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Hypoxia for MSC expansion and differentiation: the best way for enhancing TGF β -induced chondrogenesis and preventing calcifications in alginate beads

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Running title: Continuous MSC hypoxia enhances chondrogenesis and prevents calcifications

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Abstract (223 words)

We examined the respective influence of a sequential or a continuous hypoxia during expansion and TGF- β 1-driven chondrogenic differentiation of human bone marrow mesenchymal stem cells (MSCs). The differentiation was performed within alginate beads, a classical tool for the implantation of MSCs within the joint. The standard normoxic 2D (expansion) and 3D (differentiation) MSCs cultures served as reference. In order to determine the quality of the chondrogenesis, we analyzed typical markers such as type II and X collagens, *SOX9*, *COMP*, versican, and aggrecan mRNAs using PCR and we assessed the production of type II collagen and HIF-1 α by histological stainings. We simultaneously assessed the expression of osteogenic mRNAs (Alkaline Phosphatase, *RUNX2* and Osteocalcin) and the presence of microcalcifications by alizarin red and Raman spectroscopy. Chondrogenic differentiation is clearly improved by hypoxia in 3D. Best results were obtained when the entire process, *i.e.* 2D expansion and 3D differentiation, was performed under continuous 5% hypoxic condition. In addition, no calcification (hydroxyapatite, proved by RAMAN) was observed after 2D hypoxic expansion even in the case of a normoxic differentiation, in contrast with controls. Finally, a better chondrogenic differentiation of human MSCs is achieved when a reduced oxygen tension is applied during both expansion and differentiation times, avoiding the *in vitro* osteogenic commitment of the cells and subsequently the calcification deposition.

Key words: mesenchymal stem cells, hypoxia, alginate, chondrogenesis.

Abbreviations

ACAN: aggrecan gene

ACI: autologous chondrocyte implantation

ALP: alkaline phosphatase gene

bFGF: basic Fibroblast Growth Factor

BGLAP: Osteocalcin gene

BMP: bone morphogenetic protein

DAB: diaminobenzidine

COL10A1: type X collagen gene

COL2A1: type II collagen gene

COMP: cartilage oligomeric matrix protein gene

DMEM-HG: Dulbecco's modified Eagle medium with high glucose

DMEM-LG: Dulbecco's modified Eagle medium with low glucose

TGF β -1: Transforming growth factor beta 1

GAG : glycosaminoglycan

HA: hydroxyapatite

HES: Hematoxylin, Eosin, Saffron

HIF: hypoxia-inducible factor

H/H: Hypoxic (5%)/ Hypoxic (5%)

H/N: Hypoxic (5%)/Normoxic (20%)

MSCs: mesenchymal stem cells

N/H: Normoxic (20%)/Hypoxic (5%)

N/N: Normoxic (20%)/ Normoxic (20%)

OA: osteoarthritis

RP29: Ribosomal Protein S29 (housekeeping gene)

SOX9: SRY (sex-determining region Y)-box 9 gene

TGF- β : Transforming growth factor β gene

VCAN: Versican gene

1. Introduction

Articular cartilage defects such as osteochondritis or focal post-traumatic chondral lesions are a common problem in orthopedic surgery. Due to its limited capacity for regeneration and self-repair as well as the lack of available therapeutic options, chondral defects may have severe consequences (focal/diffuse OA lesions) (1). Some focal injuries can be treated by an autologous chondrocytes implantation (ACI) (2). However, the ACI might result in the synthesis of fibrocartilage/hypertrophic repair tissue and of a subsequent long-term risk of developing OA (3, 4). This current state requires improved therapeutic options for treating cartilage defects such as cell-based and tissue-based methods that can overcome the size limitations of current ACI technologies and be applied to larger defects such as diffuse OA (3).

