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INTRODUCTION

Although osteoarthritis (OA) is the most prevalent joint disease worldwide, there is still not a single disease-modifying OA drug on the market. The current treatment options usually result in poorly predictable outcomes due to the high interpatient variability in OA clinical and structural features. Indeed, some studies have reported OA phenotype heterogeneity among patients [1-3]. Recently it has been proposed to use advanced techniques to identify combinatorial biomarkers for distinguishing the different OA phenotypes [4], and also to identify patients at higher risk of disease progression, or with different underlying pathophysiologic mechanisms and risk factors [5]. This will help to improve clinical research and to develop targeted treatments and prevention strategies based on a phenotype-guided approach. The advantage of searching for targets based on differences between risk factors is the simplicity then of selecting patients for future personalized medicine.

Currently, OA research relies on the use of various animal models (mainly mice and rats, and more rarely large animals) that mimic mechanical, metabolic or inflammatory OA. However, none of these models covers the complexity and heterogeneity of the human

disease but different models likely reflect the heterogeneity of human OA. Moreover, it is difficult to compare the results of different experimental studies due to the heterogeneity of animal backgrounds and experimental protocols. Several studies have analysed global gene expression in OA samples using RNA-seq [6-8] and have generated huge amounts of datasets. However, only small subsets of data are validated and large amounts of data are commonly not investigated. To try to tackle some of these limitations, seven French academic laboratories experts in OA animal models formed a Research on OsteoArthritis Disease (ROAD) consortium to centralize many experimental steps and to put in place standard operating procedures (SOP) in order to minimize bias and increase reproducibility. The first objective of the ROAD consortium was to investigate the TGFβ pathway in various OA phenotypes. Indeed, recent findings have shown that TGFβ is a central player in cartilage homeostasis and OA pathology [9]. However, few data are available on the pathophysiological role of TGFβ family members in the different OA phenotypes. Therefore, the consortium analysed the TGFβ pathway by transcriptomic analysis in six murine models of knee OA that reproduce the main phenotypes of the human disease: surgical meniscectomy (MNX) to mimic mechanical or post-traumatic OA, hypergravity and MNX (HG-MNX) to mimic overweight-induced mechanical OA, high fat diet and MNX (HF-MNX) to mimic obesity-induced OA, seipin knock-out and MNX (SP-MNX) to mimic metabolic syndrome-induced OA, aging to mimic age-related OA, and collagenase-induced OA (CIOA) to mimic inflammatory OA.

MATERIAL AND METHODS

Animal models

Animal models and controls (ten mice/group) were generated using C57BL/6JR6 males that are known to display more severe and reproducible disease [10] and were supplied by the same company (Janvier Labs, France). *Bscl2*-/- mice (SP-MNX and SP-sham controls; C57BL/6J background) were from CEA (Direction des Sciences du Vivant/Genoscope /LABGEM). Six animals per group were calculated to be required to demonstrate significance at the 5% level with a power of 80% using the G*power software but 10 animals were included to have 6 animals with an OA score ≥ 3 at the end of the experiment. MNX was performed in one joint of 10 weeks old mice by the use of partial meniscectomy as described [11, 12] and done by a single trained operator in all laboratories. All animal procedures were approved by the local institutions' animal welfare committees and were performed in accordance with the European guidelines for the care and use of laboratory animals (2010/63/UE). Surgery and euthanasia were performed after anaesthesia with isoflurane gas, and all efforts were made to minimize suffering. Mice were housed in solid bottomed plastic cages in quiet rooms at 22° ± 1°C, 60% controlled humidity, and 12h/12h light/dark cycle. Animals were used after an adaptation period of 7 days and had free access to tap water and standard pelleted chow (except the HF model). Mice were sacrificed at week 6 after OA induction to have a comparable disease time induction although we were aware that OA severity can vary according to the model.

