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*J Immunol* published online 18 March 2015
http://www.jimmunol.org/content/early/2015/03/18/jimmunol.1402655

 Supplementary Material  
http://www.jimmunol.org/content/suppl/2015/03/18/jimmunol.1402655.5.DCSupplemental

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Profiling of the Three Circulating Monocyte Subpopulations in Human Obesity

Estelle F. Devêvre,*†§,1 Mariana Renovato-Martins,*†§,1 Karine Clément,†§,8,11 Catherine Sauté-Fridman,*†§ Isabelle Cremer,*†§,1 and Christine Poitou†§,3,*,†,‡,**

Three subpopulations of circulating monocytes have been described: CD14+CD16− (classical monocytes [CM]), CD14+CD16+ (intermediate monocytes [IM]), and CD14+CD16− (nonclassical monocytes [NCM]). We previously showed that obesity is associated with an increased proportion of IM and NCM. Our objective is to decipher the migratory and inflammatory functions of each monocyte subset in obesity-related low-grade inflammation. Twenty-six healthy, normal-weight and nondiabetic volunteers (C) and 40 obese nondiabetic (Ob) individuals were included in this study. We explored the gene expression profile of 18 inflammatory genes in each subset of C and Ob subjects and measured protein expression of the upregulated genes. We then tested their functional response to TLR signaling in both groups. We showed an increased expression of CX3CR1 in all monocyte subpopulations and of CCR2 and CCR5 in CM and IM in the Ob group. We found negative correlation between CCR2 and CX3CRII expressions and high-density lipoprotein-cholesterol, whereas CCR5 expression was positively linked to obesity-related metabolic traits. Production of inflammatory proteins upon bacterial LPS and viral ssRNA stimulation was higher in CM and NCM of the Ob group compared with the C group. Our work highlights an enhanced inflammatory phenotype of monocytes with a higher response to TLR4 and TLR8 stimulations in obesity. Moreover, it suggests an increased migration capacity of CM and IM subpopulations.

Received for publication October 21, 2014. Accepted for publication February 4, 2015.

This work was supported by a Centre de Recherche des Cordeliers, Paris Young Investigator Fellowship (to I.C.), INSERM, Université Pierre et Marie Curie-Paris 6, Université Paris-Descartes-Paris 5, Assistance Publique-Hôpitaux de Paris, and Contrat de Recherche Clinique Grant CRC P050318 (to C.P.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMI, body mass index; C, normal-weight and nondiabetic volunteer; CM, classical monocyte; FMO, fluorescence minus one; HDL-c, high-density lipoprotein-cholesterol; HS, human serum PBS; IM, intermediate monocyte; MFI, mean fluorescence intensity; NCM, nonclassical monocyte; Ob, obese nondiabetic.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402655
Monocytes in human obesity: subsets and functions

Materials and Methods

Subjects

Two groups of subjects were included in this study (Table I). The first group included 40 obese nondiabetic (Ob) individuals involved in a bariatric surgery program and recruited to the nutrition division of Pitié-Salpêtrière Hospital (Reference Center for the Medical and Surgery Care of Obesity, Institute of Cardiometabolism and Nutrition, ICAIN La Pitié-Salpêtrière Hospital, Paris, France). Patients met the criteria for bariatric surgery as described earlier (12). The second group comprised 26 healthy normal-weight and nondiabetic volunteers (C), living in the same region as the Ob subjects. The Ethics Committee (CPI Ile de France 1) approved the clinical investigations for both Ob and C individuals. All subjects gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was registered in a public trial registry (NCT00476558, NCT01655017).

For each of the experiments, we used fasting blood samples from only part of the two groups. For ethical reasons, blood withdrawal for monocyte isolation was restricted to a maximum of 10 ml per patient. This resulted in isolation of 1 × 10^3 to 4 × 10^4 monocytes per patient, which limited the number of IM and NCM after FACS sorting, and experimental design was adapted accordingly (Supplemental Fig. 1).