The mesenchymal stem cells (MSCs) are proved to be a good candidate cell source for treating cartilage defects (5, 6). Multipotent mesenchymal stem cells from adult bone marrow have the potential to renew themselves (auto-replication during expansion) and to differentiate (usually with 3D medium-dedicated conditions) into distinct mesodermal tissues including osteochondral structures(7). Differentiation of MSCs into chondrocytes has been achieved i) by adding growth factors (8), with the aid of co-cultivation with chondrocytes (9), or by transferring, virally or non-virally, dedicated chondrogenic genes (10). TGF- β englobes a multi-functional set of growth factors that have been widely used to trigger MSC chondrogenesis in 3D environments, such as pellets, hydrogels, beads, biopolymers and other composite biomaterials (11). More recently BMPs were highlighted as pivotal signals to promote osteochondral commitment in articular engineering (12, 13). However, when pushed towards chondrogenesis, MSCs tend to evolve into the hypertrophic/ osteogenic commitment (14, 15), and many approaches, including low oxygen tension (as hyaline cartilage is non-vascularized) have been investigated to modulate chondrocytic hypertrophy (16, 17).

Articular cartilage is mostly an avascular tissue, surrounded by synovial fluid delivering oxygen and the nutrients diffusing through the extracellular matrix. The lack of blood supply thus generates a hypoxic environment (chondroxia, or physioxia (15)), with oxygen saturation ranging from 1 to 8%, depending on the location and depth within the joint (18, 19). The pioneer work of Nevo *et al.* (20) demonstrated the beneficial influence of hypoxia on embryonic chicken

chondrocyte *in vitro*. Since then, many efforts have been applied in the MSC-based cartilage engineering to mimic this environment during the expansion and/or the 3D differentiation. Generally, hypoxia shows an enhancement of the chondrogenesis (21-30) and strongly inhibits osteogenesis (31, 32).

Recent papers have underlined the importance of priming MSCs by continuous or cyclic hypoxic pre-treatments in regenerative medicine (33, 34): hypoxia promotes the stemness of various types of MSCs, improves tissue regeneration through an enhancement of the MSCs viability, differentiation and paracrine activity. This study was thus designed to clarify the influence of a low oxygen concentration chronology during the hypoxic preconditioning (expansion in 2D) and/or during their chondrogenic differentiation (3D culture in alginate beads). As few studies have studied the impact of an *in vitro* sequential hypoxic preconditioning of MSC-seeded implants (24, 29, 35-41), we have compared *in vitro* the chondrogenic, hypertrophic and osteogenic profiles, this latter being characterized by the occurrence of calcifications within the cellularized implants, under normoxic, mixed and/or continuous hypoxic conditions. We used concomitantly histology and Raman scattering spectroscopy. Raman permits a finer non-destructive chemical characterization of calcium deposits rather than standard Alizarin Red staining (42).

2. Materials and Methods

2.1. Isolation. Bone marrow human stem cells were isolated from 6 informed patients (mean age $64,8 \pm 6,3$ y.o., 3 women and 3 men) undergoing total hip arthroplasty, all for hip OA. This study was approved by our local Research Institution. All procedures were done in accordance with our authorization and registration number DC-2008-263 given by the National “Cellule de Bioéthique”.

2.2. 2D expansion. The samples were placed in 50 ml tubes containing 1 ml of phosphate buffered saline (PBS) supplemented with 625 UI/ml heparin. The nucleated cells were counted and plated at a density of 50,000 cells/cm² in a T75 culture flask at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide. The proliferation medium was a Dulbecco’s modified Eagle medium with low glucose (DMEM-LG, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% glutamine, 1% penicillin-streptomycin (Gibco) and 1 ng/ml

basic Fibroblast Growth Factor (bFGF, R&D systems). The medium was unchanged for the initial 3 days, and then was changed three times per week until the cells became confluent. Then the MSCs were passaged from P0 to P3 using trypsin-EDTA (0.05%, Gibco). The chondrogenic pre-differentiation medium in P3 (just before 3D differentiation in alginate beads) was DMEM with high glucose (DMEM-HG, Gibco) containing 10% FBS, 1% glutamine, 1% penicillin-streptomycin, sodium pyruvate (110 µg/ml), 40 µg/ml proline, 10^{-7} M dexamethasone, 50 µg/ml L-Ascorbic acid-2-Phosphate and 1 ng/ml bFGF. To evaluate the effect of hypoxia during the expansion phase (preconditioning phase) one monolayer batch of MSCs was incubated in normoxia (20% oxygen, v/v) and another in hypoxia (5% oxygen, v/v) as designed in Suppl 1.

