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# Predictive modeling of therapeutic response to chondroitin sulfate/glucosamine hydrochloride in knee osteoarthritis

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***Abstract***

**Background:** In the present study, we explored potential protein biomarkers useful to predict the therapeutic response of knee osteoarthritis (KOA) patients treated with pharmaceutical grade Chondroitin sulfate/Glucosamine hydrochloride (CS+GH; Droglican, Bioiberica), in order to optimize therapeutic outcomes.

**Methods:** A shotgun proteomic analysis by iTRAQ labelling and liquid chromatography–mass spectrometry (LC-MS/MS) was performed using sera from 40 patients enrolled in the Multicentre Osteoarthritis interVEntion trial with Sysadoa (MOVES). The panel of proteins potentially useful to predict KOA patient's response was clinically validated in the whole MOVES cohort at baseline ($n = 506$) using commercially available enzyme-linked immunosorbent assays kits. Logistic regression models and receiver-operating-characteristics (ROC) curves were used to analyze the contribution of these proteins to our prediction models of symptomatic drug response in KOA.

**Results:** In the discovery phase of the study, a panel of six putative predictive biomarkers of response to CS+GH (APOA2, APOA4, APOH, ITIH1, C4BPa and ORM2) were identified by shotgun proteomics. Data are available via ProteomeXchange with identifier PXD012444. In the verification phase, the panel was verified in a larger set of KOA patients ($n = 262$). Finally, ITIH1 and ORM2 were qualified by a blind test in the whole MOVES cohort at baseline. The combination of these biomarkers with clinical variables predict the patients' response to CS+GH with a specificity of 79.5% and a sensitivity of 77.1%.

**Conclusions:** Combining clinical and analytical parameters, we identified one biomarker that could accurately predict KOA patients' response to CS+GH treatment. Its use would allow an increase in response rates and safety for the patients suffering KOA.

***Keywords:*** chondroitin sulfate/glucosamine hydrochloride, knee osteoarthritis, predictive biomarkers, proteomics

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## Introduction

To date, there is no effective pharmacological strategy to prevent osteoarthritis (OA) progression. Pain relief remains the primary unmet medical need. The efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs), both nonselective and selective cyclooxygenase-2 (COX-2) inhibitors (coxibs), remains modest, with several issues concerning their safety and tolerability.[1,2] Because of these limitations, OA pain is poorly controlled.

A valuable treatment option for knee OA (KOA) is represented by symptomatic slow-acting drugs for osteoarthritis (SYSADOA). The GAIT

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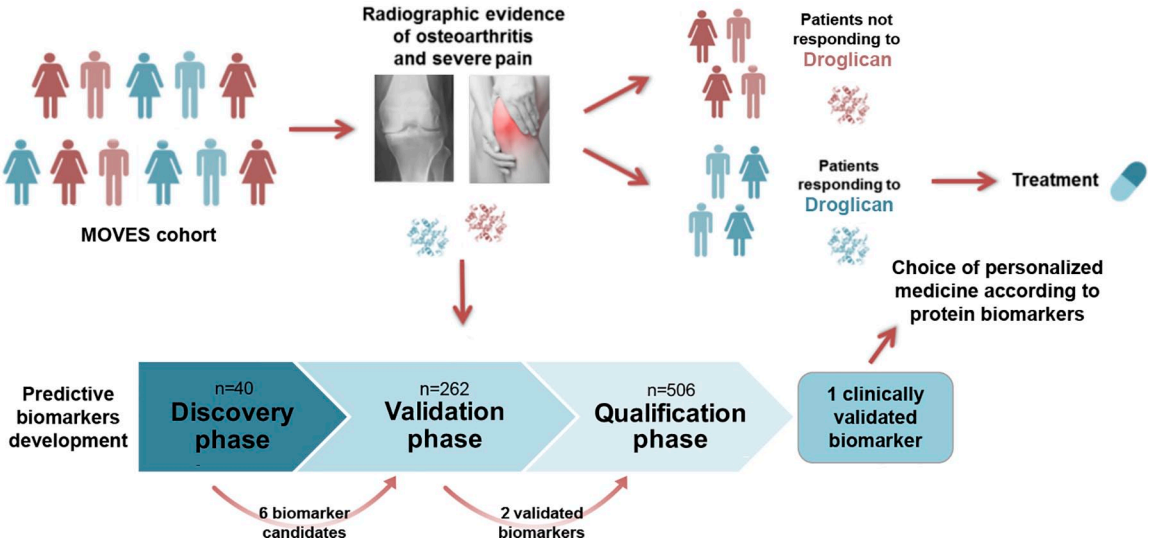


**Figure 1.** Graphical representation of the project. The different phases of the development of predictive biomarkers for knee osteoarthritis patients' stratification are illustrated.

trial was the first randomized, double-blind, placebo-controlled study to demonstrate the efficacy of the combination of glucosamine and chondroitin sulfate in the subgroup of patients with moderate-to-severe knee pain.[3] To complete that first study, the MOVES trial (Multicentre Osteoarthritis interVEntion trial with SYSADOA) was designed to confirm the noninferiority of chondroitin sulfate plus glucosamine hydrochloride (CS+GH) *versus* celecoxib (CLX) in reducing pain.[4] Both studies support that this combination of SYSADOA appears to be beneficial in the treatment of patients with KOA, offering a safe and effective alternative for those patients with cardiovascular or gastrointestinal conditions.