- **Joint instability model**

MNX was selected as the reference model of joint instability-related OA [11]. Knee joint instability was induced surgically in the right knee by medial partial meniscectomy. Surgery was performed under a binocular magnifier (X15) using a Sharpoint microsurgical stab knife. Mice were placed in dorsal position, knee flexed and right foot taped. After skin incision, the medial femoro-tibial ligament was cut, a short incision of the medial side of quadriceps

muscle was performed, the knee capsule was cleaved and the patella was sub-luxated laterally. After section of the meniscotibial ligament, the medial meniscus was gently pulled out and ¾ of its anterior horn removed. Then, the patella was replaced, the quadriceps muscle and the skin plan sutured. Control animals underwent sham surgery (ligament visualization but not dissection).

- **Hypergravity model**

Hypergravity mimics the overweight-associated mechanical strain on joints without metabolism dysregulation. In mice with MNX, hypergravity induces large OA lesions that are not observed without surgical induction [13, 14]. MNX was performed on the right knee, and mice were put back in their box for 48 hours. Then, cages were transferred in the gondolas of the centrifuge (COMAT Aérospace, Flourens, France) to maintain a permanent level of hyper gravity [14]. This device has four 1.4m-long arms that hold at their distant end a mobile octagonal gondola (56.2 × 52.0 × 59.2 cm). All gondolas are equipped with an infra-red video surveillance system to monitor the animals' condition and food/water stocks. In the centrifuge, temperature and light conditions were identical to that of control cages. At the start of centrifugation, acceleration was smoothly and gradually increased over a period of 40 sec. The final acceleration was 2 g (29.6 rpm), and animals were kept at 2 g for 6 weeks. Animals were provided with enough food and water for 4 weeks. Then, the centrifuge was transiently stopped to allow litter change, animal weighing, and chow and water supply refilling. Control mice with MNX were not exposed to hypergravity.

- **Metabolic disorder model**

Seipin (SP) knock-out mice are representative of metabolism disorder, which is a feature associated with OA [15]. *Bscl2* deficiency in mice recapitulates the main features of the phenotype of patients with Berardinelli-Seip Congenital Lipodystrophy (BSCL), including

almost complete absence of adipose tissue, hyperglycaemia, hyperinsulinemia, and insulin resistance. MNX and Sham surgery were performed in 10 week-old *Bscl2*-/- mice.

- **High Fat Diet model**

The high fat diet model reproduces the effect of obesity and dysregulated metabolism on OA onset [16]. At the age of six weeks, mice were fed with High Fat Diet (HFD, 60% of calories from fat, Ssniff, EF D12492 (II) mod. Soest, Germany) that was provided ad libitum for 10 weeks with the chow changed twice per week. A number of mice 20% higher than the final group size was included to ensure statistical power of the experimentation. MNX was performed at the age of 10 weeks. In absence of surgical induction, mice did not develop spontaneous lesions of OA. The average weekly weight gain ranged from 1 g to 1.5 g, leading to a final weight gain of 73% (mean: 14.6 g) associated with insulin resistance (HOMA-IR: +246%). Considering the large variability generally observed in the final body weight and fat mass, only animals with a final weight gain higher than 70% were analysed.

- **Collagenase-induced OA model**

The collagenase-induced model (CIOA) is characterized by low grade inflammation of the synovial membrane, leading to OA lesions [10]. A solution of 1 U/5µL type VII collagenase from *Clostridium histolyticum* (Sigma-Aldrich) was prepared in saline solution. At day 0, a small skin incision was performed on top of the patellar tendon. The knee was bended and the collagenase solution (5 µL) was injected in the intra-articular space using a 10 µL syringe (Hamilton) with a 25 gauge needle. On day 2, a second collagenase injection was performed according to the same procedure. Six weeks later, animals were sacrificed. Control animals were injected with saline solution.

- **Age-related model**

Ageing is the main risk OA factor [17]. C57BL/6JRj mice exhibit mild OA lesions in the knee at the age of 18 to 24 months [18]. Mice were housed with free access to food and water and euthanized at the age of 24 months. Control young mice were kept in the animal facility and euthanized at the age of 16 weeks.