2.3. 3D differentiation in alginate beads. Before being mixed with the cells, the alginate (Sigma, France) - hyaluronic acid (Accros, France) powder was autoclaved at 120°C for 20 minutes for sterilization. In order to create alginate gel cultures, the viable cells were suspended at a concentration of 3×10^6 cells/ml in 2% wt/vol viscosity alginate medium (16 mg alginate and 4 mg hyaluronic acid in 1 ml 0.9% NaCl solution). The cell/alginate preparation was polymerized into a solution containing 102 mM CaCl_2 solution for 10 min. Beads were washed three times with 0.9% NaCl solutions and once with basal culture medium. Beads from each monolayer (hypoxia or normoxia expansion) were divided into two parts and cultured in hypoxia and normoxia conditions resulting in four groups: normoxia/normoxia (N/N) normoxia/hypoxia (N/H), hypoxia/normoxia (H/N), and hypoxia/hypoxia (H/H) (Supplemental data 1). The chondrogenic differentiation medium was DMEM-HG supplemented with 1% glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate, 40 µg/ml proline, 10^{-7} M dexamethasone, 50 µg/ml ascorbic acid 2-phosphate and 1 mM CaCl_2 . In all conditions, the beads were treated from day 3 with ITS 1% (control) and ITS 1% + 10 ng/ml TGF- β 1 (chondrogenic condition). The beads were harvested on D28 for analysis. For sake of clarity and lack of accuracy data obtained at D3, D14 are not presented.

2.4. Histological analysis of the alginate beads. The alginate beads were fixed in a 4% paraformaldehyde solution containing 10 mM CaCl_2 and 0.1M sodium cacodylate (pH 7.4) for 4 hours at 4°C. Then they were washed overnight at 4°C in 0.1M sodium cacodylate (pH 7.4) (to irreversibly cross link the alginate matrix) containing 50 mM BaCl_2 (to irreversibly crosslink the alginate matrix) before dehydrating in ethanol, embedding in paraffin. Sections of 5 µm thickness

were stained with Hematoxylin, Eosin, Saffron (HES) to visualize the cell morphology inside the beads, while Sirius red, Alcian Blue (pH 1.3) and Alizarin Red (pH 4.2) respectively evaluated collagen content, sulfated glycosaminoglycans (GAGs) and calcific depositions.

2.5. Immunohistochemistry was performed according to LSAB®+ kit (HRP, Dako) based on avidin-biotin techniques. A type II collagen (6B3, Labvision), and HIF-1 α primary monoclonal antibodies were used at the dilution of 1/100 as we previously published (43).

2.6. RNA extraction and RT-qPCR. The alginate beads were dissolved in 55 mM sodium citrate/50 mM EDTA at pH 6.8 for 5 min. The cells were isolated by centrifugation. The RNA extraction was realized using a RNeasy Mini Kit (Qiagen). After extraction, a reverse transcription was performed on 500 ng of RNA by using Omniscript RT Kit (Qiagen). The Real Time PCR was performed using QuantiTect™ SYBR® GreenPCR (Qiagen). The levels of mRNA were compared with the levels obtained from standard curves then normalized by RP29 mRNA (housekeeping gene) (44, 45). The mRNA expressions of each gene studied were compared to ITS and expressed as fold at D28 under various oxygen concentrations. All comparisons were performed versus our control condition, normoxia/normoxia (N/N).

2.7. Primer design. The primers sequences for all genes were designed using human mRNA information published on the NCBI website. The genes examined in this study were *RP29* (housekeeping gene), type II collagen (*COL2A1*), type X collagen (*COL10A1*), aggrecan (*ACAN*), versican (*VCAN*), SRY (sex-determining region Y)-box 9 (*SOX9*), cartilage oligomeric matrix protein (*COMP*), alkaline phosphatase (*ALP*) and osteocalcin (*BGLAP*). The sequences and product sizes are listed in the Table 1 and the PCR primers were purchased from MWG-Biotech.

2.8. Raman Study. To assess/characterize the presence of calcifications (hydroxyapatite and other possible mineral phases), Raman mapping experiments were performed at D28, as we previously published (46). The laser irradiance was kept below 5 kW.cm⁻² to avoid heating of the sample. Data presented are homogeneous for each sample in the batches studied.