There are numerous options for assessing clinically relevant outcomes in OA, such as the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index,[5] Lequesne Algofunctional Index,[6] Knee Injury and Osteoarthritis Outcome Score (KOOS),[7] and the Outcome Measures in Rheumatology Clinical Trials and Osteoarthritis Research Society International (OMERACT-OARSI) Responder Index.[8–10] Biochemical measurements (in serum, urine, and synovial fluid) should be considered as additional tools to assess treatment efficacy allowing the identification of responder patients earlier during the disease progression. The right selection of KOA patient population, before starting a clinical trial, is mandatory, especially considering the heterogeneity of the disease, which comprises a number of distinct phenotypes.[11–13] Phenotype identification should be focused on those subgroups that could influence drug response, allowing targeted interventions. In the last year, several studies have been carried out in order to identify clinically homogenous KOA subgroups, such as those based on data from the OA Initiative (OAI).[11,14,15] The prediction of drug response based on the analysis of multiple clinical variables and 'omics' data is mandatory to accomplish the aim of precision medicine in rheumatology.[16,17] This will help the clinicians in decision-making for the management of KOA patients and ultimately benefit patients by matching their proteomic profiles to the most effective therapy available.

In the present study, we explored potential circulating protein biomarkers useful to predict the therapeutic response of KOA patients treated with pharmaceutical grade Chondroitin Sulfate plus Glucosamine Hydrochloride (CS+GH, Droglican®, Bioiberica), in order to optimize therapeutic outcomes in OA (Figure 1). We evaluated the ability of responder criteria based on the WOMAC index and the OMERACT-OARSI responder index to correctly classify KOA patients according to their unique protein profile at baseline. The results of this study, which define predictors of treatment response to pharmaceutical grade CS+GH, could represent a useful tool to support clinical decision-making in KOA.

## Materials and methods

### *Study participants*

Participants from the MOVES cohort were included in this study.[4] Eligible patients for this cohort were ⩾40 years of age, with a diagnosis of primary KOA according to the American College of Rheumatology, with radiographic evidence of the disease (Kellgren and Lawrence grade 2 or 3) and severe pain (WOMAC pain score ⩾301 on a 0–500 scale) at inclusion. Patients were randomized to receive 400 mg CS plus 500 mg GH (Droglican, Bioiberica) three times a day, or 200 mg Celecoxib (CLX) every day for 6 months. The primary outcome was the mean decrease in WOMAC pain from baseline to 6 months (expressed as 20, 30, 50 or 70% reduction). Patients were classified as responders (R) and nonresponders (NR) according to the WOMAC pain score (W20, W30, W50 or W70) and the OARSI-OMERACT criteria recorded at the end of the trial (after 6 months of treatment). The trial was performed according to the ethical principles of the Declaration of Helsinki and good clinical practice. All patients read and signed the informed consent, which specified the use of data/samples for research scope. The research protocol (EudraCT number: 2010-024010-61) was approved by the local Ethics Committee (Comité Ético de Investigación Clínica Parc de Salut MAR, Cataluña, Spain) and authorized by the Spanish Agency of Medicines and Medical Devices (AEMPS) (3004/RG60043).

### *Discovery phase*

The shotgun proteomic analysis was performed on serum pools from a representative group of the CS+GH cohort (40 samples) and consisted on two independent four-plex iTRAQ-based quantitative proteomic analyses. The workflow of this step is summarized in Supplementary Figure S1. Each sample for the analysis was obtained by pooling equal amounts of serum from five individuals (a total of eight pools), in order to reduce the inter-individual variability inherent to this type of samples. Four subgroups of patients were analyzed in this phase: WOMAC 20, WOMAC 70 and OARSI responders and nonresponders to CS+GH treatment, each of them including 10 patients (randomized in 2 pools of 5 patients per group).

The top-14 most abundant serum proteins were removed from the pooled samples by immunoaffinity liquid chromatography, using a commercial column (MARS Hu-14, Agilent Technologies, Palo Alto, CA, USA). Then, the concentration of proteins in the samples was quantified, and equal amounts (approximately 50 µg) were digested with trypsin. The resultant peptide extracts were differentially labeled using the iTRAQ reagents (Supplementary Figure S1), following the manufacturer's instructions (Sciex, Vienna, Austria) and a protocol previously described by our group.[18] Briefly, aliquots of the labeled samples were combined and cleaned with POROS R2 resin. The peptide mixture was resolved first by reversed phase chromatography at basic pH using a C18 column (Zorbax Extend C18, 100×2.1 mm id, 3.5 µm, 300 Å, Agilent) in a HP1200 system (Agilent, Palo Alto, CA, USA), with a flow rate of 0.2 ml/min. A total of 16 fractions were collected from each injection, which were desalted and then loaded onto a reversed phase column C18 (Integrafit C18, Proteopep ™ II, 75 µm id, 10.2 cm, 5 µm, 300 Å, New Objective, Woburn, MA, USA) to carry out a second separation at constant flow of 350 nl/min. The microfractions were collected and spotted onto MALDI plates using a SunCollect MALDI Spotter (SunChrom Wissenschaftliche Geräte, Friedrichsdorf, Germany).