Sample preparation for histology and mRNA extraction

After sacrifice, femora and tibiae from 10 knee joints (one joint/mouse) per model were dissected. Skin and muscles were removed and the knee joint was isolated by sectioning the distal extremity of tibiae and proximal part of the femurs. The tibial plateau was isolated from bone at the growth plate interface, by cutting 2-3 mm beneath the cartilage surface. The remaining soft tissues (meniscus, ligaments and synovium) were removed. The tibial plateau was immediately placed in 1mL of TRIzol® Reagent (Life Technologies), snap-frozen in liquid nitrogen, and stored at -80°C till RNA extraction. After isolation, femoral condyles were fixed in 4% paraformaldehyde for 36 hours, and then decalcified in 0.5M EDTA at room temperature for 15 days.

Histology

After dehydration in a graded series of alcohol, femoral condyles were embedded in paraffin at 60°C in a tissue processor. On average, 30 serial sagittal sections of 5 µm were cut, and three were chosen at the upper, medium and lower levels every 50 µm from cartilage surface. OA scoring was performed after Safranin O-Fast Green staining, according to the OsteoArthritis Research Society International (OARSI) recommendations [19]. For each animal, the OA score was the highest score obtained at one of the three levels. For each model, all sections were blindly scored by the same three readers.

RNA isolation

Tibial plateau samples were prepared in each consortium laboratory and then shipped for centralized RNA extraction that was performed by crushing thawed samples with ceramic beads (Precellys® Lysing kit CK28R), using a Precellys® 24 tissue homogenizer equipped with the Cryolis® cooling unit (Bertin Technologies). Samples underwent three successive lysis cycles at 6500 rpm for 15 sec, spaced by a 5 min lag phase at 4°C, before addition of 200 µL chloroform. After incubation at room temperature for 3 min, the aqueous phase was recovered, 600 µL of 70% ethanol was added, and the solution was transferred to an RNeasy® spin column (Qiagen) and the next steps were performed according to the supplier's recommendations. Total RNA was quantified with a Nanodrop® instrument and aliquots were frozen at - 80°C. RNA integrity was confirmed with the Agilent® RNA 6000 kit on an Agilent Bioanalyzer 2100®.

Transcriptomic analysis

Transcriptomic analysis was performed on Custom TaqMan® Array Microfluidic Cards (TAC) that were designed to perform 384 real-time PCR reactions on a ViiA™ 7 Fast Real-Time PCR System (Applied Biosystems®). Custom TAC were designed for the analysis of TGFβ family members (table 1). Reverse transcription was performed using 250 ng of total RNA and the High capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR was done using cDNA (150 ng) mixed with TaqMan Fast Advanced Master Mix (Life Technologies). After 40 cycles of amplification (95°C for 20 sec and then 95°C for 1 sec and 60°C for 20 sec), data were analysed with the Applied Biosystems® Relative Quantification Analysis Module. Amplification curves for each target were individually checked and baselines adjusted, when

necessary, to determine the cycle threshold (CT) values. Gene expression was normalized to the mean CT value of four housekeeping genes (*Gusb, Hprt, Rps9, Ppia*) and expressed as relative gene expression using the $2^{-\Delta CT}$ formula or as a fold change expression using the $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

Unsupervised two-dimensional hierarchical clustering was generated with mean-centred relative expression values ($2^{-\Delta Ct}$) of 91 genes per sample using XLStat software. Distances between samples were calculated based on the ΔCT values using Pearson's Correlation and average linkage method. The vertical height of the dendogram shows the Euclidean distances between samples. The two-dimensional scatter plot of Principal Component Analysis (PCA) was performed using XLStat and represents the expression pattern of ($2^{-\Delta Ct}$) sample values of the ten subgroups. When plotting the sample data points, F1 (PCA Component 1 (32.85% variance)) was used as the x-axis and F2 (PCA Component 2 (12.69% variance)) as the y-axis. Data did not assume a Gaussian distribution and were considered unpaired. The statistical analysis was performed between 2 groups for each OA model versus its respective control (MNX vs MNX-sham, CIOA vs CIOA-sham, Aged vs Young, SP-MNX vs MNX, HG-MNX vs MNX, and HF-MNX vs MNX) using the Mann-Whitney test and GraphPad 7 (San Diego, CA, USA). Data were expressed as relative expression ($2^{-\Delta Ct}$) or as fold change (fold change of gene expression in one OA sample as compared to its respective control normalized to 1) and represented as median with interquartile range. Differences were considered significant at $p<0.05$ and $p<0.01$.

