dot represents a single donor. The line indicates the mean, *p*-values indicate a statistically significant difference. Bars represent the mean, whiskers the SD. Ratios underneath the graphs indicate the male/female ratio per measurement and per material. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate. Continued on next pages

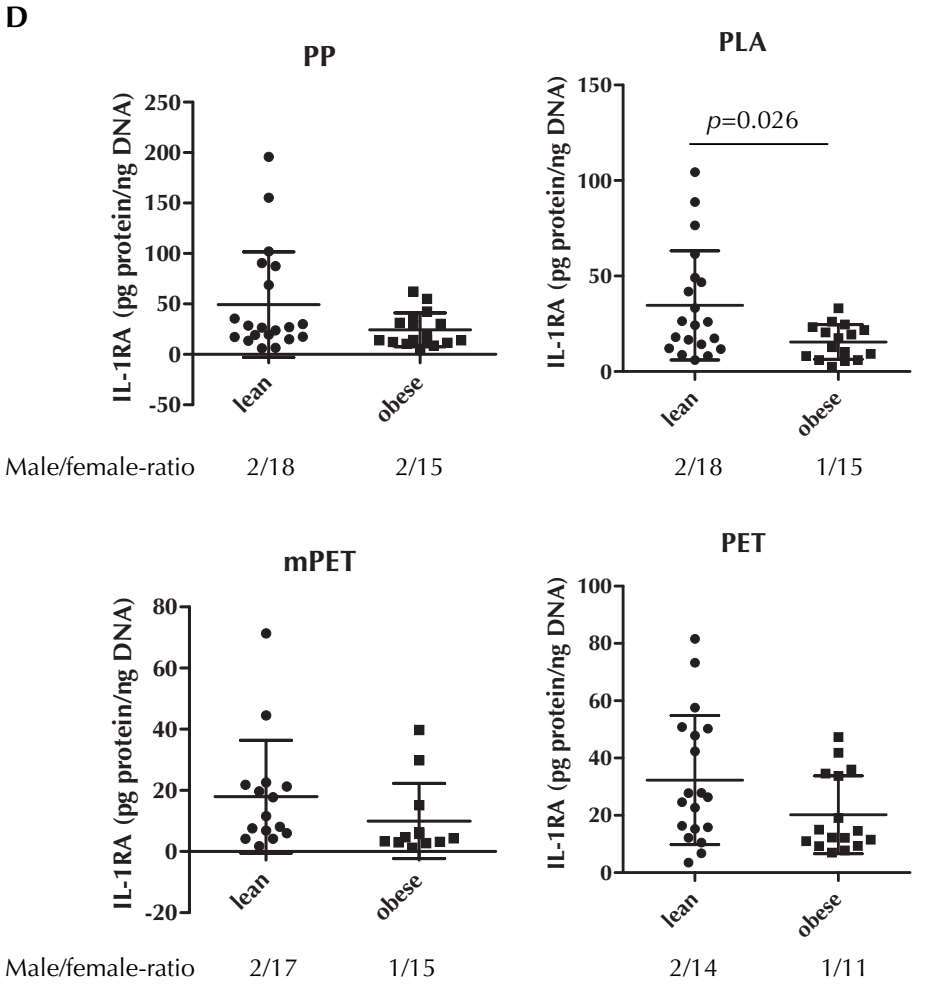


Supplementary Figure S6.2 (continued): (A) IL-6, **(B)** TNFα, **(C)** CCL18, and **(D)** IL-1RA production by macrophages seeded on different materials corrected for DNA, lean vs. obese groups shown per material. Every dot represents a single donor. The line indicates the mean, *p*-values indicate a statistically significant difference. Bars represent the mean, whiskers the SD. Ratios underneath the graphs indicate the male/female ratio per measurement and per material. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