The acquisition of mass spectrometry (MS) data was performed in positive ion mode using 4800 MALDI-TOF/TOF analyzer and 4000 series Explorer program 3.5.1. Mass spectra between m/z 800 and 4000 were acquired for each fraction using 1500 laser shots and processed with internal calibration (Angiotensin 3 fmol/spot with m/z of 1046.50 diluted in the matrix). After mass screening, precursors were automatically selected and fragmented with air in the collision chamber with energy of 1 kV. Those 25 ions with stronger intensity and signal/noise (S/N) above 80, excluding typical trypsin autolysis peaks and matrix signals, were selected as precursors for acquiring MS/MS spectra. For this process, a higher laser intensity and 2000 shots per spot were used. The identification of proteins was performed with ProteinPilot v4.5 program (Sciex) and the Paragon algorithm, using trypsin as digestion agent and iodoacetamide as fixed modification of cysteines. Each MS/MS spectrum was searched against the database Uniprot/Swissprot (2015_05 release version) for the species *Homo sapiens*. Only proteins identified with at least 95% confidence or a ProtScore above 1.3 were taken into account. The program also provided data relative to the quantification between each of the samples, and

changes were considered significant with a ratio $\geqslant 1.2$ or $\leqslant 0.8$ and a $p$-value $\leqslant 0.05$.

### *Validation phase*

In the validation phase, the specificity and sensitivity of a panel of six putative predictive biomarkers were evaluated by enzyme-linked immunosorbent assays (ELISAs) on the whole CS+GH cohort ($n=262$). Among 56 putative biomarkers from the discovery step, we selected the six best candidates to be validated following one of these criteria: proteins with the highest iTRAQ ratios or proteins modulated according to more than one responder criteria. All the ELISA kits were from Cloud-Clone Corp. (Houston, TX, USA). Serum samples were diluted in PBS 0.01 M (pH = 7.4) as follows: for APOA4, 1000-fold; for C4BPa, 10,000-fold; for ITIH1 and ORM2, 20,000-fold; for APOA2, 50,000-fold; and for APOH, 100,000-fold.

### *Qualification phase*

In the last phase of the study, proteins that were altered in the validation analyses were selected for the qualification step: inter-alpha-trypsin inhibitor (ITIH1) and α1-glycoprotein-2 (or orosomucoid 2, ORM2) as predictive marker of responsiveness to CS+GH. A total of 506 samples from the whole MOVES cohort at baseline (both groups of treatment, CS+GH and CLX) were analyzed in this phase.

### *Statistical analysis*

In the discovery phase, statistical tools from the ProteinPilot software version 4.5 were employed for the identification of the proteins, and their relative quantification between the conditions that were compared (R *versus* NR). The ProteinPilot software employs two different algorithms: one to perform protein identification (ParagonTM algorithm) and the other to determine the minimal set of confident protein identifications (Pro GroupTM algorithm). Once the identity of the protein was confirmed (Detected Protein Threshold >95%, Unused ProtScore >1.3), the ratios of the peak areas of iTRAQ reporter ions were calculated in order to compare the relative abundance of the proteins identified in the samples. Data were normalized for loading error by bias, assuming the samples are combined in 1:1 ratios. Peak areas for the iTRAQ reagent(s) and control were also corrected to remove background ion signal by applying the background correction option. Only those changes with a $p$ value $\leqslant 0.05$ and a ratio $\geqslant 1.2$ (or $\leqslant 0.8$) were considered statistically significant. The Proteomics System Performance Evaluation Pipeline (PSPEP) software was used independently to calculate false discovery rates (FDR). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012444.[19]

In the validation phase, comparisons between the two groups (R and NR) were performed by a Mann-Whitney U-test. Spearman correlation coefficients were used to describe the association between two variables (clinical, analytical and response variables) and Chi-square test to compare proportions. To evaluate the ability of serum proteins to predict drug response, areas under the curve (AUC) were computed and receiver operating characteristic (ROC) curves were plotted. A multivariate logistic regression analysis was performed to determine significant and independent contributions of specific variables, recorded at baseline by the MOVES investigation group, to drug response. Multivariate models included all covariates with associations from the univariate models with a $p$ value $\leqslant 0.20$. All reported $P$ values were two-tailed, with a $p$ value $\leqslant 0.05$ indicating statistical significance. Data from this study were analyzed using SPSS version 24 and R statistics (SPSS, Chicago, IL, USA). Our study fully complies with the TRIPOD guidelines for the development and validation of prediction models.