2.9. Statistical analysis. The results were performed for each group in triplicate for each patient. The data are expressed as a normalized gene expression (TGF- β 1 versus ITS) at D28 with various oxygen concentrations and presented as mean \pm standard deviation (SD). The significance was

determined by a non-parametric Kruskal-Wallis comparison with a Dunn's post-hoc test compared with our reference: continuous normoxia (N/N). Statistics analysis were performed with JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989-2007. A p-value less than 0.05 was considered as significant.

3. Results

3.1. Gene expression under TGF- β 1-driven differentiation of MSCs.

3.1.1. Effect of normoxia. As predicted, TGF- β 1 induced a classical 3D MSCs-driven chondrogenic differentiation (Figure 1): we noticed a high overexpression of *COL2A1* (12069 fold versus ITS condition), *COL10A1* (3802 fold), *ACAN* (174 fold), *SOX9* (7 fold) and *COMP* (64 fold). On the contrary, in our experimental conditions TGF- β 1 supplementation induces a minor overexpression of osteogenic markers like *BGLAP* (osteocalcin, 2.6 fold), *RUNX2* (1.5 fold) and even a slight decrease of *ALP* (alkaline phosphatase, 0.8 fold). *VCAN* (versican), a fibrogenic marker was only modestly overexpressed (1.6 fold).

3.1.2. Effect of sequential hypoxia. A classical terminal hypoxia (N/H) during 3D differentiation boosted *ACAN* and *COL2A1* expressions while it repressed *COL10A1* suggesting a more pronounced chondrogenic profile. An initial hypoxia during 2D expansion, followed by a reoxygenation during normoxic 3D differentiation (H/N) had no influence on *COL2A1*, *ACAN*, *COL10A1* and *VCAN* expressions, while it boosted *SOX9* and decreased *ALP* and *BGLAP*.

3.1.3. Effect of a continuous hypoxia. This particular condition promotes the best chondrogenic gene patterns by robustly and significantly improving *COL2A1*, *ACAN*, *SOX9* and *COMP* expressions, and by simultaneously repressing fibrotic (*VCAN*) and osteogenic (*ALP*, *RUNX2* and *BGLAP*). In contrast, no influence was noticed on the hypertrophic marker *COL10A1*.

3.2. Histology and type II collagen immunostaining

HES staining clearly shows that all cells present in the biomaterial are alive (purple color). These results are also confirmed by a mitochondrial activity test (test MTT) performed throughout the study showed no toxicity (Results not shown). Moreover, under normoxic conditions, the TGF- β 1 beads displayed less cellularity than others TGF- β 1 groups (Figure 2). During sequential

hypoxia, either terminal (N/H) or preceding re-oxygenation (H/N), proliferating chondrogenic islets were present.

A continuous hypoxia (H/H) markedly promoted chondrocytic-like proliferation. The Alcian Blue stain was done to appreciate proteoglycan content of the TGF- β 1-driven constructs. In continuous normoxia (N/N), the GAG (Glycosaminoglycan) content was very faint, because Alcian Blue stained only alginate. An initial hypoxia promoted a weak peri-cellular staining. On the contrary, a distinct blue staining was observed in final hypoxic condition (N/H), even more marked under continuous hypoxia (H/H), with a more pronounced extracellular matrix synthesis.

The results are similar to the Sirius Red staining, giving information about fibrillar collagen content in alginate beads. A 3D differentiation under hypoxia promoted a good collagenous synthesis (N/H), enhanced by a hypoxic preconditioning during MSC expansion (H/H). This increase was particularly observed for the type II collagen immunostaining in the H/H group.

3.3. HIF-1 α immunostaining.

Two typical patterns were observed, only depending on oxygen level during 3D differentiation, without influence of the 2D hypoxic preconditioning. In the 3D normoxic groups (N/N and H/N), the MSCs exhibited a significant cytoplasmic accretion of the HIF-1 α protein. In the hypoxic 3D culture conditions, we have observed a decrease of this cytoplasmic accumulation, due to the transport of HIF-1 α into to the nucleus indicating there was a difference in HIF-1 α expression only depending on the 3D hypoxia, without any influence of the oxygen tension (N/N and H/N) during the expansion time (2D).

