Genes and mediators of inflammation and development in osteoarthritis

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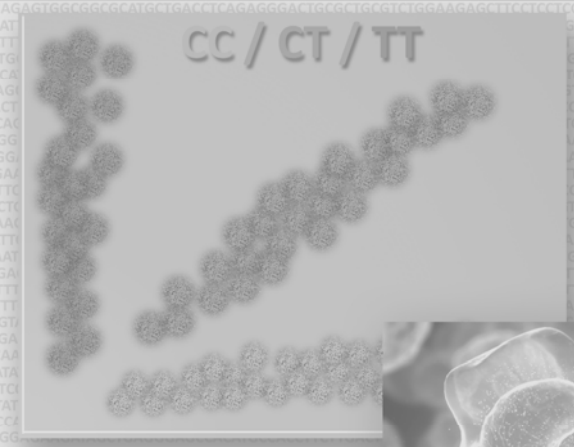

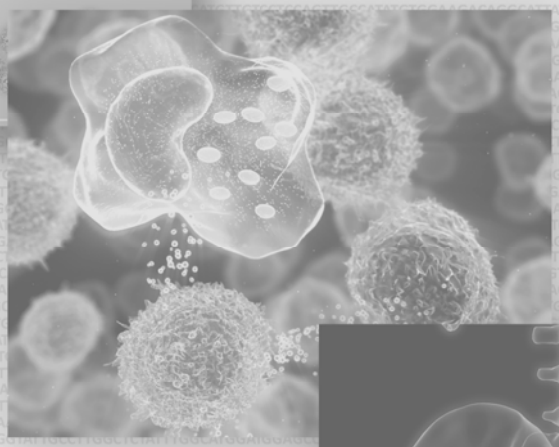

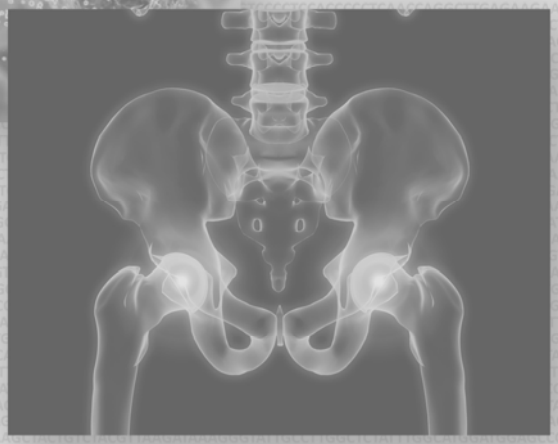

Functional characterization of type II deiodinase in human OA cartilage; assessment of *DIO2* allelic expression imbalance and immunohistochemistry of thyroid hormone signaling proteins.

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Submitted

Abstract

Objective. Functional characterization of OA risk polymorphism rs225014 at *DIO2* and respective thyroid signaling in OA and non-OA cartilage
Design. Alleles of the OA risk single nucleotide polymorphism rs225014, a T to C transition coding for a Thr92Ala substitution in type II deiodinase (D2) at *DIO2* were analyzed for differential allelic expression in mRNA extracted from OA cartilage. Immunohistochemical staining of D2 and associated thyroid signaling proteins was performed on OA and non-OA cartilage.
Results. We assessed allelic expression imbalance of *DIO2* using rs225014 in cartilage of OA patients heterozygous for the polymorphism. A significantly higher amount of expression was observed for the OA-associated C allele relative to the T allele in 17 out of 20 donors, indicating that *cis*-acting regulatory effects may underlie the association of this polymorphism to OA. Furthermore, to assess the ongoing thyroid signaling in cartilage we have used immunohistochemistry to stain anatomically "healthy" non-OA and OA affected human cartilage for D2, type III deiodinase (D3) and thyroid hormone receptors alpha and beta. We show that in OA affected cartilage thyroid hormone signaling is substantially increased and that this increase overlaps with an increasing Mankin score, indicative of increasing thyroid hormone activity with increasing cartilage damage.
Conclusion. Our analyses show activated thyroid signaling in OA cartilage, which should be considered detrimental to cartilage homeostasis. In addition, disruptions of cartilage homeostasis may be augmented by the increased expression in cartilage of the OA risk C allele of *DIO2* SNP rs225014.