RESULTS

Defining the Standard Operating Procedures. One important feature in the study design was to define the SOP after the harmonization of the experimental protocols (from animal models to transcriptomic analysis) in three consensus meetings of the ROAD consortium. A study workflow was designed (Figure 1). At each step, the analysis technique was performed in a single laboratory by the same operator to avoid experimental bias. The centralized OA scoring of the different models and controls (Figure 2A) showed that OA scores were significantly higher in all models (≥3 on a scale of 0 to 5) than in their respective control (score ≤2), although variability in control samples was observed (Figure 2B). The concentration of total RNA isolated from tibial plateau samples was not homogeneous among samples, and was significantly higher in the aged, HG-MNX and HF-MNX models than in their controls (young and MNX mice, respectively) (Figure 2C). The RIN score, which estimates RNA quality and integrity, was heterogeneous among samples, with significantly lower scores in samples from the aged and CIOA animals than from their controls (young and CIOA-sham) (Figure 2D). Among all samples, six out of the ten samples per group that met the criteria of selection were analysed by TAC. The mean CT values for the housekeeping genes were significantly higher in the CIOA, HG-MNX and HF-MNX samples than in their controls (Figure 2E). However, the mean CT values for the housekeeping genes were positively correlated with the mean CT values for all genes (Figure 2F). This indicated that the lower expression of housekeeping genes in some samples could be attributed to a lower amount of cDNA loaded in the TAC and not to a differential regulation of the housekeeping genes. We also detected the expression of genes specific for cartilage (type II collagen, aggrecan) or bone (Runx2, Sp7) in all OA models (data not shown), indicating that both tissues were represented in our samples.

TGFβ signatures according to the experimental OA phenotypes. Hierarchical clustering and average linkage clustering of the mRNA expression data in the 10 groups of mice (6 OA models and 4 controls) revealed marked differences among groups (Figure 3A). Three main subgroups could be detected: a cluster that included samples from mice with Aging-, HF-MNX-, HG-MNX-related OA; a cluster that included mainly samples from SP-sham, SP-MNX mice; and a cluster of samples from MNX, CIOA, and CIOA-sham. Sham samples did not cluster together, even though most of them are distributed in the last group with the exception of SP-sham, which is closer to SP-MNX. PCA revealed distinct transcriptional profiles among groups that allowed gathering them in three distinct clusters (Figure 3B). One (HG-MNX and HF-MNX) was clearly separated from the other two clusters that included i) CIOA, MNX and young animals, and ii) control groups (sham, SP-sham, CIOA-sham). Conversely, old and SP-MNX animals were set apart from the others.

To determine whether a specific gene signature could be associated with the different OA phenotypes, the gene expression profile of each OA group was compared with that of its control: MNX vs MNX-sham, CIOA vs CIOA-sham, Aged vs Young, SP-MNX vs MNX, HG-MNX vs MNX, and HF-MNX vs MNX. The number of significantly deregulated genes was similar in the MNX, SP-MNX, HG-MNX and HF-MNX groups (around 30 genes) (Figure 3C). Conversely, 15 and 47 genes were deregulated in the samples from CIOA and Aged animals, respectively. We identified genes that were common to two or more groups and a gene signature that was specific for each OA model (see Venn diagram in Figure 3D and table 2). Importantly, no gene was deregulated in all six OA models.