3.4. Occurrences of calcific depositions.

It is worth noting that, after a normoxic expansion, the TGF- β 1-driven differentiation led to numerous calcifications on the Alizarin Red staining, suggesting a switch towards an osteogenic commitment in both 3D normoxic (N/N) and sequential hypoxic (N/H) conditions. In fact, Figures 2 and 3Ba clearly demonstrates the presence of Alizarin Red spots surrounding the cells, indicating the presence of calcifications in the matrix. Raman spectroscopy exploration of these calcifications confirmed a typical spectrum of the hydroxyapatite (HA). The Figure 3A indicates the typical Raman spectra obtained for the HA compound and also for the paraffin embedding.

The peak at 962 cm^{-1} is a spectroscopic sign of the presence of HA in a sample and is clearly noticeable in spectrum b. The paraffin embedding may introduce some minor peaks in this spectrum (indicated by stars in Figure 3A) as some residues of paraffin slightly overlaid the HA structures.

However, the two spectra are sufficiently different and the 962 cm^{-1} peak is in a spectral region completely silent for the paraffin medium. No confusion was possible, and this distinctive feature can be integrated to generate Raman maps. The Figure 3B compares the visible observation (Alizarin red stained samples, or pictures obtained by visible light in reflection mode) and the Raman mapping of the HA compound. The Raman identification was clearly useful as with the visible light, it is very difficult to identify calcified structures due to the paraffin medium.

The vibration analysis is unambiguous and revealed that HA structures were formed with the N/N and N/H modalities for the TGF- β 1 samples. Besides, a hypoxic monolayer expansion prevented any HA calcifications in both 3D normoxic (H/N) and 3D hypoxic conditions (H/H). More than 8 areas were scanned randomly in the samples to statistically confirm the presence or the lack of HA compounds within the samples. The Raman mapping also confirmed that in our case, the Alizarin red stained deposits for N/N and N/H modalities matched to HA compounds, as seen on the Figure 3Bc.

4. Discussion

In our study, the chondrogenesis of bone marrow MSCs (bMSCs) has been induced *in vitro* by incubation of cells in a defined medium containing TGF- β 1, ascorbic acid and dexamethasone in combination with an alginate beads 3D culture. We confirmed that the chondrogenic induction of human bMSCs in alginate beads was enhanced with a reduced oxygen tension (5%) compared to a normoxic environment (38). No influence of sequential/continuous hypoxia was observed on MTT assay and DNA quantification using Hoechst (data not shown). Interestingly, a continuous hypoxia strongly upregulated mRNA overexpressions of the archetypal chondrogenic genes (*SOX9*, *ACAN*, *COMP* and *COL2A1*) and, on the other hand repressed the hypertrophic/osteogenic genes (*RUNX2* and *ALP*) as previously studied (30, 47, 48). *COL10A1* was only decreased during the terminal hypoxia as formerly reported in pellets (38, 49, 50) and it was not boosted by hypoxic priming, contrasting with some previous results (29, 38),

demonstrating a differential effect of hypoxia depending on the scaffold used. Nevertheless, it is generally accepted that hypoxia can stimulate TGF- β -driven MSCs chondrogenesis. However, the optimal oxygen tension level remains discussed (2% or 5%) (51).

Few studies assessed the specific beneficial influence of hypoxia during the expansion time. Our results with bone marrow MSCs are consistent with those of adipose-derived human MSCs under hypoxic conditions promoting the chondrogenicity (35, 52, 53), down regulating the hypertrophic phenotype (54) and increasing the GAG content (24, 52). The inhibition of *RUNX2* was also observed for adipose-derived adult stromal cells during hypoxic expansion conditions (26). Moreover, the hypoxic differentiation of MSCs in pellets, scaffolds and hydrogels was revealed to suppress some markers of the hypertrophy such as *COL10A1* and *ALP* as observed in alginate beads (25, 29, 55). After 7 days in hypoxic conditions, the human bone marrow MSCs are able to undergo a chondrogenic differentiation in alginate beads without any growth factor addition and to display a similar specific gene profile (56). However, the benefits of a hypoxic preconditioning seemed both time- and oxygen-dependent varying on the respective times of normoxic/hypoxic expansion and differentiation (53, 57).