## Results

### *Identification and validation of predictive markers of response to CS+GH*

In the discovery phase, 56 proteins showed a statistically significant modulation in at least one of the responders' group (R) to CS+GH (Figure 2a and Supplementary Table S1) compared with the nonresponders (NR). Functional analysis revealed that they were related mainly to inflammatory processes and complement activation (Figure 2b).

Six proteins were chosen for validation in the CS+GH group of the MOVES cohort at baseline ($n=262$) following the selection criteria described in *Materials and methods*: alpha-1-acid glycoprotein 2 (ORM2), inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), apolipoprotein AII (APOA2),

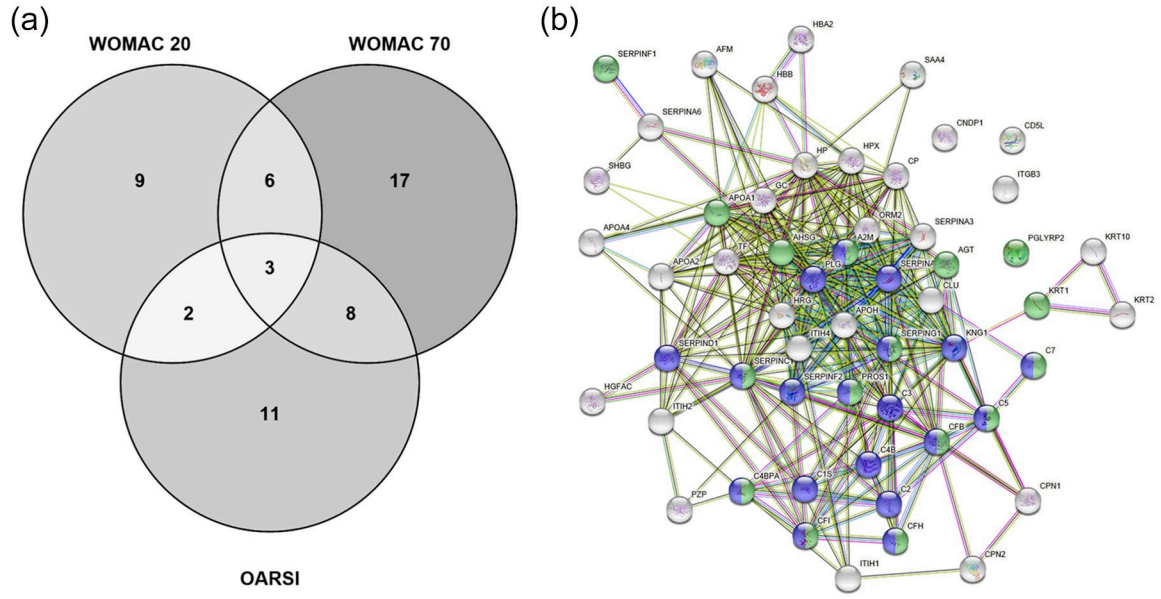


**Figure 2.** Results from the Discovery Phase. (a) Venn diagram: 56 proteins identified by shotgun proteomic analysis as significantly altered in the baseline serum of the responders to CS+GH according to the WOMAC (20 and 70) and OMERACT-OARSI criteria are represented. (b) Functional analysis: protein network visualization of the differential proteins by STRING software (http://string-db.org/). Proteins in green are involved in the regulation of inflammatory response; proteins in violet are involved in complement and coagulation cascades.

apolipoprotein A-IV (APOA4), C4b-binding protein alpha chain (C4BPa) and beta-2-glycoprotein 1 (APOH).

Nonparametric tests were performed for each group of response to CS+GH: OARSI, WOMAC20, WOMAC30, WOMAC50 and WOMAC70. Statistical analysis revealed that ORM2 showed a significant decrease at baseline in OARSI responders compared with nonresponders (19,282 ± 123,260 µg/ml ($n$ = 162) *versus* 26,158 ± 20,158 µg/ml ($n$ = 44) (Figure 3).

Nonparametric analysis showed decreased levels of ITIH1 at baseline in the OARSI R group compared with NR (1759 ± 874 µg/ml ($n$ = 162) *versus* 2169 ± 1157 µg/ml ($n$ = 44)) ($p$ = 0064, data not shown).

No statistically significant differences were found for the other response groups.

Taking into account the results obtained, we then analyzed by a blind test the levels of ORM2 and ITIH1 in the whole cohort at baseline ($n$ = 506). Interestingly, no modulation was observed in the CLX group in any of the response subgroups (Supp1ementary Figure S2).

