TREAT OA Report Summary

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Final Report Summary - TREAT OA (Translational Research in Europe – Applied Technologies for Osteoarthritis)

Executive Summary:

Osteoarthritis (OA) is the most common cause of disability in Europe and currently there are no drugs that can cure, reverse or halt the disease. TREAT-OA was a large-scale trans-disciplinary project using extensive collections of OA phenotyped subjects with available genome-wide association scan data and in vitro and in vivo state of the art technologies to further our understanding of OA. The study was highly integrated and included epidemiologic, biochemical and genetic approaches. TREAT-OA translated genetic findings into drug discovery and diagnostic development. The project was subdivided into 9 scientific and technological workpackages, a management WP and a dissemination WP.

WP 1: Novel Genes involved in OA risk and progression Genomewide scans (GWAS) for hip, hand, and knee OA were performed. Focused analysis on candidate pathways was also performed.

WP 2: Generalisability of genes and biochemical markers. Candidate variants were tested and several large scale meta-analyses showing the generalisability of candidate genes (e.g. TRPV1, SMAD3, GDF-5) and the lack of it from GWAS (BTLN2) or previous candidate gene reports (Il6, IL1). Meta-analyses of the top knee OA signals were also performed in Asian populations, showing the signal to be specific to European populations. A GWAS meta-analysis for OA biochemical markers CTX-II and COMP was carried out.

WP 3: Meta-analysis and Bioinformatics: A standardized meta-analysis protocol was developed. The first large scale meta-analyses for knee OA and hip OA were generated by TREAT-OA and have published/accepted for publication in the highest ranking rheumatology journal.

WP 4: Biochemical markers Measurements of CTX-II & COMP levels were performed on 3500 subjects from the TREAT-OA study cohorts part of TREAT-OA. The novel markers, S-CIIIIM was also measured in all cohorts. Two novel markers have been measured in the cohort with MRI and synovitis data, namely, S-C3M & S-CRPM. The diagnostic value of these markers was assessed in WP8.

WP 5: Functional genetics using in vitro models Systematic expression screens in vitro primary cells and tissues of relevance to the joint and joint associated tissues such as chondrocytes/cartilage, osteoblasts/bone, meniscal cells, and mesenchymal stem cells/synovium were carried out for all genes of interest to facilitate the investigation of their mode of action on disease aetiology.

WP 6: Functional genomics using zebrafish and chick disease models of OA Developed zebrafish and chick gain of function and loss of function models to study the mechanisms of action of discovered genes. The role of GPR22 and PRKAR2B in early development was identified.

WP 7: Functional genomics using mouse models of OA A systematic screen of the large-scale N-ethyl nitrosourea (ENU) mutagenesis in mouse was performed. The roles of selected genes identified from human genetic studies were investigated using murine OA postnatal models. Mutations were found in all of the genes studied. ENU-induced polymorphism in the 5’ UTR/promoter of Gdf5 results in increased Gdf5 expression Gdf5bp/+ mice show increased incidence and severity of OA.
WP 8: Translation into diagnostics the diagnostic value of genetic variants and biomarkers involved in risk in OA in severity and progression of OA was assessed using ROC analysis. The paper reporting the diagnostic value for predicting incidence of knee OA is in press. Sequencing projects were completed and the role of functional variants in the genes of interest has been described.

WP 9: Drug Target Screening. A reporter based BMP assay was successfully optimised for compatibility with high throughput screening (HTS). A pilot screen was performed with a chemically diverse test set of 1000 compounds. The estimated hit rate is around 0.5-1%. The second HT assay focused on the Wnt pathway, the estimated hit rate was somewhat lower 0.1-0.5%, but still sufficient to end up with a reasonable number of high quality hits. The effect of pharmacological inhibition of DOT1L was tested on human primary chondrocytes, but no effect on ECM synthesis or chondrocyte metabolism was observed.

WP11: Dissemination. Having generated over 55 peer-reviewed high quality scientific publications, and several more in preparation, TREAT-OA has also contributed to lectures and presentations and organized two summer schools (Oxford 2010 and 2011), a symposium on the molecular pathogenesis of OA in London in 2010 and a workshop on phenotyping for OA studies in Mallorca in 2010, a symposium on the role of OMICs studies in OA in London 2012 and a final conference in Mallorca in 2012. The project website has been running since 2008 and continues to be updated. www.treatoa.eu

Project Context and Objectives:

Context:

Osteoarthritis (OA), the most common joint disorder worldwide [1], is a chronic arthropathy in which cartilage loss, osteophyte formation, and subchondral bone sclerosis lead to pain, disability, and a reduction in quality of life [2]. Osteoarthritis of the large weight bearing joints (hips and knees) is a major contributor to the 57,000 knee and 55,000 hip arthroplasties undertaken each year in the United Kingdom [3] and the over 500,000 hip and knee arthroplasties per year in the US [4].

Mortality of OA: A recent large population based study has found that individuals with osteoarthritis (defined both symptomatically and radiographically) at the knee or the hip show a 55% excess in all cause mortality. [5]. Importantly, deaths from cardiovascular causes are higher in patients with walking disability due to OA (72% higher) even after adjustment for baseline covariates, indicating that there is an interplay between the underlying OA and the additional comorbid conditions which results in a higher risk of mortality. Thus, although the main clinical symptoms of OA are pain and disability, the consequences of the disease are far reaching.

Economic Burden of disease: OA is associated with a very high economic burden attributable to the effects of disability, comorbid disease, and the expense of treatment. OA represents the commonest cause of disability in Europe. This brings social, personal and economic burden as well as the costs in excess of €2 billion per year for the joint replacements performed annually in Europe. Even though direct and indirect per capita costs for OA have stabilized in recent years, the escalating prevalence of the disease, partly due to the increase in aging and obesity, has led to much higher overall spending for OA. [6]

Understanding the genetic contribution to OA has two important clinical implications. First, the identification of genes involved in disease risk can improve our understanding of the molecular mechanisms involved in the pathogenesis of OA. Understanding the role of these genes in disease pathogenesis (e.g. in vitro studies, in vivo studies in animal models opens the doors to therapeutic intervention.

Second, by selecting sets of genetic variants associated with risk of disease or with progression of OA, it should be possible to better define OA subphenotypes and to improve the risk assessment of OA in these various groups. Genetic variation can contribute to risk of OA and OA subphenotypes at various stages of the disease. Genetic factors have been implicated in several of the risk factors that contribute to OA such as inflammation, obesity, bone mineral density, skeletal shape, and are known to influence risk of hip OA, knee OA and generalized OA (see [7]. Furthermore, disease progression measured radiographically or as cartilage loss by MRI is under genetic control (e.g. [8-9]) and sensitivity to pain which is the major...
clinical outcome of OA is also strongly influenced by genetic factors [10].

Objectives:

The project, TREAT-OA, aimed to address the need for better treatment and diagnostics for osteoarthritis (OA). TREAT-OA exploited European excellence by bringing together teams for gene discovery with genome-wide association scans and linking these with translational research groups.

TREAT-OA had two main translational goals: the development of efficient diagnostics for risk and progression of OA and the identification of new targets for therapeutic interventions (Figure 1).

• TREAT-OA investigated the biological functions of novel genes to further our understanding of the molecular mechanisms involved in disease aetiology and to identify pathways for pharmacological intervention.
• TREAT-OA used genetic and biochemical marker information to develop better diagnostics to predict risk of OA and prognostic markers that can identify individuals more likely to have progressive OA.

The S&T objectives of TREAT-OA were:

1. Discovery of novel genes in population-based and case-control cohorts.

To find new genes involved in risk of OA using over 15,000 cases and 30,000 controls with genomewide association genotyping.

2. Generalisability and diversity of risk variants to populations across Europe

• The generalisability and diversity of discovered and known OA gene variants will be assessed by genotyping these in the different populations across Europe.
• To allow for additional generalisability towards other ethnic groups and geographic background we have sought collaboration with OA groups in Japan and China. This is relevant as some important genetic associations have been reported originally or exclusively in Asians.

3 Meta-analysis of results

Genome-wide association and other OA studies were combined using advanced meta-analysis techniques in order to identify the genetic variants that are most important for determining the risk of osteoarthritis phenotypes and to explore the consistency of the associations across different study populations and different phenotypes.

4 Finding genetic variants for biochemical markers

Biomarkers are an essential area of research in OA and arthritis as a whole, since they will help the medical community to determine: (i) Who is likely to get arthritis? (ii) Severity and progression of disease (iii) Response to drugs and which types of drugs are effective.

At present only two markers (hCOMP and CTX-II) are capable of predicting progression to a limited extent. Our research objective is to examine the value of established and novel (in the pipeline) biochemical markers of joint tissue metabolism to predict OA progression and identify genes that contribute to their systemic levels. Ultimately, this will lead to the selection of a panel of few biochemical markers to be included in as a diagnostic in the translational part of the proposal either used on their own or in combination with a larger panel of genes (STO8).
5 Functional genetics using in vitro models

• To develop in vitro models for OA that will facilitate the investigation of mode of action of the identified genes associated with OA and look into models perceived as relevant to the disease process. Towards this goal we will carry out systematic expression screens in vitro primary cells and tissues of relevance to the joint and joint associated tissues such as chondrocytes/cartilage, osteoblasts/bone, meniscal cells, and mesenchymal stem cells/synovium. We will screen the expression of the genes identified in the genome scans in the different joint-associated cell populations, including their regulation by pro-inflammatory cytokines such as TNF alfa and Interleukin-1, and eventually their interactions with the expression and/or activity of tissue destructive enzymes. The specific objectives are:
  • To develop in vitro and ex vivo cellular assays relevant to (mimicking and recapitulating) the biology of cartilage, bone and other joint tissue turn-over. We will develop models for formation, homeostasis and repair as well as mimicking OA disease (at least 5 different assays)
  • To produce tools for functional testing of relevant identified genes and proteins of interest (10-15 probe sets for expression studies. Antibodies, viral stocks and expression constructs for 3-6 Genes of Interest
  • To functionally validate the genes and proteins of interest with respect to their involvement in onset and progression of OA disease, more specifically their role in (healthy) tissue formation, degradation, turn-over, homeostasis and repair (functional data on 3-6 targets).
  • To assess the involvement of cytokines in regulating the processes described above and their involvement in the activation or inactivation of the genes and proteins of interest
  • To rank the outcomes of the functional assays - and select the most interesting genes of interest for further in vivo analysis and drug target screening as well as for development (where appropriate) of biological or diagnostic markers (3-6 targets).

6 Functional genomics using zebrafish and chick disease models of OA

The goal was to identify zebrafish orthologues of the human genes associated with OA, in order to identify signalling pathways associated with the newly discovered genes. Following the initial preselection of target genes in the zebrafish, a subset of those genes will be evaluated using developing chick limb in ovo.

7 Functional genomics using murine models of OA

• In order to test the function of selected genes in mouse models of OA TREAT-OA:
  • Systematically screened the large-scale N-ethylNitrosourea (ENU) mutagenesis resources established for mouse. These ENU resources were screened for mutations in genes identified for OA from the human studies. The likelihood of identifying mutations in these genes has been estimated to be &gt;99%. New mouse models were archived and made available to the scientific community.
  • In parallel, the roles of selected genes identified from human genetic studies were investigated using established postnatal models of osteoarthritis in mice caused by instability, direct cartilage loss or inflammation.
  • In addition, some genes of interest were investigated for their potential role in the development of OA by the generation of inducible, tissue specific (articular cartilage, bone) loss of function/knock-out mice or tissue specific overexpression using transgenic mice.

8 Translation into diagnostics

• The objective was to use the genes and biomarkers identified and confirmed by TREAT-OA to test their value as diagnostics using the largest collection of population-based longitudinal cohorts
• combinations of biochemical and genetic makers along with epidemiological risk factors in existing population-based cohorts were used to predict radiographic progression and clinical outcomes. The value of such markers as diagnostic products
capable of identifying individuals at high risk of developing OA and/or of progressing to severe OA was tested using ROC analyses

- In addition, next generation sequencing was used to identify the most likely functional variants in the genes identified which can be used to optimize the markers identified from GWAS.

9 Translation into therapeutic targets

The first objective was to set-up and validate two high throughput screens, suitable for the identification of candidate drugs, aimed against novel targets or pathways identified in previous work packages (WP1-7).

