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Summary & general discussion
Osteoarthritis (OA) is a frequently occurring joint disorder with great impact on the quality of life. In general, OA is described as a heterogeneous disease with degeneration of articular cartilage as main outcome (1-3). Despite extensive research on the pathogenesis of OA, there is until now no cure and treatments are primarily aimed at reducing pain. The heterogeneity of the patient population might be one of the reasons for the absence of appropriate treatments. Although the clinical symptoms are the same, i.e. structural damage and symptomatic features such as pain, the underlying processes for the development of OA may be different. The inclusion of all OA patients, instead of stratification for different types of OA patients, may lead to a ‘contamination’ of clinical trials and may explain the observed lack of efficacy of candidate drugs. Evidence starts to appear that mild inflammation and obesity-related biochemical changes are involved in OA pathology (4). It is uncertain what the relative contribution of these processes is and if they characterize a certain type of OA patients. By understanding the underlying disease mechanism, having a better patient stratification and an improved prediction of disease outcome, the efficiency of clinical trials of novel drugs can be optimized. We aim to provide insight in the role of multiple local and systemic factors contributing to the pathogenesis of OA.

Osteoarthritis as a multiple tissues disease
For a long time, OA research has concentrated on articular cartilage. Nowadays, however, a role for other tissues of the joint is appreciated as well (5-7). In the first three chapters of this thesis we discuss the possible involvement of multiple knee joint tissues in the OA process. One of these tissues is the synovium, which is in direct contact with articular cartilage. Increasing evidence indicates that inflammation of the synovium contributes to degenerative processes in the cartilage and that inflammatory cell types, such as macrophages, present in this tissue may secrete factors modifying joint homeostasis (8). In chapter 2 we investigated if the secretion of soluble mediators (e.g. cytokines, chemokines, adipokines) is different between OA and normal synovial tissue and if OA synovial tissue is able to initiate cartilage degradation. Surprisingly, the levels of secreted mediators by OA synovial tissue were comparable to that of normal synovial tissue, whereas we expected a more elevated
excretion of pro-inflammatory cytokines from OA synovial tissue. Moreover, we demonstrated that the responsiveness of OA synovial tissue to the pro-inflammatory trigger IL-1 was diminished compared to normal. An explanation of these unexpected results could be that the OA synovial tissue was derived from end stage OA patients. The long-lasting exposure to an inflammatory environment during disease progression might have led to ongoing inflammation-resolving mechanisms in the synovial tissue of late phase OA patients and may explain why the synovial tissues do not respond to a pro-inflammatory trigger anymore (9). Furthermore, we found no additional effects of the synovial tissues on the basal release of glycosaminoglycans (GAG) from the matrix, nor on the matrix metalloproteinase (MMP) activity, when co-cultured with cartilage explants. We conclude that synovial tissue from end stage OA patients is not or no longer capable of initiating cartilage degradation, however these results do not rule out the involvement of synovial tissue in an earlier phase of OA development. It should also be noted that the effect of OA and normal synovial tissue on OA derived cartilage was not investigated for its role in the progression of OA. Other groups have shown that synovitis is correlated with symptom severity, rate of cartilage degeneration and osteophytosis (10). However, the synovial response in OA is complex and data in literature are contradictory. The data in chapter 2 emphasize this complexity, which may be attributed to time-dependent changes in the immune response during disease development.

Another neglected tissue in the knee joint is the infrapatellar fat pad (IPFP). This special form of adipose tissue is located intracapsularly and extrasynovially in the joint (11). The relevance of this joint tissue in the OA process is uncertain, but it is hypothesized that IPFP contributes to the development of OA by the secretion of inflammatory mediators (12-14). In chapter 3 we examined the secretion of a special class of inflammatory mediators, named oxylipins, by the IPFP. These mediators are derived from essential fatty acids and are important signaling molecules involved in inflammatory processes and the maintenance of physiological processes through the whole body (15, 16). With an LC-MS/MS approach, a wide panel of essential fatty acids and oxylipins (e.g. prostaglandins) involved in pro-inflammatory and inflammation-resolving processes in IPFP-conditioned medium (FCM) was detected. Partial least squares discriminant analysis (PLS-DA), a multivariate statistical method,
demonstrated that OA FCM could be distinguished from normal FCM with regard to the detected essential fatty acids and oxylipins. OA FCM samples obtained with the same protocol but at another location revealed comparable error rates for OA samples in the PLS-DA model, which strengthened our findings. The observed changes were probably specifically due to OA-related alterations instead of basal systemic changes, as the increase of essential free fatty acids (precursors) levels were not necessarily associated with an increase in precursor-derived oxylipins. Although it is tempting to conclude that these changes are causative for OA development, it is very plausible that these disturbances occur as a consequence of OA and may contribute to the amplification of the disease process only. These data indicate the relevance of balanced mechanisms in the joint and provide a valuable tool for further research to the role of IPFP in the OA process.