Mononuclear cell isolation and FACS sorting of monocyte subpopulations

PBMCs were isolated from blood by centrifugation on a Ficoll-Hypaque gradient (PAO Laboratories). Monocytes were then enriched by a centrifugation on a Hyper-osmotic Percoll™ Gradient (GE Healthcare) (18). Monocyte-enriched cell fraction was further subdivided by FACS. For the gene expression experiment, cells were stained with allophycocyanin-CD14 and FITC-CD16. Doublets and dead cells were excluded, monocytes were selected based on their light scatter and CD14 and CD16 expressions, and sorted into CD14^+CD16^−, CD14^-CD16^+, and CD14^+CD16^- cells directly in RNase Lysis Buffer (Qiagen) using a FACS Vantage cell sorter (BD Biosciences). Sorted cells were kept at −80°C before RNA isolation. For cytokine production, we added a PE-elimination channel with PE-CD3, PE-CD19, and PE-CD56 to exclude potential contaminant from the monocyte gate. A total of 10,000 monocytes per subset were sorted using a FACS Aria III (BD Biosciences) into RPMI 1640 medium. Sorted monocytes were cultivated immediately. Supplemental Table I describes Abs used for these studies.

RNA extraction, reverse transcription, and cDNA preamplification

Total RNA was isolated from 9000 to 90,000 sorted monocytes. RNasey Mini Kit (Qiagen) was used according to manufacturer’s instructions including DNase treatment. RNA quality and quantity were analyzed by capillary electrophoresis (BioAnalyzer; Agilent). Equal amounts of cDNA were preamplified using the TaqMan PreAmp Master Mix (Applied Biosystems).

Real-time PCR

Preamplified cDNA samples were amplified using Made-to-Order Low Density Array System according to manufacturers’ instructions. The arrays were carried out on the Applied Biosystems 7900HT. TaqMan Low Density Array Design was used for screening in gene expression of CX3CR1, CSF-1R, SELPLG, TNF, CCR2, CCR3, CD36, ICAM1, IL-10, IL-8, IL-1β, CCL2, CCL3, TLR2, TLR4, TLR7, TLR8, peroxisome proliferator-activated receptor γ genes, and actin as endogenous controls. A detailed list of probes is given in Supplemental Table 1. Each sample was run in duplicate, and the results were analyzed with the RQ Manager software (Applied Biosystems) and expressed as relative gene expression (2^(-DDCt) method) using β-actin expression as endogenous control.

Cytokine production

To analyze cytokine production, we cultivated 1 × 10^4 FACS-sorted CM, IM, or NCM in 100 μl RPMI 1640 and 10% FBS. Each subset was stimulated with 10 ng/ml LPS, 10 μg/ml viral ssRNAs (ssRNA40/Lyo Vect; Invivogen), or media alone. After 18 h, supernatants were harvested and kept at −80°C for cytokine quantification. Monocyte supernatants were quantified for TNF, IL-1β, IL-6, IL-10, IL-12p70, CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP10), and IL-8 (CXCL8) by the human 9-flex cytometric bead array. Details about each flex set are given in Supplemental (RANTES), CXCL10 (IP10), and IL-8 (CXCL8) by the human 9-flex cytometric bead array. Each flex set are given in Supplemental Table I. Samples were processed according to the manufacturer’s instructions; acquisition was performed on an LSRII and quantification calculated with FCAP array v3 software (all from Becton Dickinson).