Introduction

Osteoarthritis (OA) is a common, degenerative disease of the articulating joint that causes pain and disability. Currently, treatment of the disease is limited to pain suppression with no drug yet available that can effectively slow down or reverse the disease process. Ultimately, affected joints need replacement thereby imposing a considerable burden on patients and on health care systems[1]. Previously, using genome wide linkage and association approaches in multiple centers across different ethnic groups, we identified *DIO2* as a gene harboring susceptibility for OA. A *DIO2* haplotype consisting of SNPs rs225014 and rs12885300 showed consistent association to OA[2]. *DIO2* codes for type II deiodinase (D2), which is expressed in specific tissues where it has a vital role in the regulation of intracellular thyroid hormone 3,5,3'-triiodo-L-thyronine (T_3) levels through deiodination of inactive thyroid hormone thyroxine (T_4)[3,4]. In the growth plate, chondrocytes express D2 in the transition to terminal differentiation which is controlled at least in part through T_3 levels[5-7]. Functional differences of *DIO2* in this process might lead to subtle differences in joint shape or bone composition, which could predispose to OA as a result of a lifelong exposure to overt biomechanical factors on the articular cartilage. Furthermore, later in life functional differences of *DIO2* might enhance the predisposition for articular chondrocytes to turn hypertrophic, thereby initiating or augmenting their release from the maturational arrest that sustains chondrocytes in articular cartilage[8]. No *in vivo* cartilage studies specifically aimed at D2 have been performed, however *DIO2* RNA transcripts were shown to be up regulated in OA cartilage as compared to healthy

cartilage[9,10]. Here we investigated the role of D2 in articular cartilage and OA by functional genomic research of patient and control cartilage.
We assessed putative differential allelic expression (DAE) of rs225014 alleles by allelic discrimination assays in OA cartilage RNA. Although the OA risk allele of *DIO2* rs225014 codes for a Thr92Ala substitution in the protein, to date no compelling evidence has been reported showing that this substitution has a major influence on protein function[11-14]. Possibly, the polymorphism acts as a *cis* regulatory element or is in linkage disequilibrium with such an element; earlier studies of OA cartilage samples have indicated the presence of polymorphic *cis* regulatory elements on alleles of OA associated genes such as *FRZB*, *GDF5* and *BMP5*[15-17]. DAE effects are present throughout the genome[18] and potentially contribute to the observed genetic associations seen for many common complex phenotypes, including OA. It could be that for *DIO2* such modest but persistent imbalances of specific allelic expression differences throughout life renders subjects more susceptible to OA.
To explore ongoing thyroid hormone signaling in OA affected and non-OA cartilage, immunohistochemical (IHC) staining of proteins involved in thyroid signaling was performed. In addition to D2 staining we studied the thyroid hormone receptors alpha (THRA) and beta (THRB), which bind activated T_3 and which can subsequently alter gene expression through activation of thyroid responsive elements on DNA. Furthermore, D3 was included in our IHC analyses since this protein is responsible for inactivation of thyroid signaling through the conversion of T_4 into inactive reverse T_3 as well as active T_3 into inactive T_2[3]. Altogether these proteins may indicate the activity of the ongoing thyroid hormone signaling.

Materials and methods

Subjects.

For DAE assessment, subjects undergoing a joint replacement as a result of primary OA were recruited at the Nuffield Orthopaedic Centre (Oxford, UK; Nuffield samples, N=7) and at the Leiden University Medical Centre (Leiden, the Netherlands; LUMC samples, N=13). Ethical approval for the study was obtained from appropriate ethics committees. Immediately upon joint replacement the affected articular cartilage was collected, frozen in liquid nitrogen and subsequently transferred to -80˚C for storage. For the immunohistochemical assessment of thyroid hormone signaling proteins 6 non-OA and 11 OA affected hip cartilage tissues embedded in paraffin were retrieved from the archives of the department of pathology, LUMC (Leiden, the Netherlands). Non-OA samples originated from subjects receiving a replacement after a hip fracture.