The second objective involved the set-up and validation of secondary cell assays for further profiling and optimisation of the hits identified in the assays developed.

References:


Project Results:

MAIN SCIENCE & TECHNOLOGY RESULTS

WP1

1. The main objective of this workpackage was to identify genetic variation that is strongly and consistently associated to the risk of OA.

The GWAS in individual studies were completed and data released for meta-analysis (WP3) and replication (WP2). Three individual cohorts were used with GWA. This analysis has identified a SNP in the Prostaglandin-Endoperoxide Synthase 2 gene region in one of the cohorts. This gene is associated with OA in the knee and was further analysed using a pooled GWA and subsequent replication in other cohorts. This analysis in two cohorts produced a list of top SNPs that were put forward for replication within the cohorts in WP 2.

2. For meta-analysis, the phenotypes should be as similar as possible. In order to standardize the phenotype data as much as possible, all phenotype information was been gathered for each cohort. This has been done for the cohorts in WP1 but also for
WP2. All the phenotype information has been evaluated and a definitive phenotype definition has been set for the different OA-types. A manuscript describing the TREAT-OA consortium and the standardization of the phenotypes was published in Osteoarthritis and Cartilage.

3. The results of the hip and knee OA TREATOA GWAS meta-analysis identified a novel loci in chrom 20 and 7 respectively and results from the meta analysis are described in detail in WP3.

4 Focused analysis on candidate genes and skeletal related pathways was carried out.

New tools are being developed to extract additional information from Genome-Wide Associations studies (GWAS) beyond the comparisons involving a SNP at a time. A promising area involves generating a single global statistic for each gene by combining association data from each SNP in its locus, hence called Gene-Based Analysis (GBA). This approach will be particularly useful for genes with multiple susceptibility polymorphisms of small effect and for genes with several low frequency susceptibility variants of relative high effect. However, there are still many standing questions that need to be addressed before GBA could be widely accepted. We have explored the feasibility of using GBA for the elucidation of OA genetics. We have decided to use an approach accounting for linkage disequilibrium between SNPs. This involved the use statistical tests that take into account the relation of dependence between SNPs defined by the LD structure of each gene. In addition, we choose to incorporate information about genetic recombination for the definition of each gene. Gene coordinates were taken from a human genome database (UCSC hg18). This information was combined with the deCODE recombination maps to define gene boundaries. Overlapping genes were combined in a single locus. About 2.5 million SNPs with p values were taken from a meta-analysis of 9 OA GWAS. Their positions in the genes were obtained from HapMap Phase II+III (rel28) as well as the genotypes used to obtain the pairwise r² matrix of LD in each locus. This was done with PLINK. Four different statistics were used to combine p values from all SNPs in each gene: linear combination test (LCT), quadratic test (QT), decorrelation test (DT) and Fisher-Satterwhite’s approximation test (FSA). Combination of the information about genomic coordinates of 18022 human genes and the recombination (sex-averaged) map gave 7125 non-overlapping loci. These loci contained from 1 to 61 genes with a mean of 2.5 genes. Median size of these loci was 170 Kb (IQR 90-300 Kb). First analyses showed that SNPs with minor allele frequency (MAF) below 5% disproportionally contributed to the complexity of the LD pattern and to a reduction of the power of statistics accounting for LD. Therefore, GBA was split in two parts: a first part for SNPs with MAF > 5%; 5% that accounted for LD, and a second part (not pursued here) for SNPs with MAF < 5%; 5% without accounting for LD. For the first part, 1.23 million SNPs with p values from the OA GWAS meta-analysis and MAF > 5% were contained in the genes (median = 117, IQR 60-216). A pilot study with 100 randomly chosen genes plus two known OA susceptibility loci (GDF5 and 7q22) showed that two statistics (LCT and FSA) performed reasonably well. They indentified the two known loci as the most associated. The other two statistics did not identify any of them. There are still obstacles for the use of GBA to extract more information from OA GWAS data. We have found that two separate analyses are required because the low frequency SNPs.

Results: A gene-set screen was performed by means of a meta-analysis across 8 genome wide association scans (GWAS) of the TREAT-OA populations for genes that are found to cause rare skeletal (dysplasia) syndromes. These “skeletal dysplasia genes” were selected from the international ontology and classification of skeletal disorders and from the OMIM database resulting in a set of 2766 independent SNPs across 142 genes. Results of the analyses are shown in Table 1. For the COL11A1 gene a significant association with hip OA (P-value 1.5 x10-5) was observed. COL11A1 is a minor fibrillar collagen which adds structure and strength to connective tissue.

Table 1. Results from focused analysis.

| SNPID   | gene location EA EAF OR P | rs1241164 COL11A1 intron T 8% 0.82 1.5x10-5 | rs7517682 COL11A1 intron A 57% 1.11 1.9x10-5 | rs2114325 TBX5 3’UTR (45kb) T 3% 1.39 2.7x10-5 | rs10144601 BMP4 3’UTR (50kb) T 19% 1.12 2.9x10-4 |
rs743682 FGFR3 intron A 10% 1.16 3.2x10^-4
rs11645645 CLCN7 intron T 49% 1.10 5.6x10^-4
rs2551381 TBX5 5’UTR (25kb) A 98% 0.68 7.6x10^-4
rs12551466 COL5A1 3’UTR (5kb) A 12% 1.14 8.7x10^-4
rs7933427 FLI1 3’UTR (40kb) A 5% 0.82 9.0x10^-4

The manuscript describing results was submitted to Arthritis & Rheumatism and is at the second round of revisions.

5. GWA meta-analyses of sCOMP and uCTX2 across studies of WP1 and WP2

Our objective was to identify new genetic variants involved in quantitative OA phenotypes such as the biomarkers sCOMP and uCTX2, by means of a large-scale hypothesis-free genome-wide association study (GWAS) meta-analysis. Marker levels were measured in TREAT~OA studies, 6 studies for sCOMP (N=3316) and 7 studies for uCTX2 (N=4554) (Table 2). For the discovery, genome wide meta-analyses were applied to these quantitative traits. Effects were estimated using a random-effects model. SNPs with P ≤ 1x10^-6 were replicated by de novo genotyping in the Cohort Hip and Cohort Knee (CHECK; N=964; Table 2). Furthermore, the discovered variants were tested for their association with OA across the TREAT~OA populations. In the overall analyses we found for serum COMP levels a SNP located within the mannose receptor C type 1 (MRC1) gene (rs691461, BETA = -0.15 P = 1.66*10^-12) without evidence for heterogeneity across the studies (Figure 1).

Table 2: Overview of the sCOMP and uCTX2 discovery studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>sCOMP samples</th>
<th>sCOMP SNPs</th>
<th>sCOMP Lambda</th>
<th>uCTX2 samples</th>
<th>uCTX2 SNPs</th>
<th>uCTX2 Lambda</th>
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<tr>
<td>Chingford14</td>
<td>159 536,984</td>
<td>1.002</td>
<td>159 544,135</td>
<td>1.003</td>
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<tr>
<td>GAR15</td>
<td>351 2,162,385</td>
<td>1.145</td>
<td>353 2,165,144</td>
<td>1.008</td>
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<tr>
<td>LLS16</td>
<td>620 2,267,101</td>
<td>1.033</td>
<td>639 2,225,148</td>
<td>1.026</td>
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<td>RS-I17</td>
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<td>- - -</td>
<td>1100 2,543,887</td>
<td>1.02</td>
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<tr>
<td>RS-II 116</td>
<td>2,543,887</td>
<td>1.01</td>
<td>1044 2,543,887</td>
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<td>TWINSUK18</td>
<td>754 3,044,064</td>
<td>0.987</td>
<td>1096 2,441,760</td>
<td>1.008</td>
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<td>VIDEO19</td>
<td>271 537,298</td>
<td>0.977</td>
<td>263 543,415</td>
<td>1.008</td>
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<td>Repl. Study Samples</td>
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<tr>
<td>CHECK</td>
<td>964 20 964</td>
<td>20</td>
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</table>

In addition, for uCTX2 we identified a SNP near the CUB and sushi domain-containing protein 1 (CSMD1) gene, at the suggestive evidence for genome wide significance level (rs1983474, BETA = 0.13 P = 8.45*10^-8; Figure 1). Furthermore, of note was association of sCOMP with a SNP within the sCOMP gene itself (rs10038, BETA = 0.11 P = 2.3*10^-5). The SNP appeared also significantly associated with hip OA (BETA=0.07 P=8.9*10^-3) whereas sCOMP gene expression was significantly different in OA affected cartilage as compared to preserved.
We explored whether the 6 loci identified in this study (Table 2) in addition to their association with sCOMP and uCTX2 levels were also associated with OA. For this, the TreatOA genome wide association scan dataset including all participants stratified for hip and knee OA was examined. Evidence for association was only found for rs10038 with hip OA ($p = 8.9 \times 10^{-3}; OR = 1.09$).

Next, we set out to examine gene expression of the annotated genes by exploring a microarray mRNA expression dataset of human matched OA and preserved articular cartilage obtained from 33 individuals that received joint replacement due to end stage OA (unpublished data). Very high expression of COMP was observed in cartilage tissues (relative levels of expression are within the highest quartile of the dataset; see Table 3). Furthermore, only for the COMP gene we observed significant differential expression in cartilage harvested near the OA lesion (OA affected cartilage) as compared to cartilage distal to the OA lesion (preserved cartilage; $p = 2.0 \times 10^{-3}$). In contrast, no differential expression between OA affected and unaffected cartilage was detected for the other genes tested, and relative expression of the MRC1 gene appeared low in cartilage.

OA populations across Europe. Genotyping of osteoarthritis candidate (gene) variants of the different OA populations across Europe. To allow powerful replication of OA candidate genes we have collected DNA samples from OA studies across Europe and were made eligible for genotyping at the Sequenom Mass Array genotyping platform by aliquoting into 384 well plates, entering of studies into the laboratory management system and Quality controls on DNA for its suitability for genotyping. In total we have now available around 7500 samples, 4 phenotypes across 5 different countries

Table 2: A selection of initial compelling OA susceptibility loci discovered by a combination of genome wide linkage and candidate gene approaches

<table>
<thead>
<tr>
<th>Genome wide linkage</th>
<th>Phenotype</th>
<th>Pathway involved</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>FRZB Hip OA</td>
<td>WNT signaling</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DIO2 GOA; Hip OA</td>
<td>Thyroid signaling</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Candidate gene Phenotype Pathway involved Reference

| GDF5 Hip OA | TGF-beta signaling | 3 |
| SMAD3 Hip OA | TGF-beta signaling | 7 |

WP2

Early genetic studies had provided a few compelling OA genes such as the growth and differentiation factor 5 (GDF5) gene1, frizzled related protein (FRZB) gene2-4 and the deiodinase, iodothyronine, type II (DIO2) gene5. These signals have been discovered by a combination of (discovery driven) genome wide scans and hypotheses driven candidate gene approaches. Replication of compelling OA candidate genes and/or pathways across the TREAT~OA studies were performed by de novo genotyping.
COL11A1 in an overall gene set analyses we aimed to assess evidence for association to a large number of recognized OA susceptibility candidate genes by means of a meta-analysis of 8 TREAT-OA genome-wide association scans (GWAS). A total of 186 OA candidate genes including 24298 SNPs were analysed in the TREAT-OA GWAS results. Subsequent replication by the novo genotyping in additional 5921 individuals were studied for top SNPs in the meta-analysis. Overall we found an association signal for the COL11A1 gene with study wide significance. The COL11A1 codes for one of the chains of type XI collagen that is involved in maintaining cartilage integrity and cohesion. Furthermore, these analyses showed additional evidence for the 7q22 and GDF5, were already widely recognized as truthfully involved in OA. Results are submitted for publication.