### *Predictive biomarkers qualification*

Next, we moved to the qualification step and we explored the predictive value of all the clinical and analytical variables recorded at the beginning of the study by the MOVES group in a Multivariate Logistic Regression Analysis (Supp1ementary Table S2). Patients with KOA participating in the MOVES trials showed no statistically significant differences, at baseline, in their overall health status as measured by both arthritis-specific and general evaluations (Supp1ementary Table S3). When we explored the possible associations between important KOA risk factors (such as gender, age, BMI) and the selected markers, we found a statistically significant association between three proteins (APOA2, APOH, C4BPa) and age (Spearman's rank correlation coefficient: –0.214; –0.157; –0.147). We also found a statistically significant association between APOA2 and BMI (Spearman's rank correlation coefficient: –0.134), while the association between APOA2 and gender showed a $p$ value of 0.058. The other associations were not significant. When we considered clinical parameters like K/L grade, stiffness or joint swelling /effusion, we found a statistically significant association between APOA2 ($p$ = 0.0004), APOA4 ($p$ = 0.0028) and ITIH1 ($p$ = 0) and K/L grade. For ORM2, the $p$ value was 0.0055. No

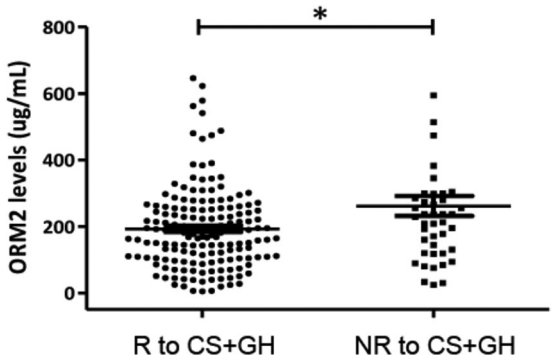


**Figure 3.** Results from the Validation Phase. Levels of ORM2 in OARSI responders (R, n = 162) vs nonresponders (NR, n = 44) in CS+GH group (*p = 0042).

association was found for the other parameters. Going deeper into the analysis, those variables that were significant in the univariate analysis were included in the regression model, while those variables that were significant in the univariate analysis but showed a strong correlation with others were discarded (Supp1ementary Table S4). Other variables that could improve the predictive power of our regression model were also added following a step-wise method. Finally, we observed that seven of the variables recorded at baseline significantly influence OARSI patients' response to CS+GH treatment (Table 1). These were five clinical [Global Assessment of Disease by Patient (GAPS), Eqpd pain score from EuroQol-55, joint effusion, precondition metabolic disorder and BMI) and two analytical (eosinophils, haemoglobin) variables. The regression model calculated including these variables showed a good predictive power [AUC = 0.806 (0.730–0.881), $p = 0.007$]. As shown in Table 1, when we add the baseline ORM2 as covariate in the model for CS+GH response, the OR was 0.996 [(0.993–0.999) $p = 0.007$]. A ROC curve was performed to quantify the overall ability of ORM2 as predictive biomarker to classify OARSI R and NR to CS+GH correctly. As shown in Figure 4, the inclusion of ORM2 levels at baseline in our predictive model increases the AUC from 0.806 up to 0.843 [(0.781–0.906) $p = 0.000$].

We also explored the possible interactions present between ORM2 and other variables in the multivariate model. We found statistically significant interactions between GAPS:ORM2 ($p = 0.0007$) and haemoglobin:ORM2 ($p = 0.0014$). However, their inclusion in the model did not improve its predictive capacity, the previous one being equally good and easier to interpret.

When we considered the seven previous baseline features that significantly influence OARSI patients' response, and included baseline ITIH1 as covariate, we found a specific interaction between response to CS+GH and baseline protein levels ($p = 0013$) thus increasing the power of the prediction model up to AUC=0.823 (Supplementary Figure S3). In ORM2 + ITIH1 model we observed a marked improvement in ROC curve from 0.806 up to 0.841 [(0.778–0.903) $p = 0.000$] (Supplementary Figure S3). However, the ORM2 model still remained the best predictive model of response to CS+GH treatment (AUC = 0.843).

## Discussion

Currently, OA is considered a disorder with different phenotypes, and characterization of the diverse subtypes of OA presents new opportunities for developing targeted therapies.[20] Biomarkers can be used not only in the process of drug development, but also in assessment of individual patient's response to treatment. On the one hand, it is believed that by implementing biomarkers for screening of drug candidates in early clinical development phases (*in vitro* and preclinically), potential safety issues can be addressed in advance, allowing more efficient and less costly trials *via* a reduction in study size and length. On the other hand, by evaluating the biomarker, clinicians will be able to conclude whether the treatment has the desired effect or not. According to the BIPED classifications,[21,22] in this study, we focused on 'Prognostic/Predictive (P)' biomarkers. A predictive biomarker is a baseline characteristic that categorizes certain patient populations that are more likely to respond to a drug therapy or to avoid specific adverse events. The primary objective of this study was to identify subpopulations of responsive subjects in order to optimize therapeutic outcomes in OA.