In chapter 4 we presented a comprehensive overview of soluble mediators (e.g. cytokines, chemokines, adipokines) in knee synovial fluid (SF) of OA and normal donors. The mediators in SF can be considered as a representative of processes in the knee joint as they are in close contact with synovial tissue, IPFP and articular cartilage. We showed with multiplex analysis that SF from OA donors contains increased levels of mediators, such as macrophage-derived chemokine (MDC), interleukine (IL)-6 and ‘regulated and normal T cell expressed and secreted chemokine’ (RANTES), compared to SF from normal donors, which is in agreement with the increased involvement of inflammatory processes in OA. In addition, principal component analysis (PCA), a multivariate statistical analysis, indicated various clusters of cytokines that probably reflect the involvement of different processes in the joint. We consider this data set valuable as a reference for future experiments to study pathophysiological pathways.

The use of more advanced technologies, such as applied in chapters 2-4, demonstrated the application of wide screening opportunities of joint tissues, which until recently did not gain a lot of attention in OA research, and emphasized the involved processes on a very small scale. The strength of these studies is that the results obtained with OA donors could be compared to that of normal donors. Tissues from normal donors are difficult to obtain and most studies in literature include a comparison with rheumatoid arthritis patients to assess for OA specific alterations. We experienced practical drawbacks, such as that all tissues were ex vivo material
and were obtained from OA patients at the end stage of the disease. The data in chapter 2 did not support the hypothesis that alterations in synovial tissue of OA patients is one of the potential mechanisms that leads to the initiation of cartilage degradation. Surprisingly, the synovial tissue obtained from OA patients turned out to secrete levels of mediators comparable to normal donors and was less responsive to a pro-inflammatory trigger. This makes us consider whether the end-phase of the disease is the most representative stage of OA for studying initiation of cartilage degeneration. It has been shown that end stage OA synovial tissue exacerbates OA features in OA-derived cartilage explants, suggesting its effect in the perpetuation of the disease (17). We realize that to study effects of synovial tissue on the initiation of cartilage degradation, tissue from an earlier stage would be more representative, but in practice this is very hard to obtain. It should be noted that the mediators secreted by IPFP and SF, which are described in chapter 3 and 4, are indicative for the end stage of the disease as well, but this may be different for earlier stages. Furthermore, the tissues may be altered due to ex vivo modifications. The data should therefore be interpreted with caution. Nevertheless, we detected differences in synovial tissues, IPFP and SF between normal and OA donors, indicating altered mechanisms in OA.

New questions are raised by the data obtained in chapters 2-4. For example, are IPFP and synovial tissue primarily active contributors to the OA process or do they get inflamed due to alterations in the joint or body? Do modifications of the tissues lead to a more pronounced stage of OA or are these modifications just indicative for the current state of the joint? Do these different types of tissues interact? Is it sufficient to target one of these tissues, or is a multiple target approach needed? To provide answers to these questions, additional research is required and we propose an integrated approach to study different potential active tissues in the OA process to understand the mechanisms and disturbed pathways. An expansion of the number of donors is needed for more extensive profiling. This possibly leads to the discovery of new targets for drug development. The results in chapter 2-4 are the first steps in the direction of approaching OA pathogenesis in a different way.
Osteoarthritis as a disease of the whole body

With regard to the above mentioned alterations on a local level, we speculated whether these changes could be induced by alterations on a systemic level. This hypothesis is encouraged by the fact that the metabolic syndrome is getting more and more attention in the Western world and is regarded as an instigator for several diseases, such as arthritis, cardiovascular diseases and psoriasis (4, 18).