DOT1L A polymorphism in the promoter of the DOT1L gene (rs12982744) has been reproducibly associated with minimum joint space width at the hip with \( p = 1.1 \times 10^{-11} \) and also genetic susceptibility to hip osteoarthritis (OA) \( (p=10^{-4}) \) across a number of TREAT-OA studies. The latter association falls over an order of magnitude short from the currently accepted threshold for genome-wide statistical significance (GWS) \( (p<5\times10^{-8}) \). By increasing the sample size of both cases and controls with additional TREA we aimed to achieve GWS for this variant. Therefore, another 4349 hip OA cases and 46903 controls of European descent were synthesized with de novo genotyped data from the UK, Estonia and other effect sizes from the previously published report for the DOT1L polymorphism rs12982744. The total number of samples included now was 8331 hip OA cases and 61970 controls. The odds ratio (OR) for the risk (C) allele at rs12982744 was computed with a fixed effects model. The C allele at rs12982744 was associated in the combined datasets with \( OR= 1.15 \) (95% CI: 1.10-1.21) \( p=2.2\times10^{-8} \) and no between study heterogeneity was observed \( (I^2=1\%) \). The association between the DOT1L rs12982744 polymorphism and hip OA is a consistently reproduced across European populations and is genome wide statistically significant (Figure 3).

Figure 3. Forest plot of study-specific estimates and fixed-effects summary odds ratio (OR) estimates and 95% confidence intervals (95%CIs) for the association between the rs12982744 polymorphism of the DOT1L gene and hip osteoarthritis.

Replication of genome wide association scans (WP1) of OA phenotypes

GWA meta-analyses of the 7q22 locus To identify novel genes involved in osteoarthritis (OA) a genome-wide association study was performed in the Rotterdam study (WP1). In workpackage 2, SNPs that associated with at least 2 OA phenotypes were replicated across TREAT-OA cases by the novo genotyping (Figure 4). Together these analyses resulted in a novel common variant on chromosome 7q22 that influences susceptibility to prevalence and progression of OA. In a second step additional replication across the 7q22 locus was performed by the novo genotyping that resulted in a cumulative sample size of 6,709 cases and 44,439 controls and a genome-wide significant locus on chromosome 7q22 for knee OA \( (rs4730250, p-value=9.2\times10^{-9}) \), thereby confirming its role as a susceptibility locus for OA.

GWA meta-analyses on hip OA across TREAT-OA populations To identify OA susceptibility loci for hip OA, a two-stage design was used for the identification of potential associations. The variants that surpassed the \( p<1\times10^{-6} \) threshold in the discovery effort were selected for further follow-up. In silico and de novo replication was sought for these signals in 8 additional studies. In the discovery stage 5,244 cases and 17,836 controls were included, in the replication stage 5,010 cases and 17,151 controls, with in total 10,254 cases and 34,987 controls in the final meta-analysis. As shown in Table 6, at the replication stage known studies (e.g. Greek and Spanish cases) were genotyped for the relevant SNP but also new studies (such as Paprika) entered as replication study of WP2. De novo genotypings of Greek, Spanish and Paprika cases were performed at LUMC (partner 4). We have performed additional Genome-wide genotyping in a total of 336 OA-cases from the Raak-study (Leiden, LUMC, partner 4) and the Rotterdam Study (ErasmusMC, partner 3), in order to be able to study generalizability of results towards endo-phenotypes, such as the biomarker phenotypes (CTX-II and COMP) as well as joint space width (identification of DOT1L).
WP3

The objectives of this WP were to combine datasets from genome-wide association and other studies using advanced metaanalysis techniques in order to identify the genetic variants that are most important for determining the risk of OA phenotypes and to provide bioinformatic support for the Consortium.

Meta-analysis of knee OA

We performed a meta-analysis of four genome-wide association (GWA) studies of 2,371 knee OA cases and 35,909 controls in Caucasian populations. Replication of the top hits was attempted with data from additional ten replication datasets. A cumulative sample size of 6,709 cases and 44,439 controls was included in an inverse-variance fixed effects meta-analysis.

Meta-analysis of hip OA Data from 8 teams were collected and QCed for the discovery effort. We accumulated 11,277 cases of radiographic and symptomatic hip OA. A meta-analysis was performed using inverse-variance fixed effects models and eight SNPs with p-value $\lt; 5 \times 10^{-6}$ were prioritized for further follow up. Ten teams with a total of additional 6531 cases and 22595 controls provided in silico (n=4) and de novo (n=6) data on the prioritized genetic variants.

Candidate-gene approach The results of the discovery effort from the conducted meta-analyses were used for the assessment of 199 published candidate genes that were obtained from Huge Navigator for knee and hip OA.

Knee OA We identified one genome-wide significant locus on chromosome 7q22 for knee OA (rs4730250, p-value=$9.2 \times 10^{-9}$), thereby confirming its role as a susceptibility locus for OA (Figure 1). The associated signal is located within a large (500kb) linkage disequilibrium (LD) block that contains six genes: PRKAR2B (protein kinase, cAMP-dependent, regulatory, type II, beta), HPB1 (HMG-box transcription factor 1), COG5 (component of oligomeric golgi complex 5), GPR22 (G protein-coupled receptor 22), DUS4L (dihydrouridine synthase 4-like), and BCAP29 (the B-cell receptor-associated protein 29).

Figure 4. Forest plot of the association between rs4730250 and knee OA

Hip OA meta analysis: One locus, at 20q13, represented by rs6094710 (MAF 4%) near the NCOA3 (nuclear receptor coactivator 3) gene, reached genome-wide significance (GWS) level with P=7.9x10-9 and OR=1.28 (95% CI:1.18-1.39) in the combined analysis of discovery (P=5.6x10-8) and follow-up studies (P=7.3x10-4). The forest plot is shown in figure 1.

Figure 5: Forest and regional plot for the association of rs6094710 and hip OA

Moreover, two loci remained suggestive associated; rs5009270 at 7q31 (MAF 30%, P=9.9x10-7 OR=1.10) and rs3757837 at 7p13 (MAF 6%, P=2.2x10-6 OR=1.27 in male specific analysis).

WP4

The main objective of the work package 4 was to identify commercially available and novel biochemical markers measured in serum or urine that may be diagnostic and/or prognostic of OA severity and progression.

To complete this aim several practical tasks were sketched out to be completed, which is schematically illustrated in table 4.1.

Task no. Aim Status

IA Identify two commercially available biochemical markers Urinary CTX-II and serum COMP were chosen, because these
markers have in several previous studies indicated potential prognostic value, however but not before had they been measured in a sample size which the TreatOA provided.

IB Technically validate those markers. Before starting the measurement of the valuable cohort samples a technical validation of the CTX-II and COMP was commenced. Some troubleshooting was necessary for the COMP due to a faulty lot delivered from the manufacturer. However the technical validation was completed.

IC Clinical validation through measurement of the TreatOA cohort samples. All samples were measured for urinary CTX-II and serum COMP. Urinary creatinine was measured as well for correction of the CTX-II measures.

ID Transfer of raw data of CTX-II and COMP to partners. After the final measurements, the raw data was transferred to the partners (cohort owners) for further analyses including association with GWAS data and patient demographics (see other work packages).

IE Explore the role of DNA methylation as biomarkers of OA severity. DNA methylation levels were obtained using the 450k Infinium CpG methylation probe array in DNA extracted from blood from 48 OA patients with different levels of radiographic severity. After quantile normalization methylation data were tested for association with radiographic severity. Results from this small study suggest that DNA methylation levels can have strong predictive value for OA radiographic severity.

IIA Identify novel biochemical markers that would potentially have prognostic or diagnostic value (II). A novel marker that was suggested was HELIX-II developed by the Synarc lab, however after scientific analyses of the target of the biomarker it was discovered that the assay did not measure what was first indicated. Synarc lab partnered with Nordic Bioscience to get access to more novel biomarkers. It was decided that serum C2M should be measured instead in all samples as this marker had shown promise in smaller studies of joint diseases.

IIB Production of the C2M ELISA kit. The C2M kit underwent production and the quality was assessed by a technical team at Nordic Bioscience before transfer to Synarc.

IIC Transfer and technical validation of the C2M assay. As Synarc had not themselves developed and produced the serum C2M assay it was necessary to complete a full validation as done with CTX-II and COMP. The assay was technically validated.

IID Clinical validation through measurement of the TreatOA cohort samples. All samples were measured for serum C2M.

IIE Transfer of raw data of C2M to partners. After the final measurements, the raw data was transferred to the partners (cohort owners) for further analyses including association with GWAS data and patient demographics (see other work packages).

IIIA Identify novel biochemical markers that would potentially have prognostic or diagnostic value (III). So far the focus had been on markers of cartilage degradation, but it was clear from the scientific discussions that biomarkers of synovial inflammation would potentially be of great value for patient prognostics and segregation. As part of the partnership between Synarc and Nordic Bioscience it was decided that more novel biomarkers developed by Nordic Bioscience for joint diseases would be prime candidate for testing in the TreatOA consortium. Following biomarkers were proposed based on scientific publications presented by Synarc/Nordic Bioscience:

• C1M, type I collagen degraded by MMPs (connective tissue degradation, inflammation depended)
• C3M, type III collagen degraded by MMPs (synovial tissue turnover)
• CRPM, CRP degraded by MMPs (chronic inflammation)
• VICM, citrullinated and MMP degraded vimentin (a potential autoantigen)
IIIB Production of the C1M, C3M, CRPM and VICM ELISA kits The kits underwent production and the quality was assessed by a technical team at Nordic Bioscience before transfer to Synarc. Production of the C1M and VICM were the last to be completed.

IIIC Transfer and technical validation of the C3M and CRPM assays As Synarc had not themselves developed and produced these two serum assays it was necessary to complete a full validation as done with C2M, CTX-II and COMP. The assay was technically validated.

IIID Clinical validation through measurement of the TreatOA cohort samples. After technical validation and approval, C3M and CRPM was measured in the VIDEO cohort to start with, as this data set included information on synovitis, which help in evaluating the value of these novel markers. After measurement the raw data was transferred to the scientific team.

IIIE Measurement of C1M, CRPM and VICM in the ERGO+ samples The ERGO samples were transferred to the Herlev site, due to organisational changes within Synarc and Nordic Bioscience. The samples were measured for these 3 markers at the end of the last project period. After measurement the raw data was transferred to the scientific team.

CTX-II, COMP and C2M meta-analysis

A manuscript for publication has been put together by the participants of WP4 to summarize the data for CTX-II, COMP and C2M and their predictive value for OA severity and OA risk.

Manuscript abstract;

Objective: to evaluate the role of three cartilage-derived biomarkers on osteoarthritis (OA): urinary C-terminal telopeptide (uCTX-II), serum cartilage oligomeric protein (sCOMP), and serum MMP degraded type II collagen (sC2M).

Subjects and methods: Samples from 3490 individuals from the Rotterdam Study, the Genetics osteoArthritis and Progression (GARP), the Chingford Study and the TwinsUK cohort were assayed using enzyme linked immune sorbent assays. Log10 of concentration levels were correlated with risk of hip, hand and knee OA, hip and knee OA severity and incidence, and progression of knee OA, adjusting for age, gender and body mass index. Results were meta-analyzed to assess overall significance.

Results: After adjusting for covariates, sCOMP was associated with hip incidence, knee incidence and prevalent knee OA and sC2M was associated with knee incidence and progression. After adjustment for multiple tests (Bonferroni p<0.002) only the association between sCOMP and knee OA remained significant (OR= 3.26 (95%CI 1.63 - 10.1) p=0.0008). Levels of uCTX-II were significantly associated with five of the eight traits studied. The strongest association was with risk of knee OA (OR= 5.72 (95% CI 3.88 - 30.5) p=7 x 10[-19]) (see table 2 below). A receiver operating characteristics (ROC) analysis showed a consistent improvement in knee OA progression from an average area under the curve (AUC) = 0.646 for age, sex and BMI alone to an AUC=0.668 including uCTX-II for prediction.

Conclusions: uCTX-II is the most predictive biochemical marker for OA. Both sCOMP and C2M showed some association with OA, thus indicating that these are descriptive of disease activity. The use of the biochemical markers may aid in the characterization of disease combined with clinical assessment to provide improved evaluation of the individual patient.

Table 2 of the manuscript. Fixed effect meta analysis results. Odds ratios (OR) refer to a log10 unit for each one of the biochemical markers. Mutlivariate analyses are adjusted for age, gender and body mass index. Bonferroni p-value corresponds to p=0.002 values under this threshold are highlighted in bold.