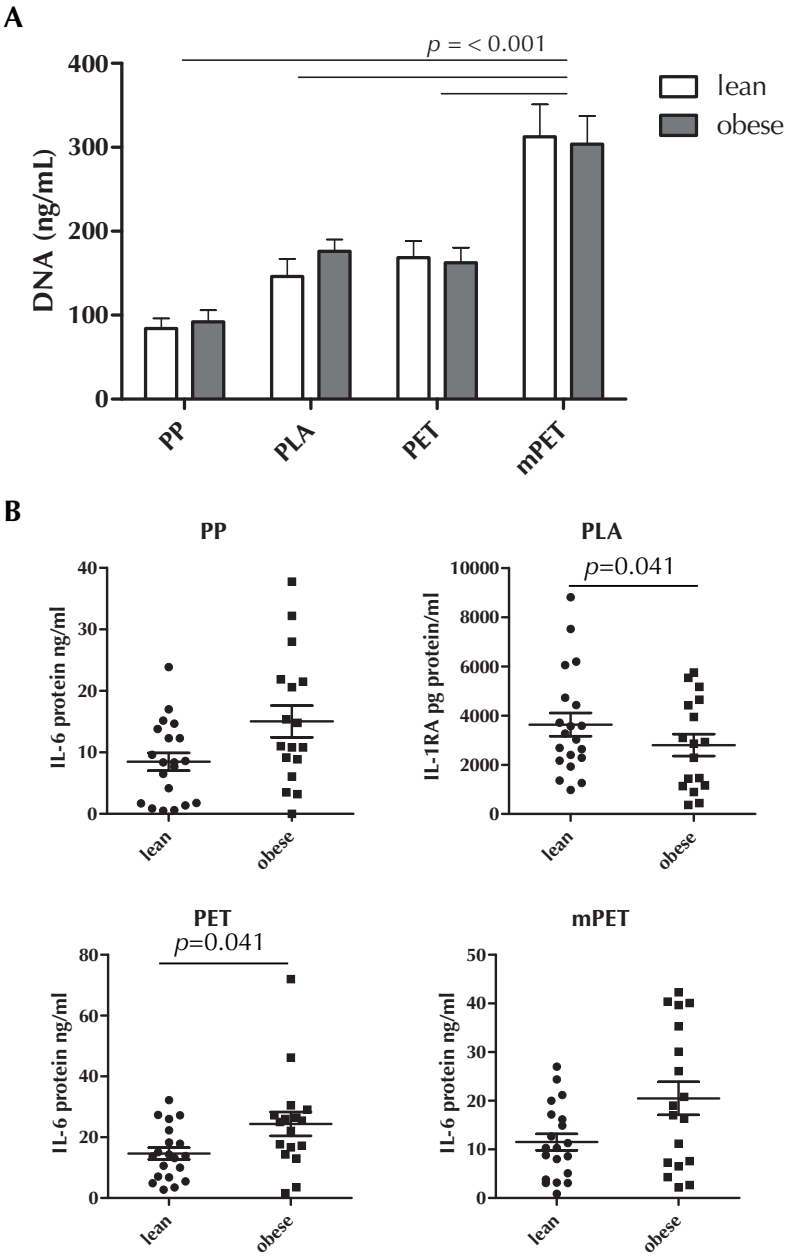
C



Supplementary Figure S6.2 (continued): (A) IL-6, **(B)** TNFα, **(C)** CCL18, and **(D)** IL-1RA production by macrophages seeded on different materials corrected for DNA, lean vs. obese groups shown per material. Every dot represents a single donor. The line indicates the mean, *p*-values indicate a statistically significant difference. Bars represent the mean, whiskers the SD. Ratios underneath the graphs indicate the male/female ratio per measurement and per material. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.



Supplementary Figure S6.2 (continued): (A) IL-6, **(B)** TNFα, **(C)** CCL18, and **(D)** IL-1RA production by significant seeded on different materials corrected for DNA, lean vs. obese groups shown per material. Every dot represents a single donor. The line indicates the mean, *p*-values indicate a statistically significant difference. Bars represent the mean, whiskers the SD. Ratios underneath the graphs indicate the male/female ratio per measurement and per material. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.