OA model-specific TGFβ signatures. To further analyse the specific gene signatures, we visualized the genes that were significantly dysregulated (fold change >1.5) in each OA model using Volcano plots. In the MNX model, gene expression profiling revealed that all 30 modulated genes were upregulated compared with control (Figure 4A). In the CIOA model, 14 of the 15 deregulated genes were significantly upregulated (Figure 4B). Conversely, in the Aging- and SP-MNX-related OA, most genes were downregulated (44/47 and 25/28 genes, respectively) (Figure 4C-D). Finally, in the HG-MNX and HF-MNX models, 70% and 71% of genes were upregulated (Figure 4E-F). Only four genes were differentially regulated between these models: *Smurf2* and *Id2* were upregulated, *Tgfbrap1* and *Lefty* were downregulated only in the HF-MNX model. Altogether, our data revealed that many TGFβ family members were deregulated in the different OA subtypes, supporting the key role of the TGFβ pathway, whatever the OA risk factor.

A *Gdf5*, *Ltbp4*, *Cd36* combinatorial gene signature for OA. Then, we split the six OA models in two groups. The first group included the OA models related to obesity or fat metabolism (SP-MNX, HG-MNX, and HF-MNX) and/or MNX. The number of shared and specific genes is shown in the Venn diagram (Figure 5A). Most of the modulated genes were common to two or three models, and few genes were specific to each model. However, only *Cd36* was deregulated in all four models. The second group included MNX and the two other most common OA models: inflammation (CIOA) and aging (Figure 5B). Approximately 50% of all deregulated genes were specific to each model and only two genes were deregulated in all three models: *Gdf5* and *Ltbp4*. Analysis of these three genes in all models and their respective controls showed that *Cd36* was significantly upregulated in MNX, SP-MNX, HG-MNX and HF-MNX samples (Figure 5C). *Gdf5* was significantly

upregulated in the MNX and CIOA models and significantly downregulated in the Aging model. *Ltbp4* was significantly upregulated in all models, but for the Aging model where it was significantly downregulated. These data suggest that *Cd36* upregulation is a hallmark of trauma-related OA, while the deregulation of *Gdf5* and *Ltbp4* is related to different OA stimuli.

DISCUSSION

The first objective of the ROAD consortium was to identify specific gene signatures for the main OA clinical phenotypes using six relevant murine models by focusing on the transcriptomic analysis of the TGFβ pathway. Although this pathway has been extensively studied in some OA murine models [18, 20, 21], it is quite impossible to compare these results from independent laboratories due to potential biases that may influence gene expression, such as mouse genetic background, age, sex, housing conditions, histopathological scoring subjectivity and inter-investigator variability. Here, we wanted to limit these potential biases by defining SOPs and by centralizing each step of data acquisition and processing, thereby minimizing the risks of failure to identify relevant targets [22-25]. The resulting data allowed the accurate comparative analysis of six models using their respective controls.

The main finding of our transcriptomic analysis is the unexpected lack of deregulated genes common to all murine models of OA, although many TGFβ family members were deregulated pointing out the critical role played by the TGFβ pathway in OA [26]. This might reflect the heterogeneity of responses to the different stimuli leading to similar symptoms, as observed in patients with OA. Differences in the expression pattern

between the different models likely relate to the peculiarities and distinct natures of the models. We are also aware that transcriptional regulation of genes may not be reflected at the protein level. Analysis of these differences at the protein level were beyond the scope of the present study but likely warrants further studies. Some genes, such as type II collagen, may be differently regulated depending on the OA model suggesting possible different timings or mechanisms of regulation that warrant further investigation. Heterogeneity may also be emphasized by the individual responses within the same model, thus highlighting the interest of classifying OA phenotypes using relevant biomarkers in the clinic. Heterogeneity might also reflect different stages of OA in the different models but this is unlikely since the OA scores are similar in all models. The absence of a common signature could also be due to the late time point (6 weeks after OA induction and 24 months of age for old mice) chosen for the transcriptomic analysis when the gene expression profile might reflect an adaptive response. However, this time point is relevant for patients in whom OA is generally diagnosed long after disease initiation.