Univariate multivariate
<table>
<thead>
<tr>
<th>Trait biomarker</th>
<th>OR 95%CI p-value</th>
<th>OR 95%CI p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hand OA risk C2MB</td>
<td>1.37 (0.76 - 2.49) 0.29</td>
<td>1.59 (0.91 - 2.74) 0.10</td>
</tr>
<tr>
<td>COMP</td>
<td>1.32 (0.38 - 3.34) 0.66</td>
<td>4.78 (1.46 - 16.5) 0.0097</td>
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<tr>
<td>C2MB</td>
<td>7.77 (4.09 - 55.6) 3.8E-10</td>
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<tr>
<td>COMP</td>
<td>6.87 (2.55 - 43.6) 1.4E-04</td>
<td>2.68 (0.96 - 7.36) 0.06</td>
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<tr>
<td>C2MB</td>
<td>5.75 (2.38 - 12.6) 4.3E-03</td>
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<tr>
<td>COMP</td>
<td>2.60 (0.26 - 9.86) 0.41</td>
<td>2.27 (0.18 - 8.41) 0.52</td>
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<td>C2MB</td>
<td>8.26 (4.7 - 13.2) 2.8E-07</td>
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<tr>
<td>hip OA incidence C2MB</td>
<td>1.28 (0.62 - 2.7) 0.50</td>
<td>1.69 (0.69 - 3.59) 0.25</td>
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<td>COMP</td>
<td>6.78 (3.97 - 24.6) 2.3E-12</td>
<td>3.74 (1.46 - 9.6) 0.0027</td>
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<tr>
<td>hip OA risk C2MB 1.33 (0.76 - 2.4) 0.32</td>
<td>5.12 (0.12 - 2.0) 0.23</td>
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<tr>
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<td>1.42 (0.43 - 3.47) 0.0423</td>
<td>1.95 (1.16 - 3.2) 0.0117</td>
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<td>3.07 (1.74 - 5.4) 1.7E-04</td>
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<tr>
<td>hank OA incidence C2MB</td>
<td>9.92 (3.12 - 98.7) 1.0E-04</td>
<td>5.85 (1.74 - 32.0) 0.0043</td>
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<tr>
<td>C2MB</td>
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<td>1.74 (1.14 - 3.0) 0.0104</td>
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<tr>
<td>C2MB</td>
<td>0.0042</td>
<td>1.69 (1.05 - 2.87) 0.0311</td>
</tr>
<tr>
<td>Knee OA C2MB 1.94 (1.23 - 3.69) 0.0042</td>
<td>1.69 (1.05 - 2.87) 0.0311</td>
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<tr>
<td>Progression COMP 10.98 (3.99 - 109.) 3.5E-06</td>
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<td></td>
</tr>
<tr>
<td>C2MB</td>
<td>1.00 (1.00 - 1.00) 1.0E-00</td>
<td></td>
</tr>
<tr>
<td>C2MB</td>
<td>12.13 (6.36 - 33.3) 3.6E-14</td>
<td>3.26 (1.63 - 7.3) 8.0E-04</td>
</tr>
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<td>C2MB</td>
<td>10.02 (7.01 - 91.4) 7.2E-37</td>
<td>5.72 (3.88 - 30.5) 7.0E-19</td>
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<td>Knee OA severity C2MB 0.93 (0.52 - 1.79) 0.82</td>
<td>0.75 (0.4 - 0.82) 0.37</td>
<td></td>
</tr>
<tr>
<td>C2MB</td>
<td>5.96 (2.53 - 33.1) 4.5E-05</td>
<td>1.45 (0.57 - 3.2) 0.43</td>
</tr>
<tr>
<td>C2MB</td>
<td>9.23 (5.61 - 77.8) 1.4E-18</td>
<td>5.72 (3.33 - 30.5) 2.6E-10</td>
</tr>
</tbody>
</table>

**PATENTABLE FINDINGS.**

We have no direct patentable findings within WP1, WP2, WP3, or WP4 since the direction of effect and the functional impact of the genes identified should be elucidated in order to allow such results. However, the results obtained within WP2 generated a selection of robust OA susceptibility genes to enter the functional work packages of TREAT–OA that have established complex biological activities of some that now allows the discovery of new druggable targets that counteract the detrimental effects of OA-genes.

**Reference List**


Workpackage 5: Summary report

Introduction. Workpackage 5 was specifically defined within the TREATOA project to bridge the gap between novel genetic findings and further in vivo studies addressing specific susceptibility genes and their pathways. Two main objectives were put forward:

1. To develop in vitro models for OA that will facilitate the investigation of the potential mode action of the identified genes associated with OA and look into models perceived as relevant to the disease process.
2. To systematically screen in vitro mostly primary cells and tissues of relevance to the joint and joint associated tissues such as chondrocytes/cartilage, meniscal cells, osteoblasts/bone and mesenchymal stem cells/synovium.

Summary of the results

Different in vitro models that allow the study of molecular processes involved in osteoarthritis pathogenesis have been set-up or optimized.

1. Cell cultures of primary human joint cells including: human chondrocytes, human cartilage explants, human meniscal cells (fibrochondrocytes), human synovial fibroblasts (mesenchymal stem cells) and human periosteal cells.
2. High-density micromass cultures of articular chondrocytes. This model allows us to compared gene expression and protein levels of molecules of interest in a tissue-like 3D setting and compare these levels to monolayer cultures in which the cells
typically show progressive dedifferentiation.

3. High-density chondrogenic differentiation cultures using primary periosteal cells. This model has been optimized as a high-throughput assay for chondrogenesis after stimulation of periosteal cells with a chemically defined medium and growth factors TGF-β1 or BMP2.

4. High-density micromass cultures of murine ATDC5 cells. This model mimics different steps in chondrogenic differentiation including final hypertrophy. From this perspective it is relevant as a chondrogenesis model as well as an osteoarthritis in vitro model. This cell line is easily transfected and can therefore be used to establish stable gain or loss-of-function clones for a gene of interest.

5. Gain or loss of fuction stable clones for genes of interest in osteoblastic MC3T3 cells, an important tool taking into account the importance of bone cells in osteoarthritis within the bone-cartilage biomechanical unit of the joint.

6. Optimization of a real-time quantitative PCR approach for allelic differentiation applicable to different cell types and species.

In task 5.1 we studied the localization and expression of genes of interest in different species and models. For this work we have specifically focused on genes and pathways identified by investigators within the TREATOA consortium. More in particular, FRZB, GDF5, DIO2, GRB10, SMOC2, BID, NEK7, DUSP6, ANP32A, DOT1L and the genes clustering at C7q22-q31 (GPR22, COG5, HBP1, DUS4L, PPKAR2B, BCAP29) have been detected at low or moderate levels in joint or joint associated cell populations including articular chondrocytes, meniscal fibrochondrocytes and synovial fibroblasts. Amongst these genes, DIO2 and to a lesser extend SMOC2 expression diminishes with dedifferentiation towards a more fibro-chondroprogenitor-like stage.

We have specifically demonstrated the presence of GPR22, ANP32A and DOT1L in joint tissues obtained from mice with or without induced osteoarthritis and in the developing skeleton. GPR22 is absent in the normal articular cartilage, but can be found when the joint is challenged with papain or mBSA. In addition, GPR22 is also found in the developing osteophyte. ANP32A is present in healthy and osteoarthritic cartilage. Damage to the cartilage seems to increase the number of ANP32A positive cells. ANP32A positivity is also found in osteophytes. DOT1L is strongly expressed in chondrocytes.

In task 5.2 we focused on “gain of function” or “loss of function” approaches for genes of interest.

1. The different teams have a longstanding interest in Wnt signaling in osteoarthritis, mostly focused on FRZB, a secreted Wnt modulator that was originally identified from a chondrogenic extract of articular cartilage. Microarray analysis of cartilage in wildtype and Frzb KO mice suggests that distinct clusters of genes associated with either the immune systems or cell cycle are differentially expressed. Further analysis of the microarrays has lead to new insights into the role of FRZB in the articular cartilage. Different online bioinformatics tools were used for analysis of the large dataset with emphasis on the identification of pathways that appear to be differentially regulated between the Frzb-/- and wild-type mice. Among the upregulated pathways integrins, cell adhesion, Wnt signalling are most striking from a biological perspective. From a biological perspective, detailed analysis of Wnt pathway components identified (compensatory) upregulation of different other extra- and intracellular Wnt antagonists. RT-PCR showed significant upregulation of sFRP1 and sFRP2. For Dkk2 we saw a trend of upregulation. This compensatory upregulation of the antagonists might result in the same activity status of the WNT pathway in wild-type and Frzb-/- mice. Besides the WNT pathway, also some ligands and receptors from the bone morphogenetic protein (BMP) pathway, which is also important in bone development and homeostasis and linked to the WNT pathway, were significantly upregulated. All together these data support the view that homeostasis in the articular cartilage is tightly regulated by balances between different signaling cascades.

2. Loss of function studies with COG5 demonstrated that COG5 loss negatively affects chondrogenesis and osteogenesis. COG5 particularly affected glycosylation processes and Wnt signaling, underlining its critical role in the fate of cells from the articular chondrocyte-bone unit in the course of OA.

3. Overexpression of GPR22 blocks molecular events in the proliferative phase of chondrogenesis, but enhances those from the hypertrophic and mineralization phases. This underlines a critical role for GPR22 in the fate of chondrogenic cells, and may be of interest regarding the hypertrophic switch of articular chondrocytes in the course of OA.

4. Immunohistochemical staining of the DOT1L protein in mouse limbs supports a role for DOT1L in chondrogenic...
differentiation and adult articular cartilage. DOT1L is also expressed in OA articular chondrocytes. Silencing of Dot1l inhibited chondrogenesis in vitro. Dot1l knockdown reduces proteoglycan and collagen content, and mineralization during chondrogenesis. In the ATDC5 chondrogenesis model system, DOT1L interacts with TCF and Wnt signaling.

In task 5.3 we specifically looked at the regulation of genes of interest by environmental factors such as cytokines. GDF5, DIO2 and SMOC2 expression is downregulated in conditions that simulate OA (IL1β and TNFα treatment). Therefore, these genes are further explored in in vivo models (see WP7). PRKAR2B expression was found to be upregulated in response to IL1β and TNFα in articular cartilage, but not in meniscal cells. Two genes (DIO2 and PRKAR2B) respond significantly to stimulation with IFNγ in articular and meniscal cartilage. The former gene is downregulated and the latter upregulated in response to this stimulus. Smoc2 shows also a downregulation in articular cartilage upon exposure to IFNγ, albeit to a lesser extend. High-density micromass cultures showed an up-regulation of the transcription of COG5, DUS4L, HBP1 and BCAP29 and a down-regulation of GDF5 in a 3D environment as compared to the monolayer culture in vitro, suggesting a role in cartilage development / homeostasis. In cartilage explants, there was only minor up regulation of GDF5, DIO2 and COG5 (≤ 2-fold), HBP, DUS4L and GRP22 were moderately upregulated (3-6 fold) and PRKAR2B was significantly upregulated (15 fold) upon stimulation with IL-1β and TNF. Under these conditions only SMOC2 was shown to be extensively downregulated (50 fold).

Finally, correlation of SNPs associated with osteoarthritis with variation in expression in the deCODE expression database was studied.

Although the eQTL dataset at deCODE Genetics utilizes expression data from tissues that are not of highest relevance to osteoarthritis (blood and adipose) it can provide evidence pointing to particular transcripts likely to be mediating the association signals. Three of the 19 identified candidate genes for OA at the 14 loci that associate with OA at a genome-wide significance level show evidence of correlation with the OA associated SNP and are either the strongest cis-variants, or good surrogates thereof, for those genes. These are the BCAP29 gene at the 7q22 locus, and the SUPT3H and GLN3 genes.

Transcript levels of the GDF5 candidate gene are not correlated with the OA associated SNP, whereas, levels of UQCC and EIF6, also located at this locus, are significantly correlated with genotypes of the OA associated SNP. Additionally, the IFRD1 gene is a possible OA eQTL gene at the 7q31 locus that is suggestively associated with hip OA.

Patentable results

KU Leuven filed a patent on “inhibitor of calcifying disorders”.