Nucleic acid isolation and rs225014 genotyping.

To enable isolation of RNA and DNA the frozen cartilage samples were powderised using a Retsch Mixer Mill 200 with continuous liquid nitrogen cooling. RNA isolation was performed using Qiagen RNAeasy Midi kits as described earlier[19], the first wash flowthrough in this isolation was used for subsequent DNA isolation. RNA was stored in precipitated state after addition of $1/10^{th}$ volume of sodium acetate (3M, pH 5.2), linear acrylamide to an end concentration of 10 $\mu g l^{-1}$ and 2 volumes of ethanol. cDNA was synthesized by use of random hexamer primers as described earlier[17]. The wash flow-

through collected during RNA isolation was used to isolate genomic DNA by addition of 1/10th volume of sodium acetate (3M, pH 5.2) and 3 volumes of ethanol. The precipitated DNA was washed and resuspended in 60 µl of milliQ water. To assess rs225014 genotypes, genomic DNA was PCR amplified using forward primer 5'-TACCACACTCTATTAGAGCC-3' and reverse primer 5'-CACACACGTTCAAAGGCTAC-3' targeted at a 586 basepair *DIO2* fragment encompassing rs225014. The PCR products were incubated with restriction enzyme RsaI at 37˚C for 3 hours and genotypes were visually called by running the digested PCR products on a 3% agarose gel showing cut (TT), uncut (CC), or both (TC) fragments of DNA. Only heterozygous subjects were included in this study.

Differential allelic expression assessment.
cDNA and genomic DNA from heterozygous samples were subjected to a 15 µl PCR amplification using 20 and 5 replicates respectively using forward primer 5'-ATGCTGACCTCAGAGGGACT-3' (cDNA) or 5'-AGTGGCAATGTGTTTAATGTGA-3' (genomic DNA) and reverse primer for both PCR reactions 5'-CACACACGTTCAAAGGCTAC-3'. Amplification products were treated with exonuclease and shrimp alkaline phosphatase to an end volume of 18 µl prior to further processing. For the Nuffield samples 1.8 µl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), 0.1 µl of HPLC-purified extension primer (5'-CACTGTTGTCACCTCCTTCTG-3') and 0.2 µl milliQ water was added to 1.2 µl of PCR product in an extension reaction. The samples were subjected to 25 cycles of extension consisting of 10 seconds at 96˚C, 5 seconds at 50˚C and 30 seconds at 60˚C, upon which the sample was cooled to 4˚C. 1 µl of extended product was added to 10 µl of Hi-Di formamide containing 120LIZ size standard (Applied Biosystems). Analysis of the fluorescence for C and T alleles (TAMRA and ROX label respectively) in each sample was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and data was loaded in Genemapper 3.1 for quality control, genotype calling and exporting fluorescence peak height values. Samples which failed to amplify in the assays or cDNA samples which were outliers in the respective samples replicate group of normalized log transformed peak height ratios were omitted from further analysis. For the LUMC samples cleaned PCR products were diluted 500 times and 1 ul was used as template in a Taqman realtime assay (C__15819951, Applied Biosystems) in a final volume of 5 µl. The dilution was aimed to reach a cT threshold after 15-20 cycles of amplification. Samples were subjected to 10 minutes of denaturation at 95°C, and 40 cycles of 92°C for 15 seconds and 1.25 minutes at 60°C on an ABI Prism 9700HT (Applied Biosystems). Reactions were followed real-time and after cycling an end measurement of fluorescence levels was performed.

Statistics.
Peak height ratio of allele C over T from each individual cDNA sample was normalized using the pooled genomic DNA samples (1:1 ratio of allelic presence) to account for technical variation in fluorescence of the labels. Per centre, the log transformed normalized peak height ratio per cDNA sample replicate series were analyzed for differential allelic expression by a two-tailed Mann-Whitney non parametric test (SPSS Version 16.0) against all genomic DNA samples.

Histological assessment.
HE and toluidine blue staining were used to score all samples according to Mankin *et al.*[20] by 3 observers blinded towards the clinicopathological data. In this scoring system ranging from 0 (no signs of OA) to 15 (total destruction of cartilage layer) samples are scored for microscopic OA features such as decreased proteoglycan content reflected by decreased toluidine blue staining, hypo- or hypercellularity, clonal expansion of chondrocytes, tidemark crossing by blood vessels and cartilage (micro)fractures.