Protein expression

Flow cytometry using 8-fluorophores was performed on monocyte-enriched PBMCs. To prevent nonspecific binding, we blocked FcRs using 2% human serum PBS (2% HS) for 10 min. Abs were diluted in the same buffer and incubated 30 min in the dark at 4°C. Monocyte subsets were selected as described earlier. Fluorescence minus one (FMO) was used to determine the cutoff between cells expressing FITC-CSF-1R, PE/CF594-CXCR5, PerCP/Cy5.5-CXCR2, or PE/Cy7-CXC CR1 and negative cells (Supplemental Fig. 2). Expression of TLR4 and TLR8 was determined separately on whole blood. Whole blood was FcR blocked in 2% HS for 15 min. Anti-TLR4 Ab or matching isotype control was added to cells before a mix of allophycocyanin-CD14, allop hycocyanin/CD14, CD14^+CD16^−, CD14^-CD16^+, and CD14^+CD16^- cells directly in RNase Lysis Buffer (Qiagen) using a FACS Vantage cell sorter (BD Biosciences). Sorted cells were kept at −80°C before RNA isolation. For cytokine production, we added a PE-elimination channel with PE-CD3, PE-CD19, and PE-CD56 to exclude potential contaminant from the monocyte gate. A total of 10,000 monocytes per subset were sorted using a FACS Aria III (BD Biosciences) into RPMI 1640 medium. Sorted monocytes were cultivated immediately. Supplemental Table I describes Abs used for these studies.

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Statistical analyses

Data are expressed as means ± SEM. Comparisons of mean for quantitative variables between groups were performed using the nonparametric Mann–Whitney U test. Relationship among the different quantitative variables was determined by calculating the Spearman correlation coefficient. For all analyses, a p value < 0.05 was considered statistically significant. Statistical analyses were done with GraphPad Prism 5.0 (GraphPad Software).

Results

Specific gene expression profile in monocyte subsets in obesity

In accordance with previous findings (12), monocyte absolute numbers were increased in each subset of the obese subject group, as well as the proportion of IM and NCM (Fig. 1). To unravel the molecular signature of monocytes in obesity, we compared expression of 18 candidate genes related to monocyte functions in 3 monocyte subpopulations isolated from C and Ob subjects before and after gastric surgery–induced weight loss (Fig. 2). We found that each monocyte subset displayed a specific gene expression signature. Regarding molecules involved in chemotaxis, mRNA expression of CX3CR1 was significantly increased (2- to 2.5-fold) in the Ob group compared with the C group in the CM and IM, and with a trend in the NCM. After weight loss, the expression of CX3CR1 decreased in all monocyte subsets. In obese CM and IM, CCR2 and CCR5 were significantly 2- to 2.5-fold overexpressed. Decreased mRNA expression was observed after weight loss for CCR5 in the CM and for CCR2 in the IM. The expression of CCL2 chemokine was increased by 3.6-fold in the IM of Ob subjects and decreased to levels comparable with C group after weight loss. We found no difference in CCL3 expression in Ob group. Peroxisome proliferator-activated receptor γ, which regulates fatty acid storage and glucose metabolism, was increased in the NCM of Ob individuals and in the CM after weight loss.

For genes involved with the pathogen recognition function, we found a 2- to 4-fold increased expression of TLR4 and TLR8 in all monocyte subsets of the Ob group. Conversely, the expression of TLR2 and TLR7 was similar in both groups. After weight loss, the expression profiles of these TLRs were not significantly modified.
For genes involved in cytokine production, we found increased expression of IL-1β in NCM and of TNF in IM in Ob individuals. No significant difference in NCM expression level of IL-1β was observed after weight loss, whereas IM expression level of TNF tended to return to normal value.

Taken together, these results show modified mRNA expression of genes related to monocyte functions in obesity. We observed specific characteristics for each monocyte subset for genes involved in cytokines production, chemotaxis, fatty acid storage and glucose metabolism, and pathogen recognition. Indeed, TLR4 and TLR8 overexpression could be considered as a molecular signature of monocytes in obese patients.

While observing only a slight increase, however not statistically significant, in TLR4 and TLR8 expressions at the protein level in monocytes of obese patients compared with lean subjects (Fig. 3A, 3B), we demonstrated that monocyte subsets from obese patients compared with lean subjects have increased ability to secrete inflammatory molecules upon stimulation with LPS or viral ssRNA respective ligands of TLR4 and TLR8.

We found that some cytokines involved in the inflammatory response were increased in the monocyte subsets (Fig. 3C). CMs were characterized by an increased production of IL-1β in response to LPS and an increased production of TNF and IL-10 upon ssRNA stimulation in the Ob group compared with C. The IM subset did not differ in terms of cytokine production in response to LPS or ssRNA. Finally, the NCM subset displayed higher production of IL1-β upon ssRNA stimulation.