WP6

The initial goals of the WP6 were:

1. to develop zebrafish and chick gain of function and loss of function models for OA related genes and pathways identified by human genetic approaches.
2. to identify zebrafish orthologues of the human genes associated with OA to identify signaling pathways associated with the newly discovered genes (WP1-4).
3. to investigate, in vivo, the function of those genes in skeletal development and synovial joint formation by developing a chick model. Following the initial preselection of target genes in the zebrafish, a subset of those genes will be evaluated using developing chick limb in ovo.

Summary of progress

During the last reporting period we have explored in depth the LOF and GOF phenotypes for GPR22 and PRKAR2B. We discovered that deregulation of GPR22 leads to defects in cilia structure. Indeed, overexpression of Gpr22 results in
lengthening while downregulation in shortening of the cilia. This appears to be caused by the effect that Gpr22 might have on the phosphorylation status of ciliary proteins. This hypothesis will be further tested by the analysis of the phosphorylation status of cilia proteins using phosphospecific antibodies and eventually mass spectrometry for the phosphorylated protein. For the PRKAR2B gene, we discovered that partial inactivation of the gene results in defective neural tube development. We focused our work on linking PRKAR2B to PKA signaling and a manuscript has been submitted describing our work. We have also continued our studies on the function of Smoc2. Downregulation of Smoc2 in the zebrafish model revealed that Smoc2 is important for hematopoiesis and the development of the myeloid lineage but it is not involved in lymphopoiesis.

WP 6.1 Zebrafish model: gain and loss of function experiments

During the final reporting period we focused our effort on three genes and the zebrafish model since the information gained from our latest experiments revealed that while it is justified to continue the zebrafish work, as proposed in the original grant application, the current result did not merit further work in the chick model system (see chick package description further on).

PRKAR2B

This gene encodes a negative regulatory subunit of Protein Kinase A (PKA) and in the zebrafish is represented by the prkar2aa homologue. We examined the mRNA prkar2aa expression pattern by whole mount in situ hybridization. prkar2aa expression was initially ubiquitous, though at 16 somites higher transcript levels were observed in the posterior notochord and tail. At 22 hpf, transcripts were mainly restricted to the head and notochord and at 35 and 48 hpf, prkar2aa transcription was observed exclusively in the head and notochord.

Studies of gene function using morpholino-based silencing approach showed that at 24 hpf, prkar2aa morphants were normal. During the later stages of development however prkar2aa morphants had a phenotype, first distinguishable around 36 hpf. Morphant embryos exhibited a thin notochord, flattened somites and fail to elongate along the anterior-posterior axis. During differentiation, notochord precursor cells become either vacuolated and reside in the center of the notochord or epithelial, forming the peri-notochordal basement membrane (PBM), surrounding the notochord. The PBM is composed of three layers; the outer and the middle layers consist mainly of collagens while the inner is rich in laminin. All layers formed normally in morphant PBM. The middle layer however appeared to contain an increased number of collagen fibers. Morphant vacuoles were notably smaller, suggesting that notochord vacuole forming cells were specifically affected by prkar2aa knockdown.

Next we wanted to examine how prkar2aa inactivation affected PKA signaling. We hypothesized that if prkar2aa knockdown resulted in increased PKA signaling, embryos injected with a subphenotypic amount of morpholino would become sensitized to a treatment with a low dose of the PKA activator Forskolin. Control embryos and embryos injected with a subphenotypic dose of prkar2aa morpholino were exposed to a low concentration of Forskolin from 25 hpf onwards. At 54 hpf, morphant embryos treated with Forskolin were significantly shorter than both control embryos exposed to Forskolin alone or untreated morphants.

The relatively mild prkar2aa morphant phenotype confirms that PKA activity is only partly affected by the morpholino. This could be explained by partial gene silencing, and likely by redundant functions of different PKA regulatory subunits. prkar2aa is ubiquitously expressed at early stages, though when the phenotype develops, its transcription is restricted to the notochord.

GPR22

G-Protein coupled receptor 22 (GPR22) is an orphan receptor with unknown target. The work on GPR22 focused on the mechanism of action by which the deregulation of GPR22 expression levels could affect the length of the cilia in Kupffer’s vesicle (KV). We showed previously that deregulation of Gpr22 affected cilia length and now we have focused on the molecular basis of this phenotype. We did carry out ultrastructural analysis of the cilia. While the work is ongoing, the current results
indicate that the normal 9+0 structure in the cilia of KV is affected in the morphant fish. Specifically the number of axonemes is reduced to 7.

Further investigation revealed that Gpr22 protein was present in the ciliary fraction of the CL4 cell line supporting our notion that Gpr22 might have a very specific function in the cilia proper rather than in other parts of the cell.

The master gene regulating ciliogenesis is the transcription factor FoxJ1 which is, in turn regulated by FGF/Wnt signaling network. We could show that neither FoxJ1, nor Wnt was affected by the deregulation of Gpr22 since the expression pattern of Fgf8 or Wnt8a was not affected either by the loss or gain of function of GPR22 suggesting that GPR22 regulated signaling is independent of the currently known main players regulating cilia formation. Deregulation of GPR22 induces defects in other cilia in zebrafish as well. For instance the formation of otholites, uniquely dependent on the presence of function cilia, was defective in the GPR22 morphants. Other ciliated cells present in neuromast did show defects as well. Downregulation of GPR22 resulted in a reduced number and poor migration of these cells as visualized by the vital stain for FM1-43- the specific marker of neuromasts.

SMOC2

We noticed during the earlier stages of the work that smoc2 morphants had defects in the formation of blood islands. We have therefore focused on the analysis of the hematopoetic lineage. The expression of runx1, a marker for the early erythropoetic stem cell was significantly reduced in the morphants (red arrows) while the expression of gata1 (erythroid precursors) was reduced (Q-PCR data).

Next, we investigated different hematopoetic lineages by analyzing the expression of acknowledged marker genes for these differentiation processes. The expression of mpo (heterophil granulocytes) and l-plastin (macrophages) was reduced. We have also investigated the vascular development and blood flow using transgenic reporter lines flk1 and flt4 respectively. The blood vessel formation proceeded normally in the smoc2 morphants but the blood flow was severely impaired. Using the erythrocyte reporter line, we could show that the erythrocytes were not evenly distributed but remained in two areas of the embryo, around the heart and around the site of the initial synthesis suggesting that the blood flow was absent in the morphant.

Our current data thus suggest that smoc2 morphants have no hemangioblasts and hematopoetic stem cells at 5-9 somite stage. Interestingly, while the myeloid lineage is defective, there are no apparent defects in lymphopoiesis. Also the blood flow is severely impaired suggesting a defect in the pumping function of the heart.

Wp. 6.2. Genetic models in zebrafish

In order to generate in vivo models for most of the Cog5 cluster genes, we used two different methodologies to establish mutant zebrafish lines. Using the TILLING method, we have generated four mutant alleles in three of the Cog5 cluster genes. Specifically, these are:

Gpr22a P339S
Gpr22a K307M
Cog5 W375R
Hbp1 T337M.

All mutants were analyzed in different transgenic backgrounds, in order to allow visualization of chondrocytes and osteoblasts. We have analyzed possible premature osteoblast differentiation and premature collagen type switching. Furthermore, and since all available mutant alleles proved to be homozygous viable, we have carried out a detailed analysis of adult mineralization patterns. However, up to now none of the assays has allowed to single out one of the candidate genes as
being the one most likely to be causatively linked to osteoarthritis-like features. We are maintaining homozygous mutants for extended time periods to allow further analyses at later stages of life.

In addition, and using the recently developed TALEN technology, we have generated a deletion mutant for Gpr22a. We focused on this gene at a late stage of the project (end of 2012/beginning 2013) based on the consensus within the consortium that Gpr22a constitutes the most interesting gene for several reasons including in vitro studies of all candidates. The mutant is still being analyzed, as it is possible that a late-onset phenotype will develop.

WP 6.3 Chick model

We have invested experimental time into setting up the chick model for the electroporations in order to deliver one of the genes of interest (Cog5) using the lentivirus delivery system. The reason not to use RCAS in that case was that the size of the insert exceeded the permitted 2.2 kb limit.

In parallel we have investigated the consequences of the overexpression of Smoc2 in the developing chick limb.

The experiments revealed that overexpression of Smoc2 in chick limb did not result in a detectable phenotype. We checked subsequently for the expression of Smoc2 using a newly generated construct with HA tag added to the C terminal part of Smoc2. While we could detect low levels of Smoc2 in the lysates of chick DF1 fibroblasts, we could not detect the protein in the limb extracts. This was possibly the reason why we did not detect a phenotype. Several attempts have been made to rectify that situation but we did not succeed in overexpression Smoc2 from the RCAS vector at any significant level. Consequently we have decided to clone Smoc2 into the lentiviral vector, known to have high expression levels, and deliver it using electroporation to the chick limb. This work will continue beyond the TREA-OA project.

We have cloned GPR22 into the RCAS vector but, due to the vector instability induced by the presence of the insert containing GPR22, we did not succeed in generating a construct that would have a nonmutated GPR22 in the vector. Consequently we have abandoned this strategy and will move to the lentivirus based approach. The other genes were not pursued in the chick model system since the zebrafish data did not warrant this approach- as stated in the initial grant proposal. We have however, in the anticipation of positive results from zebrafish, designed and generated the shuttle vectors containing the miRNA silencing constructs for several genes. These constructs, if necessary, will be used in the future studies of gene function in the chick skeletal development.

Highlight clearly significant results

- PRKAR2B affects the formation of vacuole forming cells within the notochord
- GPR22 does not affect ciliogenesis by interfering with FGF or Wnt signaling but rather has a direct effect of the phosphorylation status of the proteins involved in cilia genesis
- SMOC2 is associated with hematopoiesis during zebrafish development
- Zebrafish mutant lines generated

WP7

Introduction. Workpackage 7 was specifically defined within the TREATOA project to understand the role of novel genetic findings in in vivo mouse models. Two main objectives were put forward:

1. To test the role of selected genes in osteoarthritis in mice and to develop new genetic mouse models for OA that will facilitate the investigation of the pathogenesis of OA and provide models in which novel therapeutic targets can be tested.
2. To screen the large-scale random N-ethylNitrosourea (ENU) mutagenesis resources established for mouse (WP7.1) and test
selected genes in established postnatal models of OA triggered by instability, direct cartilage damage or inflammation in mice. (WP7.2).

Summary of the results. The Oxford group performed mutation scanning of the mouse ENU DNA archive. We initially chose to study genes which have been shown to be associated with OA: Growth and differentiation factor 5 (Gdf5) (Miyamoto et al., Nat Genet. 2007. 39:529-33), Cartilage intermediate layer protein (Clip) (Valdes et al., Arthritis Rheum. 2004. 50:2497-507) and Asporin (Aspn) (Kizawa et al., Nat Genet. 2005. 37:138-44) or genes which have been identified as genes which may be associated with OA: Secreted modular calcium-binding protein 2 (Smo2) (Vannahme et al., Biochem J. 2003. 373:805–814) and lodothyronine-deiodinase type 2 (Dio2) (Meulenbelt et al., Hum Mol Genet. 2008. 17:1867–1875). A genome-wide association study identified an osteoarthritis susceptibility locus on chromosome 7q22 (Kerkhof et al., Arthritis Rheum. 2010) which contains six genes: G protein-coupled receptor 22 (Gpr22); protein kinase, cAMP-dependent, regulatory, type II, beta (PRKAR2B); HMG-box transcription factor 1 (HBP1); component of oligomeric golgi complex 5 (COG5); dihydrouridine synthase 4-like (DUS4L); and B-cell receptor-associated protein 29 (BCAP29). The archive has been screened for mutations of Gpr22, Bcap29 and Hbp1, Prkar2b and Cog5. Mutations (nonsense and/or missense) were found in every gene screened (10 genes, 1-5 mutations per gene).

We have resurrected ENU mouse models for OA – Growth differentiation factor 5 (Gdf5), Cartilage intermediate layer protein (Clip), lodothyronine-deiodinase type 2 (Dio2), G protein-coupled receptor 22 (Gpr22), and component of oligomeric golgi complex 5 (Cog5).