Immunohistochemistry.
Antibodies and protocols used for antigen retrieval and blocking are listed in Table 1. Immunohistochemistry (IHC) was performed according to standard procedures as described previously[21]. Visualization was performed by Powervision incubation followed by incubation in 0.05% diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide and hematoxylin was used to counterstain the slides.

Table 1. Characteristics of antibodies used in the IHC analysis of thyroid signaling proteins.

Antibody	Manufacturer	Type	Target protein	Positive control	Antigen retrieval	Block	Dilution
COLX	Quartett (X53)	Monoclonal	Collagen Type X	Growth plate	prot-K & hyaluronidase	none	1:100
D2	Custom[1]	Polyclonal	type II deiodinase	Thyroid Gland	citrate	none	1:8000
D3	Custom[1]	Polyclonal	type III deiodinase	Placenta	proteinase-K	10% nonspecific goat serum	1:5000
THRA	Genetex (GTX16846)	Monoclonal	Thyroid Receptor α	Stomach	Tris-EDTA	none	1:50
THRB	Genetex (GTX17898)	Monoclonal	Thyroid Receptor β	Colon	none	10% nonspecific goat serum	1:400

[1]Antibodies were kindly provided by Prof. Dr. T.J. Visser[22] (Department of Endocrinology, Erasmus University MC, Rotterdam, the Netherlands).

Immunohistochemical staining for D2, THRA and THRB in the superficial, middle and deep cartilage layers was scored for nuclear and cytoplasmic localization whereas D3 was scored for cytoplasmic and extracellular staining by scoring 0 (no staining), 1 (weak or moderate staining) or 2 (strong staining).

Results

Differential allelic expression analysis
Characteristics of the Nuffield and LUMC OA cartilage samples are listed in table 2, all individuals included in this study were heterozygous for rs225014. The relative abundance of rs225014 alleles was analyzed by use of a SNaPshot extension reaction (Nuffield samples) or Taqman realtime PCR assay (LUMC samples) on the amplified target region encompassing rs225014. Table 2 lists the relative ratios of the T and C alleles for rs225014 for each cDNA sample, with the pooled genomic DNA ratios serving as the 1:1 allelic reference. Six of the 7 Nuffield samples demonstrated a higher expression of the C allele and this was a significant observation ($P \leq 0.01$) for 4 of these 6 samples. In an overall analysis of all 7 Nuffield samples allele C was significantly more abundantly present in the

cDNA samples (P<0.01). All 13 LUMC samples demonstrated a significantly higher expression of the C allele (Table 2, Figure 1). Overall therefore, 17 of the 20 samples studied (85%) demonstrated a significantly higher expression of the C allele of SNP rs225014 relative to the T allele. The mean percentage of relative difference was 17% and 36% for the Nuffield and LUMC samples, respectively. The DAE observations were consistent between different skeletal sites and between the two genders. None of the tested genomic DNA samples showed significant deviations from allelic balance when tested against the remaining genomic DNA samples (data not shown).

Table 2. Characteristics of the 20 OA cartilage samples studied and the results of the allelic expression analysis.