Although there are no blood tests specific for OA, certain tests can help rule out other causes of joint pain, such as rheumatoid arthritis. In recent years, a number of studies have attempted to use proteomic approaches for the discovery of new

**Table 1.** Multivariate logistic regression analysis including those variables recorded at baseline and resulted significantly associated with CS+GH response in the univariate analyses.

| Variable | R ($n$ = 162) | NR ($n$ = 44) | $p$ Value | OR | IC 95% |
|---|---|---|---|---|---|
| BMI | 30.762 ± 5.97 kg/m$^2$ | 31.950 ± 5.52 kg/m$^2$ | 0.013 | 0.911 | 0.847–0.980 |
| GAPS | 69.72 ± 16.77 | 64.27 ± 17.70 | 0.000 | 1.057 | 1.027–1.089 |
| Eosinophils (blood) | 3.00 ± 1.81 mm$^3$ | 2.26 ± 1.32 mm$^3$ | 0.010 | 1.551 | 1.113–2.162 |
| Haemoglobin (blood) | 8.78 ± 0.69 g/dl | 8.45 ± 0.69 g/dl | 0.000 | 4.194 | 1.996–8.809 |
| Eqpd score pain | 2.21 ± 0.41 | 2.34 ± 0.48 | 0.002 | 0.170 | 0.55–0.553 |
| Metab dis (prev) | 18.5% | 13.6% | 0.026 | 3.317 | 1.158–9.502 |
| Joint effusion | 5.6% | 11.4% | 0.047 | 0.222 | 0.050–0.980 |
| ORM2 | 192.82 ± 123.26 µg/ml | 261.58 ± 201.58 µg/ml | 0.007 | 0.996 | 0.993–0.999 |

BMI, bone mass index; GAPS, global assessment of disease by patient; Eqpd score pain, Eqpd score pain from EuroQol-55; Metab dis (prev), precondition Metabolic Disorder; R, responders; NR, nonresponders; OR, odds ratio; IC, confidence interval. Where appropriate, mean values ± standard deviation are shown.

biomarkers for early OA diagnosis.[23–25] To date, many different biomarkers have been tested,[26] but none has yet been qualified for OA. Most studies have focused on testing whether the biomarkers can differentiate OA patients from healthy controls (diagnostic biomarkers) or whether they are associated with disease progression (prognostic biomarkers). However, despite the great need for developing biomarkers that can be used for treatment response or patient stratification/phenotyping, to date there are no studies that investigate their value to predict response. To date, there is no evidence of existing treatment guiding markers for such disease. We consider that this is the first study arising from a randomized clinical trial in KOA in which one of the main objectives was to evaluate a treatment selection marker. To our knowledge, this is the first work to combine proteomic tools (iTRAQ labeling and nanoLC-MALDI-MS) with clinical parameters to discover potential biomarkers for predicting drug response in KOA patients. In this study, we followed the workflow summarized in Figure 1 according to the guidelines accepted by the FDA and EMA for biomarker development: from the preliminary phase of biomarker discovery to the qualification phase going through verification and validation steps. The main results obtained are illustrated in Figure 5. Briefly, a shotgun proteomics strategy has been followed to identify circulating proteins with biomarker value for predicting the response of KOA patients to CS+GH treatment. With this objective, a proteomic screening was carried out on a representative set of serum samples from the MOVES cohort. In the first phase of the study, 56 proteins with different expression patterns in the sera of responders and nonresponders to CS+GH were successfully identified. Among them, we selected proteins to be validated on the basis of their iTRAQ ratios and their reproducibility (proteins altered in more than one subgroups of response). Thus, six proteins were chosen as putative predictive biomarkers for CS+GH treatment (Figure 5). Due to the results obtained in the validation phase, two proteins were selected for the qualification step using samples from the whole MOVES cohort: ORM2 and ITIH1. ORM2 is a protein involved in a pivotal process strictly related to OA pathophysiology: inflammation.[27] ITIH1 has been recently described as candidate circulating protein biomarker useful to support the diagnosis of radiographic KOA.[24] The results obtained confirmed the specificity of ORM2 in predicting response to CS+GH treatment, showing no