The Leuven group has previously studied two genes in which polymorphisms were identified as susceptibility factors for osteoarthritis: frizzled related protein (FRZB) and growth and differentiation factor 5 (GDF5) (Loughlin et al. PNAS 2004; 101(26): 9757-9762) (Miyamoto et al. Nature Gen 2007; 16(18):2226-2232). We have demonstrated that Frzb-/- mice show increased cartilage damage in induced models of osteoarthritis (Lories et al., Arthritis Rheum 2007; 56(12):4095-103). The gain of function experiment using adenovirus FRZB have failed to inverse the phenotype. Surprisingly, overexpression of FRZB in an inflammation model increases synovitis and exsudate in the joint.

Further experiments into the Wnt signaling pathway and the role of Wnt antagonist FRZB include osteoarthritis models in Lrp5-/ mice. These animals lack an essential Wnt-coreceptor. Lrp5-/ mice have an increased susceptibility for cartilage damage in different induced models of OA. This resulted in a report published in Rheumatology.

The brachypodism mouse (A/J Gdfbp-J) has been backcrossed from the A/J background onto the CD1/swiss background. Thereby, we have established a new transgenic mouse model (CD1 Gdf5+/bp-J mice) in which the papain, collagenase and meniscal instability model have been induced. In addition, gait, bone density and muscle composition were studied. The data support a role for GDF5 in joint biology affecting cartilage, joint stability and subchondral bone. Data indicate that the haploinsufficiency is also associated with distorted collagen fibers in the bone but cannot be directly linked to increased cartilage damage in different models. These data have been published in Annals of the Rheumatic Diseases. Miyamoto and co-workers demonstrated that a functional polymorphism in the 5’ UTR of GDF5 is associated with susceptibility to osteoarthritis. In a screen of the ENU mutagenesis resource we identified a A/G polymorphism in the 5’ UTR of Gdf5. Luciferase reporter assays show that the Gdf5 promoter variant luciferase construct increases expression compared to wild-type whilst the human variant known to be associated with OA susceptibility decreased expression. The Gdf5 5’ UTR polymorphism mouse was rederived and the mice were aged for six months to assess for OA development. Mutant mice were found to excrete a decreased amount of the cartilage breakdown product, CTX-II, compared to their normal littermates suggestive of a protective effect.

In addition, the Dio2 gene, GPR22 (from the chr 7 cluster) and ANP32A genes have been identified by the geneticists as susceptibility genes for osteoarthritis (Meulenbelt et al. Hum Mol Genet. 2008 Jun 15;17(12):1867-75) (Kerkhofs et al, Arthritis Rheum. 2010 Feb;62(2):499-510) (Valdes et al, Arthritis Rheum, 2009 Jul;60(7):2046-54).
Dio2-/- mice have been obtained from Dr. Douglas Forrest (NIH) and after backcrossing into the appropriate mouse strains, were tested in the different induced mouse models of osteoarthritis that are available in our group. A phenotypical analysis of the skeleton is performed and Dio2 deficiency results in delayed closure of growth plates in the long bones in the outbred strain. In addition the effects of Dio2 deletion on senescence of cells and epigenetic regulatory mechanism are investigated in collaboration with TREATOA partner Leiden (I. Meulenbelt & P. Slagboom). We have established a treadmill running protocol and collected a number of samples for epigenetic and transcriptomic analysis. In addition a colony of mice is further studied for effects of Dio2 deletion on aging and spontaneous osteoarthritis development. These experiment were performed up to the age of 12 months. Samples for epigenetic and transcriptomic analyses have been obtained and forwarded to the partners in Leiden. Their current dataset and our in vivo data suggest the presence of a small and unique gene signature associated with forced exercise and Dio2 loss of function. In addition, different models of OA have been performed in the Dio2 ko mice and are currently in the final stages of analysis. Absence of Dio2 appears to result in discrete protection against OA, resulting in reduced severity scores. A strategy to develop inducible cartilage specific Dio2 transgenic mice has been developed in collaboration with Dr. V. Lefevre (Cleveland, USA) and Dr. A. Zwijsen (KU Leuven) and Dr. J. Haigh (U Gent). The targeting construct was developed in collaboration with DIO2 experts in Harvard, USA (Dr. P. Reed Larsen). Embryonic stem cell recombination was successful and blastocysts injection will be performed in september 2013.

Anp32A-/- were obtained from P. Opal (Northwestern). Adenoviral overexpression of Anp32a does not result in changes in the normal healthy knee joint of mice. The Anp32A-/- mice are currently also tested in the different induced models of OA and analyses are in their final stages.

In collaboration with the Thakker team we obtained a Cog5-/-, a Gpr22-/- mouse and raised ENU mutated GPR22 mice. We also obtained a mutated Dot1l allele that was further bred to obtain a floxed allele of Dot1l in mice useful for conditional and tissue selective deletion. These mice are now all available in the lab and resulted in novel (funded) projects that extend beyond the duration of TREATOA.

Smoc2-/- mice have been generated by the KU Leuven but have an early developmental phenotype leading to intrauterine death. For the in vivo model experiments heterozygotes are currently used in novel (funded) projects.

WP8

The objective of this WP was to assess the value of such markers as diagnostic products capable of identifying individuals at high risk of developing OA and/or of progressing to severe hip and knee OA. We carried out receiving operator characteristic (ROC) analyses to assess the diagnostic value of biomarkers derived from WP4 and genetic markers derived from WP1-3 for predicting risk of OA or of OA severity or progression. The manuscript that combines all factors for risk of developing OA is currently in press at the Annals of the Rheumatic Diseases. The role of individual biomarkers and of combined genetic markers was also assessed. In addition, whole genome sequencing was carried out aiming to identify functional variants that would improve risk prediction. All the objectives of the WP were accomplished.

SEVERITY STUDY OF KNEE OA

Three independent genetic variants (rs143383, rs4730250 and rs11842874 mapping to the GDF5, COG5 and MCF2L genes) have been implicated reproducibly and convincingly in the risk of knee OA with genomewide statistical significance (p<5x10^[-8]). We assessed the role of these variants in determining patellofemoral and tibiofemoral Kellgren Lawrence (K/L) grade in knee OA cases. 3474 knee OA cases from the UK with sky-line and weight-bearing antero-posterior knee X-rays were selected based on the presentation of K/L grade ≥2 at either the tibiofemoral or patellofemoral compartments for one or both knees. Patients were genotyped for the above three variants. The association between tibiofemoral K/L grade and patellofemoral K/L grade was assessed in each cohort adjusting for age, gender and body mass index. Regression coefficients
were then meta-analysed. No significant association was found between the rs4730250 and either of the severity traits. The rs11842874 mapping to MCF2L was found to be nominally significantly associated with patellofemoral K/L grade (β=0.09 (95% CI 0.01-0.17) p=0.027). The GDF5 SNP rs143383 was found to be associated with tibiofemoral K/L grade (β=0.05 (95% CI 0.02-0.08) p=0.0011). This association was statistically significant independently in the case control-cohorts and in the population based cohort. These data indicate that within individuals affected by radiographic knee OA, the genes implicated in risk of knee OA have a modest but significant effect on radiographic severity after adjustment for the major risk factors. This is not confounded by the role of genes in defining risk of knee OA since we have selected only individuals affected with knee OA and in sub-analysis, only those affected with tibiofemoral knee OA. Our data show a continuous effect between gene dosage, and presumably via the reduced expression of the chondroprotective factor, the extent of joint damage detected by X-rays. The observation suggests that chondroprotective growth factors may be of therapeutic use to prevent progression of joint damage in incident OA cases.

RISK PREDICTION USING BIOMARKERS ONLY

Fixed effect meta analysis results were also carried for biochemical markers, as described under the report for WP4. p=0.002 values under this threshold are highlighted in bold. Both COMP and CTX2 are significantly associated with risk of knee OA. CTX2 was consistently associated with progression of knee OA. The summary AUC for all cohorts is shown in Table 8.2:

Table 8.1. Receiver Operating Characteristics (ROC) analysis in four study cohorts for the use of uCTX2 levels in predicting progression of knee OA. The area under the curve (AUC) for three models is shown for all four study cohorts. Model 1 includes only age, gender and BMI. Model 2 includes only uCTX2 and Model 3 includes age, gender, BMI and uCTX2.

Model 1 Model 2 Model 3
age+BMI +(sex) uCTX2 age+BMI +(sex)+ uCTX2 AUC dif.
cohort AUC 95% CI AUC 95% CI AUC 95% CI
Rotterdam 0.696 (0.65 - 0.75) 0.607 (0.55 - 0.67) 0.706 (0.66 - 0.76) 0.010
GARP 0.636 (0.56 - 0.71) 0.628 (0.56 - 0.7) 0.672 (0.6 - 0.74) 0.037
Chingford 0.657 (0.62 - 0.7) 0.601 (0.56 - 0.65) 0.669 (0.63 - 0.71) 0.012
TwinsUK 0.599 (0.53 - 0.67) 0.615 (0.55 - 0.69) 0.626 (0.56 - 0.7) 0.027
avg 0.64692 0.668299 0.021

COMBINED GENETIC AND BIOCHEMICAL MARKERS FOR RISK OF KNEE AND HIP OA:

RISK PREDICTION USING GENES

Table 8.2 Genetic variants used to generate the genetic risk score for diagnostics in WP8

Gene SNP DNA change trait associated p-value in Caucasians RAF2 controls risk

allele hip gene knee gene
GDF5 rs143383 T/C knee OA 8 x10–9 61.9% T - +
COG51 rs4730250 A/G knee OA 9 x10–9 19.3% G - +
MCFL2 rs11842874 A/G Hip or knee OA 9.2 x10 –9 91.5% A + +
PTHLH1 rs10492367 A/C Hip OA 1.5 x10–8 21.0% A + -
SPT3H rs10948172 G/A Hip / knee OA in men 7.9 x10–8 28.6% G + +
TP63 rs12107036 A/G TKR in women 6.7 x10–8 53.6% G - +
FILIP11 rs9350591 C/T THR 2.42 x10–9 10.9% T + -
GLN31 rs11177 A/G Hip or knee OA 7.24x10-11 39.0% A + +
DOT1L rs12982744 C/G Min jsw and hip OA 1.1 x 10-10 60.2% G + -
ASTN2 rs4836732 C/T THR 6.1 x 10-10 49.7% C + -
FTO rs8044769 C/T TKR in women 6.8 x 10-8 51.3% C - +
CHST11 rs835487 A/G THR 1.6 x 10-8 34.0% G + -

Case control study of hip and knee OA incorporating genes. The top hits from published and submitted GWAS and from the most recent WP3 meta-analysis have been genotyped in the above listed samples. The first genotypes have been delivered and have been analysed in the GOAL study: GOAL study 770 controls vs 1615 knee OA cases (K/L&g;=2) independent from all discovery cohorts. Controls are free

Knee OA: The diagnostic ability of combined genetic (Table 8.2) and biochemical markers (sCOMP and uCTX2) on risk of knee OA for the Chingford cohort were: age+BMI = AUC [95% CI] for the model 1: 0.673 [ 0.63 - 0.716 ] age+ BMI+genes+ biomarkers = AUC [95% CI] for the model 2: 0.706 [ 0.663 - 0.748 ] The inclusion of genetic and biochemical markers to age and BMI raises the area under the curve (AUC) from 0.673 to 0.706.

Hip OA: The diagnostic ability of combined genetic and biochemical markers (uCTX2) on risk of hip OA is for the TwinsUK cohort was age+BMI = AUC [95% CI] for the model 1: 0.717 [ 0.63 - 0.805 ] age+BMI + hip genetic risk + biomarkers= AUC [95% CI] for the model 2: 0.76 [ 0.676 - 0.844 ]

To put these results in context, AUC values for a diagnostic/prognostic test from 0.5 to 0.7 represent low accuracy, values from 0.7 to 0.9 represent tests that are useful for some purposes, and values &gt;0.9 represent diagnostic/prognostic tests with high accuracy (Wians FH Clinical Laboratory Tests: Which, Why, and What Do The Results Mean? Lab Med; 2009, 40: 105-113) By including biochemical and genetic markers it is possible to reach levels of some clinical utility in the prediction of risk of knee and hip OA

ANALYSIS OF PREDICTIVE VALUE OF BIOMARKERS AND GENETIC MARKERS ON PROGRESSION OF OA

We then performed a ROC analysis on CTX-II and knee OA progression. An example ROC curve for knee OA progression for the Chingford cohort is shown:

The summary AUC for all cohorts was:

Receiver Operating Characteristics (ROC) analysis in four study cohorts for the use of uCTX2 levels in predicting progression of knee OA. The area under the curve (AUC) for three models is shown for all four study cohorts. Model 1 includes only age, gender and BMI, Model2 includes only uCTX2 and Model 3 includes age, gender, BMI and uCTX2.