	Donor	Gender	Age[1]	Joint	cDNA[2] (%)	Genomic[3] (%)	Relative ratio C/T	P-value[4]
Nuffield samples	1	Female	71	Left Knee	11 (55)	3 (60)	0.97	0.269
	2	Female	72	Left Knee	11 (55)	5 (100)	1.28	<0.01
	3	Female	65	Right Knee	20 (100)	5 (100)	1.14	0.052
	4	Female	75	Right Knee	20 (100)	4 (80)	1.24	<0.01
	5	Female	53	Left Knee	19 (95)	2 (40)	1.07	0.268
	6	Male	88	Right Hip	17 (85)	5 (100)	1.29	<0.01
	7	Male	61	Right Knee	19 (95)	5 (100)	1.18	<0.01
LUMC samples	8	Female	70	Right Shoulder	18 (90)	N/A	1.30	<0.01
	9	Female	79	Left Hip	19 (95)	5 (100)	1.26	<0.01
	10	Male	61	Right Hip	20 (100)	5 (100)	1.29	<0.01
	11	Female	59	Right Hip	16 (80)	4 (80)	1.34	<0.01
	12	Male	71	Right Hip	19 (95)	4 (80)	1.32	<0.01
	13	Female	75	Left Knee	20 (100)	5 (100)	1.33	<0.01
	14	Female	78	Right Hip	19 (95)	5 (100)	1.25	<0.01
	15	Female	75	Left Hip	20 (100)	5 (100)	1.60	<0.01
	16	Female	79	Right Knee	20 (100)	5 (100)	1.38	<0.01
	17	Male	56	Left Hip	17 (85)	5 (100)	1.36	<0.01
	18	Female	62	Right Hip	18 (90)	5 (100)	1.41	<0.01
	19	Female	79	Right Hip	17 (85)	5 (100)	1.42	<0.01
	20	Female	62	Shoulder	17 (85)	5 (100)	1.42	<0.01

[1] *Age at time of joint replacement*
[2] *cDNA PCR measurements passing quality control (max. 20)*
[3] *Genomic PCR measurements passing quality control (max. 5)*
[4] *Mann-Whitney non parametric test (cDNA's per individual versus genomic DNA samples per centre)*

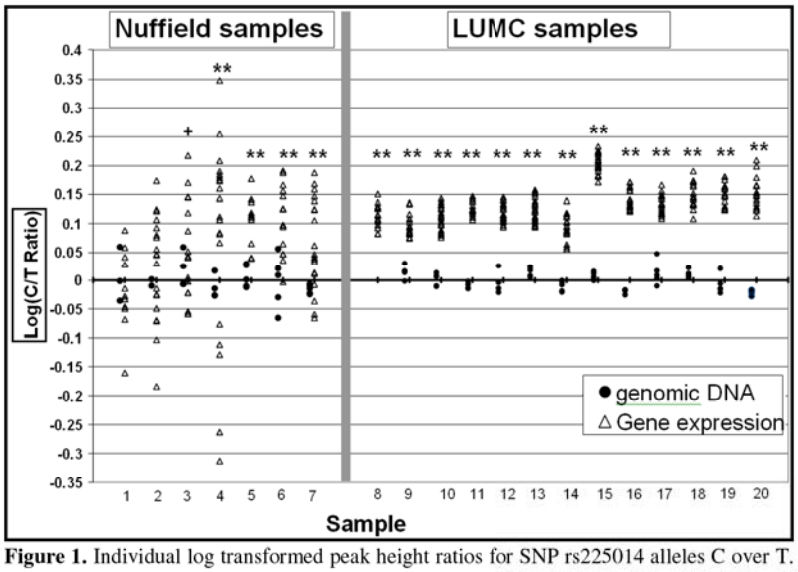

Figure 1. Individual log transformed peak height ratios for SNP rs225014 alleles C over T. Genomic ratios are indicated by black circles whilst mRNA ratios are indicated by triangles. + P < 0.1, ** P < 0.01

Immunohistochemical assessment of thyroid hormone signaling

To assess ongoing thyroid hormone signaling in OA cartilage as compared to non-OA cartilage we stained hip cartilage sections for D2 as well as for D3, THRA and THRB. Characteristics of the donors used are shown in Table 3. The non-OA subjects who received a joint replacement as a result of a fracture were on average significantly older than subjects who received a joint replacement as a result of OA. Using the Hematoxilin and Eosin (HE) staining and toluidine-blue staining (Figure 2A, A' and B, B' respectively) a Mankin score was assessed for each sample to quantify the OA damage. Four out of five non-OA samples had both HE and toluidine blue slides available and a mean Mankin score of 2.4 ranging from 0 to 7 whereas 11 OA samples had a statistically significant higher mean Mankin score of 9.5, ranging from 5 to 12 (Table 3). For the OA samples we observed increased collagen type X staining in all layers of the cartilage, indicating that chondrocytes in the OA samples have turned hypertrophic (Figure 2C, C'). Immunohistochemical stainings for THRA stained nuclear and equally throughout all samples and was not included in further analyses. Most nuclei and cytoplasm of chondrocytes throughout the different layers of cartilage in OA samples stained strongly for D2 whereas only some of the nuclei and cytoplasm in the non-OA cartilage stained positive for D2 (Figure 2D, D'). D3 staining showed increased numbers of positive cells in the OA affected areas of samples as compared to non-OA samples (Figure 2E, E'). For THRB most samples stained positive, however, the OA affected samples showed a more pronounced staining of nuclei and cells throughout all layers of the cartilage (Figure 2F, F').