Another important finding is the identification of the combinatorial *Gdf5-Cd36-Ltbp4* signature that might discriminate distinct subgroups of OA phenotypes. Indeed, *Cd36* was upregulated in all mice that underwent surgical MNX. CD36 is a membrane-bound protein and the receptor of thrombospondin-1, fatty acid translocase (FAT), platelet glycoprotein 4 (PG4) and scavenger receptor class B member 3 (SCARB3). It is expressed in adipocytes and mesenchymal stromal cells isolated from fat tissue, and its expression level correlates with poor differentiation into the chondrogenic lineage [27]. CD36 expression is increased at sites of cartilage injury and co-localizes with developing hypertrophic chondrocytes and the aggrecan NITEGE neo-epitope [28]. In patients with OA, CD36 expression has been significantly associated with the presence of osteophytes, of joint space narrowing, and

higher Kellgren-Lawrence score [29]. Moreover, in chondrocytes from patients with OA, expression of thrombospondin 1 (a CD36 ligand) is strongly decreased concomitantly with the increase in CD36 expression [30]. More recently, the anti-inflammatory and analgesic effects of serum albumin in patients with knee OA was related to inhibition of CD36 in synoviocytes, macrophages and chondrocytes [31]. In addition, our study suggests that CD36 might be a specific biomarker of post-traumatic OA. CD36 expression should be thoroughly investigated in cartilage and bone samples from patients with different OA phenotypes.

We also found that *Gdf5* expression was deregulated in three of the six OA models under study. It was previously shown that a loss-of-function *GDF5* gene mutation results in joint fusions, and a single-nucleotide polymorphism is associated with higher susceptibility to OA [32]. GDF5 deficiency has also been associated with abnormal ligament laxity and subchondral bone remodelling [33]. Several genome-wide association studies (GWAS) have reported the significant association between knee OA and the *GDF5* locus [29, 34-36]. Very recently, a GWAS using the United Kingdom OA Biobank cohort reported that *GDF5* genetic variants were the strongest predictor of knee pain [37]. In the present study, *Gdf5* expression was upregulated in the CIOA and MNX models that are characterized by ligament laxity and pain [10, 38]. Our data strongly suggest that GDF5 expression is a biomarker of painful OA phenotypes, as also suggested by genomic studies in humans.

Finally, we found that *Ltbp4* was deregulated in all six OA models (Figure 5C), although it was not identified as a deregulated gene common to all models in the statistical analysis (Figure 3D). In the bioinformatic analysis, SP-MNX samples were compared with MNX samples (Figure 4) to investigate the impact of the genetic background on OA. Conversely, in the data presented in Figure 5C, all groups were analysed independently of their

control. LTBP4 is a key molecule required for the stability of the TGFβ receptor (TGFβR) complex via interaction with TGFβR2, thereby preventing its endocytosis and lysosomal degradation [39]. However, LTBP4 has not been associated with cartilage or OA and unlike its paralogues, LTBP4 is not regulated during chondrogenic differentiation of mesenchymal stromal cells [40]. Like *Gdf5*, *Ltbp4* expression was decreased in old mice and not upregulated as observed in the murine models of induced OA. This suggests that spontaneous aging-related OA might involve different mechanisms.

In conclusion, the originality of the present study was to rely on relevant murine models of OA to understand the complexity of OA phenotypes in humans through investigation of the TGFβ pathway and based on rigorous SOPs. We did not identify a unique gene signature common to all six OA phenotypes. This highlights the huge heterogeneity of the animal models and the need of caution when extrapolating results from one model to another. But this also highlights that the diversity of the mouse models likely reflects the heterogeneity in human OA. Further studies are needed to validate these potential signatures.

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AUTHOR CONTRIBUTIONS

All authors were involved in revising critically the manuscript and approved the final version. MM: Data analysis, manuscript writing; DN: Experiment design, data analysis, manuscript writing; HKE, DM, MR, EH, XH, DC, MCS, CJa, JYJ, MHLP, PR, JS, CV: Experimental work; FR, CJo, JG, FB: Experiment design, manuscript writing.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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