Supplementary Figure S6.3 (A) The amount of DNA as indication of the number of attached macrophages to the biomaterials. DNA is shown as ng/mL for polypropylene (PP), polylactic acid (PLA), monofilament polyethylene terephthalate (PET), and multifilament polyethylene terephthalate (mPET) for the lean (open bars) and obese (dotted bars) donors. Bars represent mean ± S.E.M. *n* = 20 donors/ group, three samples/ per material/ per donor, p-value indicates a significant difference.

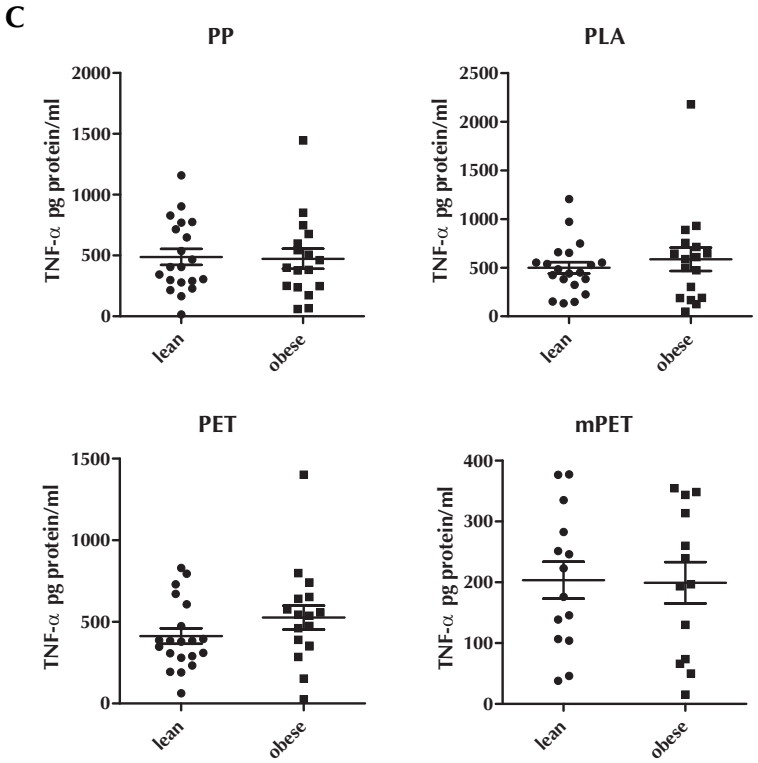


Figure S6.3B, C, D, E Comparing macrophages from lean and obese donors cultured on different materials regarding **B)** IL-6 production and **C)** TNFα production and **D)** CCL18 production and **E)** IL-1RA production in ng/ml after 3 days of culture. Every dot represents a single donor. Line and whiskers indicate mean ± S.E.M., p-values indicate a statistically significant difference. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate. Continued on next pages.