Monocyte subsets are characterized by stronger responses upon TLR4 and TLR8 stimulation in obesity

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Chemokine production analysis (Fig. 3D) revealed an increased CM production of CCL5 when stimulated by LPS in the Ob group compared with C. The IM subset did not differ in terms of chemokine production in response to LPS or ssRNA. Finally, the NCM subset of Ob subjects displayed higher secretion of CCL5 upon ssRNA stimulation.

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all subsets of the Ob group (Fig. 4). Thus, the overexpression is significant only for CCR2, CCR5, and CX3CR1 in the CM and IM subsets and for CX3CR1 at the surface of NCM subset (Fig. 4 and Supplemental Fig. 2). Taken together, these findings suggest higher migration ability and increased survival of monocytes in Ob compared with C subjects. Indeed, the highest increased
expression of CCR5 and CCR2 in the CM and the IM imply that those two cell subpopulations could have higher migration capacity than the NCM (19).

To evaluate the clinical relevance of increased expression of chemokine receptors, we performed correlation analysis between protein expression of CCR2, CCR5, CX3CR1, and CSF-1R and phenotypes related to corpulence (body mass index [BMI]) and metabolic status (lipid and diabetes-related traits) listed in Table I. The complete linkage clustering of correlation coefficients of obese clinical outcomes and protein expression levels of CCR2, CCR5, CX3CR1, and CSF-1R showed that for each receptor the expression level of CM and IM segregated from the one of NCM (Fig. 5). We found that monocyte surface expression of CX3CR1 and CCR2 was negatively correlated with high-density lipoprotein–cholesterol (HDL-c), a protective marker of cardiovascular risk. The strength of the negative correlation was particularly strong for the expression of CCR5 and CCR2 in the CM and the IM imply that those two cell subpopulations could have higher migration capacity than the NCM (19).

![Image](https://example.com/image.png)

**FIGURE 4.** Protein expression (CCR2, CCR5, CX3CR1, and CSF-1R) in CM, IM, and NCM of obese nondiabetic (Ob) and normal-weight and nondiabetic volunteers (C). (A) Surface protein expression was measured by flow cytometry; fold increase was calculated by dividing MFI of each individual by the average MFI of C and one Ob subject (Ob). (B) Representative examples of CCR2, CCR5, CX3CR1, and CSF-1R surface staining of one C and one Ob subject (Ob). n = 10 C, n = 12 Ob. Statistical comparisons between each group were obtained by the nonparametric Mann–Whitney U test (*p < 0.05, **p < 0.01, ***p < 0.001).

**Discussion**

Recent studies in healthy individuals have characterized gene expression profiles of the three monocyte subsets (11, 16, 17). However, little is known about these different subpopulations in inflammatory-related diseases. In this study, we show that the three subsets display specific modified gene expression and protein expression profiles of inflammatory molecules, as well as differential functional response to the TLR signaling in obesity, a well-known low-grade (sterile) inflammatory pathology.

It is well established that monocytes migrate into sites of inflammation in response to several chemokines and obesity is closely associated with an enhanced number of adipose tissue macrophages (7). We showed that a common characteristic of the three subsets of monocytes in obesity is the overexpression of CX3CR1, suggesting their increased potential to migrate in response to CX3CL1, the chemokine ligand for CX3CR1. This result is consistent with the demonstration by Shah et al. (20) that CX3CL1 is secreted by adipose tissue and mediates monocyte adhesion to human adipocytes. They concluded that CX3CL1 is a novel adipose tissue chemokine in humans and may modulate the recruitment of monocytes into adipose tissue. Consistently, Polváky et al. (21) demonstrated that the CX3CL1-CX3CR1 chemokine system is implicated in the development of metabolic visceral adipose tissue inflammation in obese mice. This is further supported by several studies performed in murine models (22, 23) and clinical investigations in humans (24–26) that suggest a role