Two oxygen-sensitive transcription factors, the hypoxia-inducible factor 1 α and 2 α (HIF-1 α and HIF-2 α), play a crucial role in the chondrogenicity of hypoxia. HIF-1 α was shown to improve MSC chondrogenesis via interactions with the SOX9 promoter (58). In addition, these hypoxic related chondrogenic and anti-catabolic effects disappeared with the addition of an HIF-1 α inhibitor (52, 59). In our continuous and terminal hypoxic conditions, HIF-1 α was translocated into the nucleus, as observed by the lack of cytoplasmic immunostaining, and then targeted the hypoxia-inducible elements helping the cells to switch towards the glycolytic metabolism. This indicated that HIF-1 α is not implicated in preventing calcifications, but only in promoting chondrogenesis.

The most original result in our study remained the lack of calcification/ossification inherent to the hypoxic preconditioning during the MSCs expansion, as previously reported in hydrogels and pellets (55). Interestingly, the calcifications were absent even if the 3D chondrogenic differentiation was performed under normoxia following a hypoxic expansion, this is contrasting with the results of Sheehy et al. (55). A beneficial influence of early hypoxia on calcium deposition during ATDC5 2D culture has already been reported (50) by using Alizarin red

staining. To the best of our knowledge, the qualitative RAMAN characterization, authenticating the hydroxyapatite crystal formation, has never been formerly used during *in-vitro* MSC chondrogenic differentiation studies.

The real interest for the hypoxia-mediated expansion and chondrogenic differentiation might be to improve the clinical results of *in vivo* cartilage repair and/or regeneration. Under normoxic expansion/differentiation, the MSCs, independently of their origin (bone, adipose or synovial tissue) do not undergo hypertrophic path when they are implanted intra-articularly in a cartilage environment in preclinical or in clinical conditions (3). Surprisingly, although hypoxia stimulated adipose MSCs chondrogenic commitment, it failed to enhance chondrogenesis after *in vivo* implantation in the rabbit knee (35). Besides, after a normoxic expansion/differentiation, a subcutaneous implantation of chondrogenic pellets issued from both bone marrow and adipose MSCs in SCID mice led to enhanced matrix calcifications along with a vascular invasion and even micro-ossicle ossification (47, 60). Synovium-derived pellets, exhibiting a lower ALP activity compared to bone marrow and adipose MSCs, showed only small-calcified areas after a subcutaneous transplantation (61).

Conversely, differentially hypoxic preconditioned adipose MSCs incorporated in hydrogels were injected subcutaneously in nude mice. After 5 weeks, the GAG and the type II collagen were present in the cartilage as aggregates and no calcification was mentioned (35). Similarly, after a transient hypoxic culture in a dedicated environment (2 weeks 5% O₂ followed by 1 week at 1% O₂), human embryonic stem cells were cultured in pellets under normoxia. These constructs, expressing chondrogenic markers, were implanted subcutaneously in nude mice for 28 days: the pellets remained phenotypically stable with hyaline ECM, without dedifferentiation or tumorigenesis (37). These results are consistent with those of Leitjen et al. (41), demonstrating that hypoxia-preconditioned alginate implants (human bMSCs) remain cartilaginous after implantation, whereas normoxia preconditioned implants underwent calcification, vascular invasion and subsequent enchondral ossification.

In conclusion, we have shown that combining a 2D expansion and a 3D differentiation (alginate beads) under 5% hypoxic condition increases TGF-β1-driven human bone marrow MSC chondrogenesis by up regulating the chondrogenic genes, (e.g. aggrecan and type II collagen) and by down regulating the osteogenic genes. A hypoxic priming during expansion seems crucial to

prevent the development of *in vitro* calcification/ossification. A continuous hypoxic conditioning should be promoted in the clinic to standardize the GLP dedicated to cartilage engineering, like proposed for Wharton's Jelly MSCs (62).

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Authors' contributions:

- Christel HENRIONNET: Collection and assembly of data, data analysis and interpretation, manuscript writing
- Gai LIANG: Collection and assembly of data, data analysis and interpretation, manuscript writing
- Emilie ROEDER: Data analysis, manuscript writing
- Manuel DOSSOT: Data analysis and interpretation, manuscript writing
- Hui WANG: Final approval of manuscript
- Jacques MAGDALOU: Final approval of manuscript
- Pierre GILLET: Data analysis and interpretation, final approval of manuscript
- Astrid PINZANO: Conception and design, financial support, data analysis and interpretation, manuscript writing

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