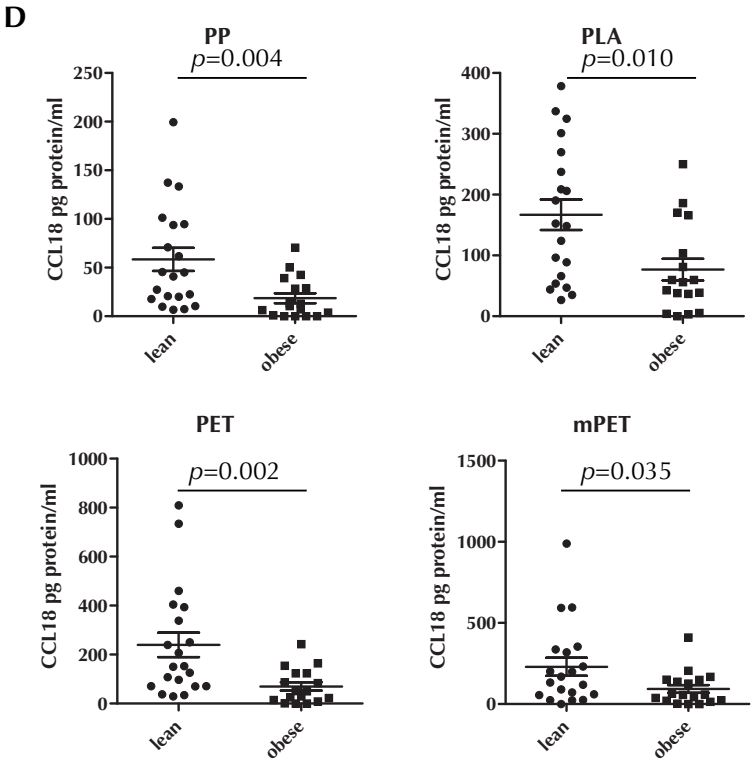


Figure S6.3B, C, D, E (continued) Comparing macrophages from lean and obese donors cultured on different materials regarding **B)** IL-6 production and **C)** TNFα production and **D)** CCL18 production and **E)** IL-1RA production in ng/ml after 3 days of culture. Every dot represents a single donor. Line and whiskers indicate mean ± S.E.M., p-values indicate a statistically significant difference. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

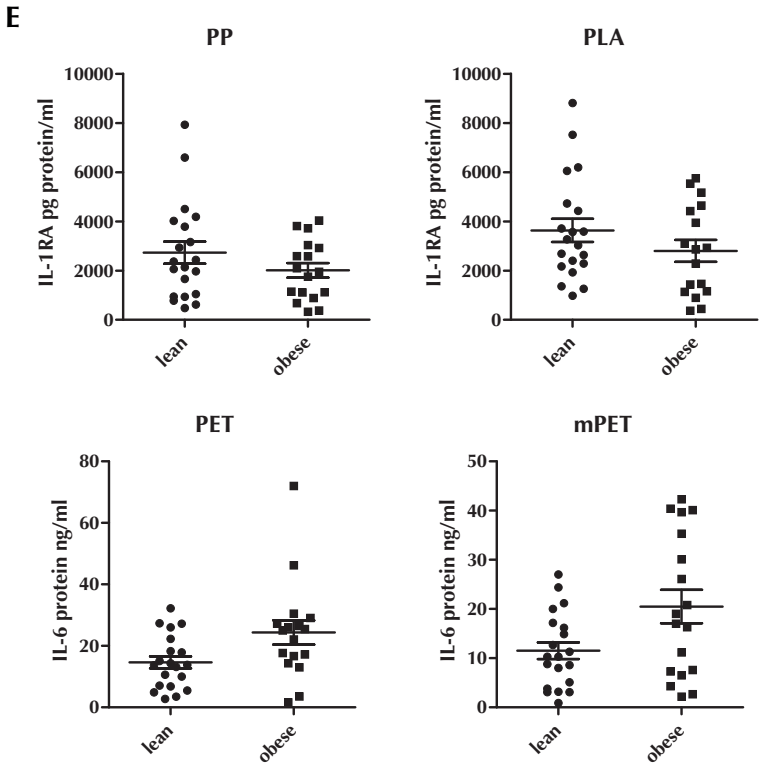
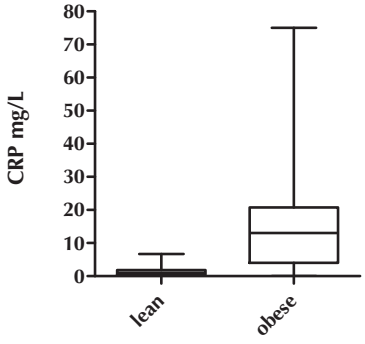


Figure S6.3B, C, D, E (continued) Comparing macrophages from lean and obese donors cultured on different materials regarding **B)** IL-6 production and **C)** TNFα production and **D)** CCL18 production and **E)** IL-1RA production in ng/ml after 3 days of culture. Every dot represents a single donor. Line and whiskers indicate mean ± S.E.M., p-values indicate a statistically significant difference. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.



Supplementary Figure S6.4: C-reactive protein (CRP) levels in mg/L in plasma of lean subjects vs. obese patients. The middle line in box represent the median and whiskers the minimum and maximum; lean (0-7 mg/L) and obese (0-75 mg/L).