Model 1 Model 2 Model 3

age+BMI +(sex) uCTX2 age+BMI +(sex)+CTX2 AUC dif.
cohort AUC 95% CI AUC 95% CI AUC 95% CI
Rotterdam 0.696 (0.65 - 0.75) 0.607 (0.55 - 0.67) 0.706 (0.66 - 0.76) 0.010
GARP 0.636 (0.56 - 0.71) 0.628 (0.56 - 0.7) 0.672 (0.6 - 0.74) 0.037
Chingford 0.657 (0.62 - 0.7) 0.601 (0.56 - 0.65) 0.669 (0.63 - 0.71) 0.012
TwinsUK 0.599 (0.53 - 0.67) 0.615 (0.55 - 0.69) 0.626 (0.56 - 0.7) 0.027
avg 0.647 0.668 0.021

We then combined genes and biomarkers. The results are now in a manuscript currently in press at the Annals of the
Prediction model for knee osteoarthritis incidence including clinical, genetic and biochemical risk factors

Objective: To develop and validate a prognostic model for knee osteoarthritis (KOA) in a general, elderly population, and determine the value of different risk factor groups to prediction. Design: Three prospective population based cohort studies. Setting: General Population This is the first longitudinal risk prediction model for KOA including different risk factor groups. The risk model is a first step towards risk prediction to identify individuals at high risk for OA-development. This study may result in direct changes of daily practice for medical doctors. In particular, in most European countries it is not common practice for radiologist to report minor degenerative changes in the knee as this is not classified as knee osteoarthritis yet. However, we show in this study that these doubtful minor degenerative features are by far the best predictor of future knee osteoarthritis, a result validated in two independent studies. A genetic risk score has moderate predictive value, but this is especially true in younger subjects (<65 years), suggesting a more important role of genetic factors in early onset KOA.

Population characteristics In total, 474 incident knee OA cases and 2154 controls had data available for all risk factors in RS-I. The baseline characteristics of all three studies are shown in Table 8.3. The mean follow-up time is ~9 years in RS-I, ~10 years in the Chingford Study and ~4 years in RS-II.

Table 8.3. Baseline characteristics of RS-I, RS-II and the Chingford Study

<table>
<thead>
<tr>
<th></th>
<th>RS-I</th>
<th>RS-II</th>
<th>Chingford Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>N incident KOA cases</td>
<td>474 69 188</td>
<td>69</td>
<td>188</td>
</tr>
<tr>
<td>N controls</td>
<td>2154 856 535</td>
<td>856</td>
<td>535</td>
</tr>
<tr>
<td>% women</td>
<td>54% 54% 100%</td>
<td>54%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean age (yrs) (sd)</td>
<td>65.1 (6.4) 63.2 (6.6) 53.7 (5.9)</td>
<td>63.2 (6.6)</td>
<td>53.7 (5.9)</td>
</tr>
<tr>
<td>Mean BMI (kg/m2) (sd)</td>
<td>26.1 (3.4) 26.9 (3.8) 25.2 (3.9)</td>
<td>26.9 (3.8)</td>
<td>25.2 (3.9)</td>
</tr>
<tr>
<td>Mean follow-up time (yrs) (sd)</td>
<td>9.4 (2.2) 4.1 (0.6) 10.3 (0.7)</td>
<td>4.1 (0.6)</td>
<td>10.3 (0.7)</td>
</tr>
</tbody>
</table>

KOA = knee osteoarthritis; BMI = body mass index; RS = Rotterdam Study; N= number of study subjects

Risk prediction models The results of the univariate analysis for the relationship between risk factors and incident knee OA in RS-I and the multivariate analyses are shown in Table 8.4. As can be seen in the full multivariate model (MV3), the strongest associations were observed for the baseline KL score of 1 (OR 6.97) gender (OR 1.66) BMI (OR per standard deviation 1.28) knee pain (OR 1.62) and hand OA (1.44). This means for example that a subject with hand OA has a 44% increased risk of developing radiographic knee OA in the next ~9 years compared to a subjects without hand OA.

Table 8.4. Univariate and multivariate association models to assess the relationship between risk factors and incident knee OA in RS-I

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR 95%CI P</th>
<th>OR 95%CI P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (%F)</td>
<td>2.13 1.72-2.64 4.3x10-12 1.66 1.25-2.19 3.9x10-4</td>
<td>1.66 1.25-2.19 3.9x10-4</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>0.99 0.89-1.10 0.84 0.89 0.79-1.00 0.06</td>
<td>0.84 0.79-1.00 0.06</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>1.41 1.28-1.55 5.0x10-12 1.28 1.15-1.42 7.3x10-6</td>
<td>1.28 1.15-1.42 7.3x10-6</td>
</tr>
<tr>
<td>Knee pain1</td>
<td>1.81 1.42-2.32 2.4x10-6 1.62 1.22-2.16 0.001</td>
<td>1.62 1.22-2.16 0.001</td>
</tr>
<tr>
<td>Disability index1</td>
<td>1.08 0.98-1.19 0.11 0.95 0.84-1.07 0.41</td>
<td>0.95 0.84-1.07 0.41</td>
</tr>
<tr>
<td>General health1</td>
<td>0.95 0.63-1.42 0.79 0.99 0.62-1.58 0.96</td>
<td>0.95 0.62-1.58 0.96</td>
</tr>
</tbody>
</table>
Validation of the risk prediction models

The Hosmer-Lemeshow $\chi^2$ statistics for goodness-of-fit showed a good calibration for all risk prediction models (Table 8.5). AUCs of the separate risk factor groups are depicted in Table 8.5. The area-under-the-curve was higher in RS-I than RS-II or Chingford for all models. This is to be expected because the risk prediction model was created in RS-I. A model including only age, gender and BMI resulted in an AUC of 0.66 in RS-I (Table 8.5). The addition of questionnaire based predictors did not add predictive value (similar AUC). Subsequently adding the genetic risk score to the model also did not improve the model much (AUC of 0.67) whilst addition of radiographic variables increased the AUC up to 0.79 in RS-I. This increase in AUC is explained by the baseline KL score, not by the risk factors hand and/or hip OA (data not shown). We then tested a “minimal model” including age, gender, BMI, knee pain and baseline KL-score 0/1 for its predictive value. We found that this model was very similar to the full model (including the 13 tested variables), with an AUC of 0.79 in RS-I. In RS-II and the Chingford Study similar trends in AUCs were observed as in RS-I, with the AUCs being smaller compared to the AUCs in RS-I. The minimal model performed well in the two validation cohorts, with an AUC of 0.86 and 0.76 (RS-II and Chingford respectively).

In a subset of RS-I (135 cases, 794 controls), data was available on a biochemical marker, uCTXII levels. Risk prediction models were created in the same way as mentioned before in RS-I and were tested for discrimination in RS-I, RS-II and the Chingford Study (Table 8.6). Comparing the genetic risk score with uCTXII levels, the same results were obtained, namely no significant increase in AUC compared to MV2. The AUC for the genetic risk score only was 0.65 (95%CI 0.62-0.70) in subjects aged &lt; 65 years whilst it was only 0.55 (95%CI 0.51-0.59) in subjects aged ≥65 years Subjects aged &lt;65 years of age were similar to subjects aged ≥65 years in terms of gender ($P=0.70$) and BMI ($P=0.88$) but had on average 1.0 years longer follow-up time ($P=4.4\times10^{-33}$) and less frequent a baseline K/L score of 1 (35% versus 42%, $P=1.9\times10^{-4}$) (data not shown). In summary we developed different types of risk prediction models to obtain an estimate of the discriminative power of very basic risk factors such as age, gender and BMI and the additive value of less conventional risk factors like a genetic risk score and uCTXII levels.

Table 8.5 Validation of the risk prediction models: calibration and discrimination

<table>
<thead>
<tr>
<th>Model Discrimination: AUC (95%CI)</th>
<th>Variance explained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal (RS-I)</td>
<td>External (RS-II)</td>
</tr>
<tr>
<td>Gender, age and BMI (1)</td>
<td>0.66 (0.64-0.69)</td>
</tr>
<tr>
<td>1 + “questionnaire” variables (2)</td>
<td>0.66 (0.64-0.69)</td>
</tr>
<tr>
<td>1+2+ genetic risk score (3)</td>
<td>0.67 (0.64-0.70)</td>
</tr>
<tr>
<td>Full model (4)</td>
<td>0.79 (0.77-0.82)</td>
</tr>
<tr>
<td>Minimal Model (5)</td>
<td>0.79 (0.77-0.81)</td>
</tr>
</tbody>
</table>

AUC: area under the curve; CI: confidence interval; RS-I: Rotterdam Study I; RS-II: Rotterdam Study II; Model 1: gender, age,
Table 8.6. Discrimination, expressed as AUC, for risk prediction models including uCTX-II in a subset of RS-I

RS-I RS-II Chingford Study

AUC (95%CI) AUC (95%CI) AUC (95%CI)

Age, gender and BMI (1) 0.60 (0.55-0.66) 0.60 (0.52-0.67) 0.61 (0.56-0.66)
(1) + “questionnaire” variables (2) 0.64 (0.59-0.69) 0.60 (0.52-0.67) 0.63 (0.58-0.68)
(1) + (2) + uCTXII (3) 0.66 (0.61-0.71) 0.64 (0.57-0.71) 0.65 (0.60-0.70)
(1) + (2) + (3) + radiograph variables + genetic risk score 0.77 (0.72-0.81) 0.85 (0.81-0.89) 0.74 (0.69-0.79)

RS-I: Rotterdam Study-I; RS-II: Rotterdam Study-II; BMI: body mass index; AUC: area-under-the-curve; CI: confidence interval

D8.6 - NEXT GENERATION SEQUENCING AND ANALYSIS OF FUNCTIONAL VARIANTS

Whole genome sequencing project at deCODE Genetics

deCODE whole genome sequencing (WGS) project encompasses WGS, chip typing and imputation efforts of a large number of variants and individuals that allows immediate and direct association analysis of all the variants identified through WGS.

The WGS is funded entirely by deCODE but TreatOA support for some bioinformatic analyses of this hugely complex data with respect to OA is billed to the WP8 as well as replication effort genotyping and analysis.

Hand OA: We have identified one locus on chr1 that is associated with severe hand OA using this method. This locus is highly significant in Iceland with OR 50.6 and P = 9.8 × 10^-10. It is very rare in Iceland with freq. 0.02% but the variants are present in other populations and work is ongoing investigating whether it is also associated with hand OA in these populations. This locus is included in a manuscript describing genome-wide association study on severe hand OA, with TreatOA and many individual members as coauthors.

Generalized OA: We have identified two rare non-synonymous SNPs, in JAK1 (0.2% freq.) and in NUDT12 (0.4% freq.), that associate with generalized OA (many joints). Both SNPs are present in Swedish OA samples, however, these only have information on TKR and THR. This observation needs additional replication effort in samples that have information on generalized OA.

Total knee replacements (TKR) and total hip replacements (THR): We have focused on variants that are associated with TKR or THR, mostly because we have replication samples available in-house for direct genotyping and association testing of the same phenotype.

We selected 23 SNPs and indels that associate with TKR or THR and are either loss of function variants or are predicted to cause damage to the protein. Selection criteria: LOF P values < 0.001 and non-syn SNPs P < 0.0001. None of these variants were genome wide significant.

We genotyped these variants for replication in the Swedish samples. Thirteen variants turned out not to be polymorphic in the
Swedish samples; these were in 0.014% to 0.69% frequencies in the Icelandic samples. 5 SNPs remain interesting after the replication effort; similar odds ratios as in the Icelandic discovery set and p-values in replication samples 0.2 (except for 1 SNP = 0.7 selected from OAall phenotype but showed evidence for association with TKR and THR). However, none reach the genome-wide significance level in the combined analysis of the Icelandic samples and the Swedish samples. These variants are in the following genes: C2orf65, C4orf47, ABCC6, SLFN12L and MYBBP1A. Additional replication effort is needed in order to confirm these associations.