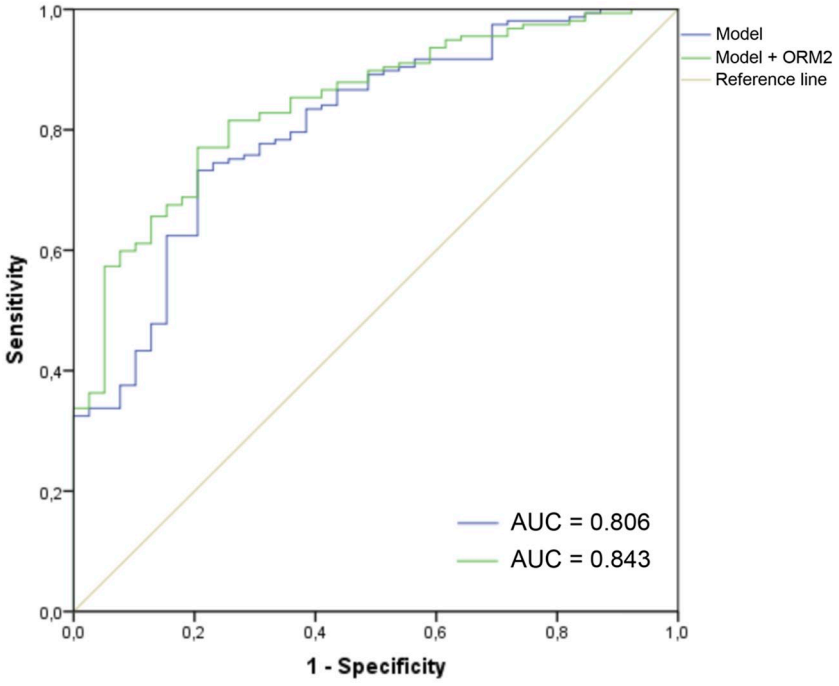


**Figure 4.** Predictive model of response to CS+GH. ROC curve for CS+GH and OARSI response group, created using values predicted by logistic regression with markers considered as predicted variables, and with or without ORM2 as covariate. The best trade-offs in Model + ORM2 between specificity and SENSITIVITY were 82.70% and 66.70%, respectively.

statistically significant modulation in the Celecoxib group (Supp1ementary Figure S2).

To complete the clinical validation of our study, we moved to the last step of predictive modeling of therapeutic response in KOA, in which we combined commonly available clinical and analytical variables with proteomics measurements in order to stratify KOA patients into responders and nonresponders to CS+GH treatment. Using ROC curve analysis and prediction modeling, we showed that serum concentration of ORM2 at baseline combined with seven variables (five clinical and two analytical) could efficiently predict patients' response to pharmaceutical grade CS+GH with a specificity of 79.5% and a sensitivity of 77.1%, respectively.

To our knowledge, this is the very first study investigating the serum ORM2 differences among responders and nonresponders to CS+GH, measured retrospectively using samples collected at baseline. Our results provide a clear evidence for the role of ORM2 in KOA and its potential value as a molecular signature to predict which patients will benefit from CS+GH treatment. Serum ORM2 levels are significantly different between patients with and without symptomatic amelioration that potentially can serve as a differentiating factor from the predictive biomarker perspective. Orosomucoid 2 (ORM2), also known as alpha-1-acid glycoprotein 2 (AGP2), is a member of the acute-phase protein family. There are two isoforms of ORM in human (ORM1 and ORM2) being the constitutive level of ORM1 much higher (fivefold) than ORM2. ORM2 functions as transport protein in the blood stream. It is considered one of the most important drug-binding proteins in plasma and may have important pharmacokinetic implications in clinical therapy. It is well recognized that changes in ORM concentration could potentially alter the free fraction of many drugs in plasma or at their target sites, and eventually affect their pharmacokinetic disposition and pharmacological action, which leads in many cases to treatment failure.[28] Moreover, given that an increasing number of drugs have been shown to bind preferentially to an ORM2 variant, a better understanding of this unique interaction may provide great benefit for drug discovery and development.[29] In this context, we could speculate that the higher

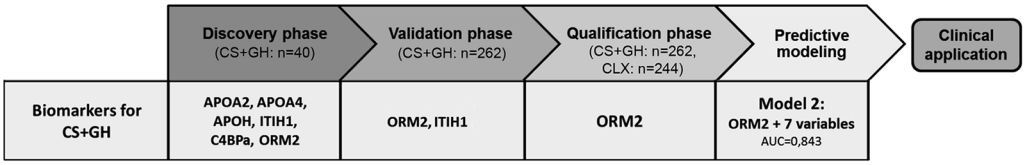


**Figure 5.** Predictive modeling of therapeutic response in knee osteoarthritis. Steps from the discovery phase to clinical application.

concentration of ORM2 founded in the NR group could, in part, explain the worse response of these patients to CS+GH treatment. However, further experiments should be done to confirm this hypothesis. ORM2 also modulates the activity of the immune system during the acute-phase reaction, although its function in peripheral tissues needs further investigation. ORM2 is increased under certain stress conditions. It is not specific to OA, being also elevated in other inflammatory diseases like rheumatoid arthritis or Crohn's disease.[30] Therefore, its association with CS+GH response could explain the pleiotropic effects of CS, which has shown an effect in different chronic inflammatory conditions.[31] At first glance, ORM2 could be considered a descriptive biomarker because it reflects the state of the disease but is not directly involved in OA pathogenesis. An increase of serum ORM level has been observed in obese humans.[32] Further studies demonstrated that this increase is correlated with BMI, body fat mass, serum leptin and glucose level, suggesting that ORM might participate in the regulation of energy balance.[33,34] Sun and colleagues confirmed the role of ORM as a negative feedback molecule in energy homeostasis and a novel target for the management of obesity and related metabolic disorders.[35] In this scenario, ORM2 could acquire a more important value as mechanistic biomarker, since alterations in metabolic pathways and energy production are well documented features of metabolic OA phenotype.[15,36,37]