Table I. Bioclinical characteristics of obese and control subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ob</th>
<th>C</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>40</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SEM), y</td>
<td>41 ± 2</td>
<td>37 ± 2</td>
<td>0.211</td>
</tr>
<tr>
<td>BMI (mean ± SEM), kg/m²</td>
<td>43.2 ± 0.7</td>
<td>21.0 ± 0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>36/4</td>
<td>24/2</td>
<td></td>
</tr>
<tr>
<td>Glycemia (mean ± SEM), mmol/L</td>
<td>5.4 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Insulinemia (mean ± SEM), mU/ml</td>
<td>15.6 ± 1.4</td>
<td>7.8 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol (mean ± SEM), mmol/l</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.3</td>
<td>0.883</td>
</tr>
<tr>
<td>HDL-c (mean ± SEM), mmol/l</td>
<td>1.2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL-c (mean ± SEM), mmol/l</td>
<td>3.2 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>0.197</td>
</tr>
<tr>
<td>Total triglycerides (mean ± SEM), mmol/l</td>
<td>1.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP (mean ± SEM), mg/L</td>
<td>9.8 ± 1.2</td>
<td>&lt;5</td>
<td>0.001</td>
</tr>
<tr>
<td>Total WBCs (mean ± SEM), 10⁶/l</td>
<td>7.4 ± 0.3</td>
<td>6.4 ± 0.6</td>
<td>0.054</td>
</tr>
<tr>
<td>Lymphocytes (mean ± SEM), %</td>
<td>32.7 ± 1.2</td>
<td>30.1 ± 2.2</td>
<td>0.323</td>
</tr>
<tr>
<td>Neutrophiles (mean ± SEM), %</td>
<td>57.4 ± 1.3</td>
<td>60.0 ± 2.2</td>
<td>0.440</td>
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<tr>
<td>Monocytes (mean ± SEM), %</td>
<td>7.1 ± 0.3</td>
<td>5.8 ± 0.4</td>
<td>0.022</td>
</tr>
<tr>
<td>Total monocytes (mean ± SEM), 10⁶/l</td>
<td>0.5 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Statistical comparisons between each group were obtained by the nonparametric Mann–Whitney U test.

CRP, C-reactive protein; LDL, low-density lipoprotein–cholesterol.
of CX3CR1–CX3CL1 axis in the development of atherosclerosis and cardiovascular events. In this scenario, CX3CL1 functions as a chemoattractant or as an adhesion molecule, facilitating monocyte and T cell transmigration in atherosclerotic lesion areas (27–29) and is also able to regulate monocyte differentiation and survival (30).

We also observed increased expression of CCR2 and CCR5, which have been involved in the recruitment of monocytes in atherosclerotic plaques in murine models (31, 32), in CM and IM of obese subjects. We previously observed that conditioned medium from visceral adipose tissue of obese subjects or the addition of CCL5 induced adhesion and transmigration of human blood monocytes through adipose tissue endothelium (33). A recent study on obese women demonstrated higher chemotactic activity of monocytes from obese subjects in response to CCL2 and CCL5 (19). These findings together with our data suggest that higher levels of macrophages found among adipose tissue during obesity (19) could be a result of increased migration of monocytes expressing high levels of CX3CR1, CCR2, or CCR5 in response to chemokines secreted by activated endothelium and adipose tissue. Furthermore, our results suggest higher chemotactic ability specifically of the CM and IM subsets. It suggests that the NCM would be less involved than the other two subsets in recruiting monocytes to atherosclerotic plaques and decreased migration toward the adipose tissue.

Interestingly, we found a strong negative correlation between HDL-c serum level and CX3CR1 and CCR2 expression. On the same line, others reported that in vitro incubation of monocytes with reconstituted high density lipoprotein reduces CCR2 and CX3CR1 expression (34). These data suggest that decreased HDL, a common abnormality observed in obesity, could be directly linked to changes in monocyte phenotype and function.