Table 3. Characteristics of samples used in immunohistochemical analysis of thyroid signaling proteins.

Donor (OA)	Sex	Age	Mankin score	D2[1] nuc./ cyto	D3[1]	THRB[1] nuc./ cyto	Donor (noOA)	Sex	Age	Mankin score	D2[1] nuc./ cyto	D3[1]	THRB[1] nuc./ cyto
1	F	62	5	1/0	2	1/1	***12***	M	93	0	1/0	1	0/1
2	F	68	7	2/2	2	2/2	***13***	F	79	1	1/2	2	1/1
3	M	51	9	2/1	2	1/1	***14***	F	79	2	1/1	1	1/1
4	M	54	9	1/0	0	1/1	***15***	F	79	2	2/2	2	1/1
5	F	61	9	2/2	2	2/2	***16***	F	71	7	1/1	1	2/2
6	F	59	10	2/1	2	2/1	***17***	F	82	n/a	1/1	2	1/1
7	M	39	10	2/2	2	2/2	***Mean (SD)***	-	***80.5 (7.1)****	***2.4 (2.7)****			
8	F	72	11	1/2	1	1/1							
9	F	38	11	2/2	2	2/2							
10	M	80	11	2/1	2	2/2							
11	F	79	12	2/2	2	2/2							
Mean (SD)	-	***60.3 (14.2)***	***9.5 (2.0)***										

*t-test p-value < 0.01

[1] nuc: nuclear staining score cyto: cytoplasmic staining score; 0 no staining, 1 weak to moderate staining, 2 strong staining.