Erasmus and LUMC:

Partners 3 and 4 performed additional RNA-sequencing in 200 samples of the RAAK-study (LUMC, Leiden, Partner 4) and the Rotterdam Study (ErasmusMC, partner 3), in order to be able to identify and study functional effects of rare variants with large effect sizes.

The RAAK study is a unique biobank of healthy and diseased blood and joint tissues (for example cartilage, bone and tendon) of OA patients that underwent a joint replacement surgery (RAAK study). DNA and RNA have been isolated from the preserved and affected areas of the respective tissues in order to apply genetic and transcriptomic profiling with respect to the OA pathophysiological process but also for OA specific eQTL analyses, differential allelic expressions and identification of rare variants..

KCL: projects encompassed both WGS, chip typing ; Sequencing Study samples:

SET1: 150 severe knee osteoarthritis cases, All female, Age at TKR 62; K/L 3 or 4; BMI <32, Mean age = 57.5 years Avg BMI = 28.4 kg/m2 150 Controls from the GOT2D consortium selected to have higher BMI and higher age, Mean age = 66.5 years Avg BMI = 29.5 kg/m2 The controls in this case were called separately

SET2: From the TwinsUK cohort 60 cases (K/L >=2 no restriction on age or BMI), 370 radiographic OA controls Cases and controls were called in the same run.

Whole genome Sequencing (WGS). The sequencing of both case and control samples was by whole genome shotgun sequencing generated as on HiSeq machines, Illumina HiSeq 6-8x.

12 variants were selected for de novo replication in 1493 controls and 1147 cases. Two of the 12 assays failed. One of the variants was significant both in the discovery and replication sets, this is rs2295295 mapping to the RUNX2 gene achieving an overall OR= 0.70 (95% CI 0.59-0.84) p=8 x 10^-5. The RUNX2 gene encodes the a transcription factor involved both in chondrocyte hyperthrophy and osteoblast maturation

Association Analysis SET 2. All Variants mapping to gene regions (+/- 50kb) were selected. In total 3,065,041 SNPs regions were included. Genomic control Lambda = 0.96 The QQ-plot and Manhattan plots for association with severe knee OA in set 1 are shown below. 3124 variants had a p<0.001. Of these we selected 27 variants which are part of the Illumina exome chip assay that focuses on rare functional variants in gene regions. Replication was carried out using data from the exome chip 350 cases, 1167 controls. The results of the meta analysis if the 22 variants for which the genetic effect was in the same direction is shown below

Exome chip. Single point analysis on exome chip from 350 knee OA cases, 1167 controls. was conducted using PLINK (Purcell, 2007) association and frequency tests. 350 cases, 1167 controls. The results mapping to previous GWAS hits are shown below A number of rare aminoacid changes were identified two of which are likely to damage the protein function , mapping to genes identified by GWAS
In particular our results confirm that aminoacid variants in some of the GWAS hits are significantly more common in knee OA cases than in controls.

WP9

The first objective (D9.1) of WP9 was to set-up and validate two high throughput screens, suitable for the identification of candidate drugs, aimed against novel targets and or pathways identified in previous work packages (WP1-7). Candidates selection was done on the basis protein/enzyme class (Kinase, GPCR, etc), understanding of the pathway(s) they are involved in, pathway specificity, freedom-to-operate and amenability to develop a biochemical or cellular assay. Identification of GDF5, SMAD3, SMOC2 and DOT1L throughout WP1-7 pointed out the importance of the developmental pathways BMP (GDF5, SMAD3, SMOC2) and Wnt (DOT1L) in human osteoarthritis. These novel findings together with the established role of BMP and Wnt during endochondral bone formation, a process which is believed to be recapitulated in OA and is responsible for the phenotypic change (anabolic-> catabolic) observed in chondrocytes, made up a strong argument to develop high throughput assays for these two pathways. Unfortunately none of the individual candidates qualified for HTS development. This was mainly because of a (1) lack of enzymatic activity (COG5, BCAP29, HBP1), (2) difficulties in setting up high throughput screens because the ligand or substrate is not know (GPR22, an orphan GPCR; DUS4L poorly characterised) and/or patents on screening technology (cell lines expressing high levels of GPR22), (3) not specific (PKA, kinase involved in a wide range of cellular processes) or (4) validation studies pointed out that inhibition has an adverse effect on development of OA (DOT1L). As a consequence the focus in WP9 shifted from target based to a pathway based screening.

For the development of the BMP and Wnt assay, the following approach was used: (i) the optimisation of a BMP/Wnt sensitive cell based reporter assay for high throughput screening (ii) assay validation using a test set of chemically diverse drug like compounds. Because the overall objective of the high throughput screen is to identify a diverse set of modulators the assay was set-up both in absence as well as in the presence of a suboptimal ligand concentration. By doing so it should be possible to pick up pure agonists (absence) as well as enhancers and inhibitors (presence of ligand) of both pathways.

In case of BMP a stable cell line, C2C12 BRA, carrying multiple repeats of a BMP response element (BRE) coupled to a luciferase reporter gene was selected to set up the assay. In a first phase, the assay was successfully adapted for high throughput screening. The assay was scaled down to 384-well format and further optimized in terms of incubation time, DMSO and ligand concentration, to set up a high quality screening assay (z-factor ≥ 0.5). To validate the applicability of the assay to pick up hit molecules, we also performed a pilot screen using a chemically diverse test set of 1000 compounds drug like compounds. The estimated hit rate was around 0.5-1% in the presence of ligand, indicating that upon screening a whole library we expect to pick up a reasonable number of high quality hits using this approach. In the absence of ligand we could not identify any hits in the test set, making it more unlikely that BMP mimetic will be identified during a high throughput campaign. A similar approach was applied for the Wnt pathway and although the estimated hit rate was somewhat lower 0.1-0.5%, we also expect to find a reasonable number of high quality hits in case of the Wnt pathway.

The second objective (D9.2) involves the set-up and validation of secondary cell assays for further profiling and optimisation of the hits identified in the BMP and Wnt assays described above. Since the reporter screens are done in a generic cells line, the output of such a high throughput screen should be seen as collection of pathway modulators, from which we still need to select those compounds that produce the desired effect on chondrocytes preferably using human articular chondrocytes. To support the selection of these compounds, we configured a panel of assays that allows the evaluation of the effect of novel compound on the various aspects of chondrocyte biology in osteoarthritis. I.e. matrix production, proliferation, inflammation and de-differentiation in a concentration dependent manner. The various assays were set-up and validated using prototype inhibitors/enhancers, where available, across a panel of donors.

In addition to the primary ‘hit’ selection, these assays can of course also be used to evaluate the biological activity during compound optimisation. A disadvantage, however, is the use of primary cells, which cannot be kept constant throughout the
optimisation cycles (years). Therefore, we also explored the use of chondrogenic cell lines to setup ‘surrogate assay’ that closely mimic the biology of the primary cells, but also deliver robust data over a long period of time. Different read-outs and formats have been evaluated using two different cell lines, but the results were not satisfactory both in terms of sensitivity of read-out as well as response of cells; and reinforced the importance of using primary cells to study the effect of compounds on chondrocyte metabolism/phenotype.

The third objective (D9.3) involved the use of specific stable mutant zebrafish lines generated in WP6.1 for drug screening i.e. to investigate if a compound, or class of compounds can recover a mutant phenotype. This deliverable was originally introduced as a complementary approach to the cell based assay (D9.2) and represented an elegant way to confirm on target activity in a more complex environment. In agreement with the deliverables of WP6, several lines were made available, however, as the research direction in WP9 shifted from target based to pathway based screening, it was no longer appropriate to pursue the evaluation of the target specific mutants for drug screening purposes. As a consequence, no further research efforts have been done in this direction and also explain why the person months allocated to this task have not been claimed. Since D9.3 represents a complementary approach to the cell based assays (D9.2) the premature termination of D9.3 has no major impact on the overall objectives of the WP9.

In conclusion we successfully developed two cell-based high throughput assays, against pathways that play an important role in the development of OA (BMP and Wnt), as well as the necessary tools (panel cell assays) to support the identification (profiling) of hit series that exert a beneficial effect on chondrocytes metabolism/phenotype and monitoring of their biological activity during further optimization cycles.

Potential Impact:

Societal impact: OA is the most prevalent joint disorder worldwide, affecting 40% of people over the age of 70. It comprises a group of overlapping disorders resulting in joint failure. Individuals with osteoarthritis (defined symptomatically and radiographically) at the knee or the hip have a 55% excess in all-cause mortality even after adjustment for other risk factors. OA is associated with a very high economic burden attributable to the effects of disability, comorbid disease, and the expense of treatment. Although typically associated with less severe effects on quality of life and per capita expenditures than rheumatoid arthritis, OA is nevertheless a more costly disease in economic terms because of its far higher prevalence, estimated to be around 12% of the adult population. A recent US-based study has found that OA contributes substantially to health care expenditures. OA was estimated raise aggregate annual medical care expenditures by $185.5 billion. At the same time, the burden of OA is increasing. Even though direct and indirect per capita costs for OA have stabilized in recent years, the escalating prevalence of the disease, partly due to the increase in aging and obesity, has led to much higher overall spending for OA.

Overall, the nature of the research carried by TREAT-OA into osteoarthritis should have great benefits for society in the long term and help reduce the economic burden across the EU of this disease.

Scientific impact: TREAT-OA has made a substantial contribution to the understanding and diagnosis of OA and furthered European scientific excellence in this area as follows:

1. It generated an international research network that can continue to foster scientific excellence in the field of OA
2. Whole genome for OA risk alleles were identified by genomewide association scans
3. It generated information on the generalisability of genetic and biochemical factors that affect risk of disease both in Europe and other populations.
4. It developed novel biochemical and genetic diagnostic markers for disease incidence, risk, severity and progression which can be used by the medical community to study response to drugs more cost-effectively via clinical trials.
5. The in vitro and in vivo assays developed by TREAT-OA provide a comprehensive technology platform to obtain new insights.
into the process of human OA
6. The cell based assays developed are ready for high throughput screening
7. The development of new animal models for OA enables the validation of new therapeutic agents for OA

Workforce statistics

In this respect the project has enhanced understanding of the mechanisms of the disease and the potential for treatment across all stages. In the process of the research project, the Consortium members have engaged in many different dissemination activities, including events aimed at the scientific community and fellow researchers across the world, as well as events aimed more for the general public, or lay audiences.

By implementing the research agenda, the Consortium hope to have influenced policy makers to the extent that understanding of the progress and management of the disease can affect decision making in the health care professions.

In terms of employment impact, this is difficult to assess as each beneficiary has different requirements and internal strategies. The overall aim of the project was not to influence employment ratios, and the personnel were of necessity highly skilled scientists, many of whom already worked in the field.

Overall the gender balance was good, with more women working on the project than men, though still with more men in positions of work package leaders/ scientific co-ordinators.

The academic partners work mainly in institutions where gender balance is encouraged, and with equality as an overarching policy, so no specific gender actions were necessary within the project itself. The industrial partners did put gender actions in place, also as a part of their overall employment policy, and found these to be reasonably effective.

Main Dissemination

Publications

The main thrust of the dissemination has been the publication, in peer-reviewed journals, of numerous papers. In total 85 have been published or were in press at the close of the project. A full list of publications is available on www.treatoa.eu

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Symposia

In addition to these activities, Treat OA has organized and hosted two major symposia on osteoarthritis, in 2010 and 2012, both at the Royal College of Physicians, London.

1st February 2010: Breaking boundaries in osteoarthritis

This included sessions on the Biology of molecular signaling pathways, Human genetics and biomarkers of OA, and Epidemiological and clinical aspects of OA, with speakers from the TreatOA Consortium and other leading researchers.

https://www.regonline.co.uk/custImages/280000/285136/FinalProgramme.pdf

12th April 2012: OMICS and Ageing; a case study on osteoarthritis
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Workshops and courses

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These 4-day courses were held at Erasmus Medical Centre;

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They concentrated on the following subjects
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List of Websites:

- www.treatoa.eu
- Professor Tim Spector - Co-ordinator, King’s College London
- tim.spector@kcl.ac.uk

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