Although the value of the diagnostic and prognostic information that ORM2 provides could be limited, this protein clearly showed its specificity for predicting drug response to CS+GH treatment, especially when combined with other clinical and analytical variables (Table 1). Among the seven variables that were included in our predictive model, some seem especially relevant for predicting CS+GH response in KOA patients. Patients with a precondition of metabolic disorder respond better to pharmacotherapy (OR = 3.317), dislipaemia being the most frequent condition among the OARSI response subgroup (67%). Conversely, patients with higher BMI present higher values of ORM2, in accordance with previous data by Alfadda and colleagues,[33] and show a worse response to CS+GH treatment (OR = 0.911). Our data also demonstrated that eosinophils and haemoglobin levels in blood directly correlate with treatment response (OR = 1.551 and OR = 4.194, respectively). In the OA field, an understanding of the potential significance of results obtained in routine blood tests, including the parameters eosinophils and haemoglobin, is often difficult, due mainly to the substantial variability across distinct laboratories and countries. In this context, our results support those from Walker and colleagues about the need to advise clinicians as to when to monitor OA patient's haemoglobin levels could be appropriate.[38] Another important point raised by this study concerns the presence of joint effusion at the beginning of the trial. KOA patients presenting joint effusion are likely to not respond adequately to CS+GH treatment (OR = 0.222). On the contrary, global assessment of disease by patient (GAPS) values directly correlate with CS+GH response in the OARSI subgroup (OR = 1.057), as shown in Table 1.

In this study, we also evaluated the ability of responder criteria based on the WOMAC index and the OMERACT-OARSI responder index to correctly classify KOA patients according to their unique protein profile at baseline. We compared the results of the analyses based on WOMAC and OMERACT-OARSI index to determine whether the application of different criteria influences data interpretation. The OARSI task force proposed that pain should be the primary outcome variable in trials of OA agents based on symptoms.[39] For this reason, the primary outcome measure of the MOVES trial was defined as the mean decrease in WOMAC pain subscale from baseline to 6 months,

expressed as 20, 30, 50, and 70% of decrease. In this study, we also considered the OMERACT-OARSI set of responder criteria for evaluating KOA patients' response to symptomatic therapies as suggested by Pham and colleagues.[8] These latter criteria are based on a combination of percentage and absolute changes in one or more variables including pain, physical function, and patient global assessment.[8] Of the 262 randomized patients, 188 achieved a WOMAC20 response at the end of the study after 6 months of treatment with CS+GH. Higher response levels have been more difficult to achieve: 172 achieved WOMAC30, 129 achieved WOMAC50, and 65 achieved WOMAC70 response, respectively. Finally, 171 of the 262 randomized KOA patients achieved an OMERACT-OARSI response after 6 months of treatment with CS+GH. Statistically significant differences have been detected at this level.

Briefly, our predictive or 'prescriptive' model provides a forecast of the potential for a KOA patient to respond, favourably or unfavourably, to the specific treatment object of this study. Undoubtedly, the classification model described in this study present several advantages. The use of clinical and analytical parameters routinely available strengthens the practical relevance of our analyses to stratify KOA patients. The implementation of ORM2 assay for predictive protein biomarker determination should not limit the clinical applicability of our classification model for the management of KOA patients. Identifying homogenous subgroups of responders and nonresponders to CS+GH, by the combination of clinical and analytical information, might improve treatment allocation for these patients. However, our study also presents some limitations. The cross-validation of our prediction model in another cohort of KOA patients treated with CS+GH would be desirable. Another critical point to be kept in mind is that the present results have been obtained with pharmaceutical grade CS+GH. Hence, our results cannot be generalized to lower quality compound mixtures of different source and grade of purity, generally present in nutraceuticals such those commercially available as dietary supplements in the United Kingdom and the United States,[40] or to the individual components themselves. Furthermore, using combinations of structural and protein biomarkers in stratification for intervention would be of great impact to aid targeted intervention in KOA. In this study, it was not possible to address this aspect due to the shortness of the follow-up period and the questionable structural effect of both treatments, which are symptomatic drugs for OA.

## Conclusions

In conclusion, this study has succeeded in classifying groups of KOA patients characterized by specific clinical and analytical characteristics that could efficiently respond to CS+GH. Overall, the results obtained at baseline indicate that ORM2 is a useful biomarker for predicting drug response to CS+GH in KOA patients.

## Authors Contributions

VC, FJB, JV, MH, HM, and CRR conceived and designed the study. VC, JM and PFP realized shotgun experiments. VC, MCE, LGR, LL, BR and MTSD collected samples and realized validation experiments. IRP, FP and VC performed statistical analysis. VC and CRR supervised all the experiments and drafted the article. All authors performed review/editing of the manuscript and approved the final version before submission.

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## Conflict of interest statement

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## Supplemental material

Supplemental material for this article is available online.

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