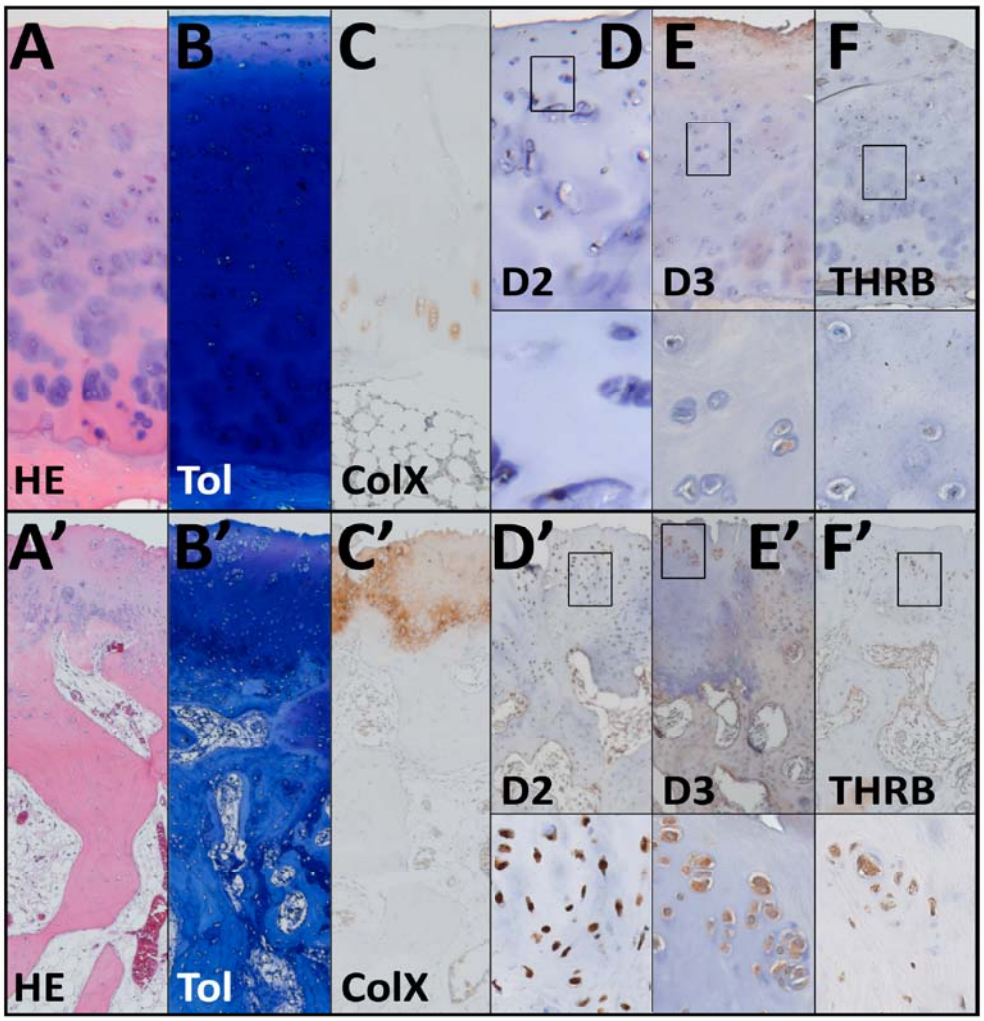

Figure 2 A-F non-OA cartilage samples & **A'-F'** OA cartilage samples**. A/A'** HE staining of non-OA (A) and OA (A') sample. **B/B'** Toluidine-blue staining of non-OA (B) and OA (B') sample. **C/C'** Collagen type X staining of non-OA (C) and OA (C') sample. **D/D'** D2 staining of non-OA (D) and OA (D') sample. **E/E'** D3 staining of non-OA (E) and OA (E') sample **F/F'** THRB staining of healthy(**F**) and OA (**F'**) sample. Tol-toluidine blue ColX-Collagen Type X. Magnifications 50x, insets 200x.

Together the increased presence of thyroid signaling proteins throughout all layers of the cartilage indicates that in OA cartilage thyroid signaling is upregulated. We semi-quantitatively assessed the staining presence and intensity for these three proteins throughout the different cartilage layers (Table 3) and observed that samples with higher Mankin scores were on average inclined to stain more abundantly and at higher intensity for these proteins and that, as opposed to the non-OA group in which mainly superficial staining was observed, the stainings in the OA group were present in all cartilage layers. The age of the subjects had no obvious relation to the Mankin grade or to the staining intensities within the groups of OA and non-OA subjects.

Discussion

Using transcript SNP rs225014 we were able to demonstrate that *DIO2* is subject to highly significant and consistent differential allelic expression (DAE) in OA cartilage with the OA risk C allele being more abundantly expressed in OA cartilage than the T allele. A large majority of the individuals studied demonstrated deviation from allelic balance indicating that the polymorphism itself or a polymorphism in strong linkage disequilibrium (LD) with it is the *cis*-acting regulatory polymorphism. This may act by influencing the transcription rate of *DIO2* or the stability of the *DIO2* mRNA. Previously, in smaller samples sizes the RNA expression of *DIO2* was shown to be up regulated in OA cartilage[9,10] and it is possible that this is partly accounted for by homozygote carriers of the OA risk C allele who, from our previous genetic studies, would be expected to be more prevalent in an OA cohort. It is possible that homeostatic feedback mechanisms ensure that the expression of the *DIO2* encoded protein D2 is balanced in response to different genotypes, and that challenges to this homeostasis mean that the response generated by the OA risk allele C is relatively strong and causes an aberrant thyroid signal in the cartilage. Investigation of *DIO2* DAE in non-OA cartilage samples may elucidate whether the observed allelic imbalance depends on the conditional use of *cis* regulatory elements in response to OA cartilage disease activity or whether it is independent of this and therefore functions as a conventional risk factor. Putatively underlying the observed association of the risk allele to OA is the possibility that during early development the increased expression of the C allele might lead to subtle changes in joint morphology and aberrant joint loading, thereby predisposing to OA. Alternatively, the maturational arrest of the chondrocytes may diminish as a function of age and allow for activation of genes not active in healthy cartilage. A greater expression of the risk allele C might increase the speed of cartilage degradation towards clinical outcomes as described earlier[2,23]. The fact that in the Netherland's LUMC study all samples showed significant DAE, whereas in the UK Nuffield study 4 of 7 samples showed significant DAE might be ascribed to the more sensitive technique used in the LUMC study. It is reassuring to note however that the use of two different techniques to measure allelic expression imbalance both highlighted relative increased expression of the C allele of rs225014. Since both studies comprised an analysis of Caucasian individuals from Northern Europe it is unlikely that genetic differences would account for the fact that not all of the UK samples demonstrated DAE. Having demonstrated DAE at *DIO2,* deep sequencing of this gene and of its proximal regulatory elements is now merited to identify additional variants that might also regulate *DIO2* expression and contribute to OA susceptibility.