We observed that CSF-1R was overexpressed in the NCM of obese subjects, however not at a significant level. CSF-1R (also known as CD115, c-fms, and M-CSF receptor) is a tyrosine kinase protein high-affinity receptor for CSF-1 and IL-34. Its ligands, IL-34 and CSF-1, have a pivotal role in regulating survival, proliferation, and differentiation of mononuclear phagocytes (9, 35, 36). CSF-1R is notably expressed by adipose tissue in obese subjects (37, 38). The highest expression of CSF-1R in the NCM could reflect a longer life span compared with the other subsets. Interestingly, we found a positive association between overexpression of CSF-1R in NCM and homeostasis model assessment–estimated insulin resistance, a surrogate of insulin resistance, suggesting that CSF-1R could be considered as characteristic of metabolic alteration, especially insulin resistance, in obese subjects.

TLRs are members of a family of pattern-recognition receptors that detect components of microorganisms, playing a critical role in the innate immune system by activating inflammatory signaling pathways (39). Studies highlighted the involvement of TLR2 and TLR4 in metabolic functions, as well as in innate immune responses, in obesity (40, 41). An interesting finding from this study is a higher activation of TLR4 signaling in response to LPS of CM subset. TLR4 could be stimulated by circulating bacterial LPS or fatty acids, whose levels are commonly increased in obesity (40). We demonstrate for the first time, to our knowledge, a higher activation of TLR8 signaling in response to ssRNA in NCM and CM from obese patients compared with lean subjects. TLR8 is an endosomal TLR that binds viral ssRNAs (17). Although there are no evidences showing increased levels of circulating nucleic acids in obesity, we can suggest that TLR8 present in monocytes from obese subjects could detect circulating nucleic acids or some not yet known ligands other than viral ssRNAs. Moreover, in accordance with increased TLR8 overexpression and stimulation, we found that monocytes display high levels of proinflammatory cytokines in obese patients, particularly IL-1β for NCM.

**FIGURE 5.** Correlation among CCR2, CCR5, CX3CR1, and CSF-1R protein expressions of each monocyte subset with obesity-related clinical parameters. Data are from 10 normal-weight and 12 obese subjects. Correlations were estimated by the nonparametric Spearman test. Distance between protein expression of each subset was estimated by complete linkage clustering. Heat-map colors are representations of each correlation coefficient, and numbers indicate p values (*p < 0.05, **p < 0.01, ***p < 0.001). CRP, C-reactive protein; HOMA-IR, homeostasis model assessment–estimated insulin resistance; LDL-c, low-density lipoprotein-cholesterol.

**FIGURE 6.** Presumed functions of monocyte subsets in obesity-related low-grade inflammation. We propose a model where proinflammatory monocytes of obese subjects would sense circulating factors through TLR4 and TLR8. In response, the CM and NCM subsets secrete more inflammatory cytokines. Expressing more CCR5 and CCR2, the IM and CM could be able to migrate more toward tissues where they could differentiate into macrophages. Both phenomena contribute to sustain low-grade inflammation associated with obesity, whereas NCM could stay longer in the circulation and assume “patrolling” function.
In conclusion, our findings demonstrate that the three monocyte subsets from obese patients display a more inflammatory phenotype with a higher response to TLR4 and TLR8 stimulation, and a chemokine receptor expression profile that suggests a higher capacity to migrate for CM and IM and increased survival for NCM (Fig. 6). Indeed, therapeutic strategies targeting inflammatory processes or TLR signaling could be a promising approach to reduce inflammatory, metabolic, and cardiovascular complications linked to obesity.

Acknowledgments
We thank Rohia Allali, Valentine Lemoine, and Dr. Florence Marchelli (Center for Research on Human Nutrition, Pitié-Salpêtrière Hospital) who were involved in patient recruitment and sampling. We thank Dr. Sarah Church for English proofreading and helpful comments on the manuscript. We thank Assistance Publique-Hôpitaux de Paris and the Director of Clinical Research for the Department of Clinical Research and Development for promoting and supporting this clinical investigation.

Disclosures
The authors have no financial conflicts of interest.

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