Our DAE results suggest that the increased expression of the risk C allele of rs225014 might underlie the association of the polymorphism to OA. Relevant to the increased signaling of the allele is whether in OA the presence of the D2 protein in cartilage is aberrant. To assess D2 protein levels in OA cartilage as compared to non-OA cartilage we performed immunohistochemical analysis of the protein in cartilage specimens obtained from OA and non-OA joints. To get a more complete overview of ongoing thyroid hormone signaling we also stained these sections for the thyroid hormone inactivating protein D3 and THRA as well as THRB, which are responsible for activation of gene transcription through thyroid responsive elements on the DNA. THRA was present in all samples and cartilage layers, indicating at least a baseline thyroid signaling activity; however we did not observe

differences between the OA and non-OA samples. In bone, THRA and THRB isoforms appear to have different properties[24], however little is known about their roles in cartilage. To establish the roles of these specific receptors in cartilage additional research is warranted. We observed increased protein expression of D2, D3 and THRB through all layers of osteoarthritic cartilage, indicative of increased thyroid hormone signaling in OA cartilage as compared to non-OA, "healthy" cartilage from hip fracture patients. This increased signaling may preferentially act through the THRB receptor. Although it is unclear whether the observed changes in thyroid signaling are causal or merely a marker of the ongoing OA disease process, the up-regulation of thyroid signaling would be detrimental to cartilage homeostasis: the increased presence of thyroid hormone related proteins in cartilage could antagonise the maturational arrest of chondrocytes and promote phenotypic changes resembling those observed in the growth plate[23], contributing to cartilage loss. The increase in presence of the thyroid hormone inactivating protein D3 may be considered as a response to the D2 induced increased levels of active thyroid hormone T_3. In some of the superficial layers of the cartilage from the non-OA patients moderate D2, D3 and THRB staining is observed, indicating that random variation of thyroid hormone proteins is present, possibly as a result of the aging process or as a reflection of the higher availability of oxygen in this layer that may predispose it to dedifferentiation. The OA samples show a more abundant staining in all layers of the cartilage and in cellular components. One of the non-OA samples that stained positive for D2 had a Mankin score of 7, which is indicative of moderate cartilage damage, although OA was not recorded in the medical history of this subject. We were unable to stain the thyroid hormones T_4 and T_3 in the cartilage sections to confirm the ongoing thyroid signaling by these hormones directly. This may be attributable to the fact that the concentration for effective signaling of thyroid hormones can be very low. Future analysis of additional samples with both affected and preserved cartilage regions as well as non-OA, "healthy" cartilage samples at different ages may help to elucidate whether the observed increased thyroid signaling precedes cartilage damage or whether this occurs once the damage is present.

Overall, our analyses showed that activated thyroid signaling in OA cartilage may play a role in OA etiology, and that this may be augmented by the increased expression in cartilage of the OA risk C allele of *DIO2* SNP rs225014. Given the role of activated thyroid hormone T_3 during endochondral ossification, where it enhances the terminal maturation of chondrocytes, the described increased presence of D2 protein and increased expression of the *DIO2* risk allele in OA cartilage should be considered detrimental to cartilage homeostasis.

Acknowledgements

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