The metabolic syndrome comprises a profile including a combination of obesity, hypercholesterolemia, hypertension, dyslipidemia and impaired glucose tolerance. It has been demonstrated that the metabolic syndrome is associated with features of OA and, therefore, may be relevant in search for new targets (19-21). Since decades it is known that obesity, an important component of the metabolic syndrome, is a strong risk factor for the development and the progression of OA. It has been argued that the gain of fat mass leads to an increased loading of the knee joint, resulting in cartilage degradation. The fact that obese people also have an increased risk to develop OA in non-weight bearing joints, gives reason to consider features of the metabolic syndrome to be involved (22). This interesting new concept was studied in chapter 5. The intake of a high fat diet (HFD) by human c-reactive protein (hCRP, a marker for inflammation (23, 24)) transgenic mice led to an increased OA development compared to chow-fed control mice, which was not correlated with body weight or fat mass. Interventions with rosuvastatin (cholesterol-lowering) and rosiglitazone (a peroxisome proliferator-activated receptor (PPAR)-γ agonist, anti-diabetic) suppressed OA to a level comparable to that of control mice. As these drugs have different modes of action, but do have in common that they exert anti-inflammatory actions, we conclude that low grade inflammation (metabolic stress) plays a major role in the development of OA in this mouse model. Additionally, we found a correlation between OA grade at end point and the change in hCRP levels shortly after the start of the study. Extrapolation of these data to the human situation suggests that the responsiveness to a metabolic challenge can be used as a marker to estimate the individual susceptibility to develop OA later in life. Therefore, correlations between OA development and the metabolic susceptibility of a person should be explored in further investigations.
In chapter 6 we demonstrated the effect of hypercholesterolemia, another feature of the metabolic syndrome, on the development of OA. Several epidemiological studies have shown an association between OA and elevated cholesterol levels or atherosclerosis (a cardiovascular disease for which elevated cholesterol levels display an increased risk of disease development) (25-28). The elevated intake of cholesterol has been suggested to cause a certain grade of inflammation in mice and men (29). In this study, a dose-dependent effect of the intake of cholesterol on the development of OA in female APOE*3Leiden cholesteryl ester transfer protein (CETP) transgenic mice (a mouse model which resembles human lipoprotein metabolism, in contrary to wild type mice, and develops hyperlipidemia and atherosclerosis (30-32)) was observed. A moderate association between OA, cholesterol levels and atherosclerosis indicated that cholesterol is involved. However, the data also suggested that other processes evoked by the intake of cholesterol are involved in the pathogenesis of OA. Indeed, levels of some inflammatory markers were elevated due to the high intake of cholesterol in the diet. Again, we demonstrated that statins had a suppressive effect on the development of OA features. This cannot be fully subscribed to their cholesterol lowering capacities, as ezetimibe treatment (a cholesterol-lowering drug with a different mode of action) reduced OA development to a lesser extent while its lowering of cholesterol was comparable. Like in chapter 5, there was no role for increased mechanical forces due to body weight, as mice receiving cholesterol in their diet had body weights comparable to control mice.

In the study in chapter 7, we aimed to better understand the mechanism of HFD-induced OA. We showed that the intake of a very HFD by male APOE*3LeidenCETP mice led to the development of OA, but less severe than expected. Moreover, OA progression in the HFD group was even less than in control mice receiving chow diet. There were no effects of various interventions on OA development compared to the HFD group. We have several indications that can explain these results. The first is that there was no fructose in the drinking water, in contrast to a previous study with male APOE*3LeidenCETP mice on HFD, which developed severe OA. Fructose leads to a shift from HDL (‘good’ cholesterol) to VLDL (‘bad’ cholesterol). The hypothesis that a shift in lipoprotein profile is essential for the induction of OA, deserves more investigation as it would indicate a highly specific pathway for the pathogenesis of
OA. Furthermore, serum amyloid A levels (an acute phase protein) in the plasma of these mice were highly variable at the start of the study, which may have influenced their response to the HFD. An interesting observation in this study is that mice on a control diet already developed substantial OA. This gives reason to believe that an altered lipoprotein profile, compared to wild type mice, increases the sensitivity of these mice to develop OA.

The susceptibility of male and female mice also needs to be addressed. In general, male mice are more prone to develop features of the metabolic syndrome and to develop features of OA, which is confirmed in the hCRP mice described in chapter 5 (33-35). However, female APOE*3Leiden.CETP mice are more susceptible to develop high serum cholesterol levels and consequently more features of atherosclerosis (chapter 6), which is not observed in their male counterparts, even though they had an even higher cholesterol intake (data not shown). Based on these observations, it would be interesting to examine the effects of a HFD in combination with cholesterol on the development of female APOE*3Leiden.CETP mice.

In chapters 5 and 6 we demonstrated that features of the metabolic syndrome (obesity and hypercholesterolemia) induced the development of OA in mice. The low grade systemic inflammation evoked by these metabolic alterations may be held responsible for the initiation or progression of OA processes in the joint, but we cannot exclude the involvement of other factors. The effects of statins on the development of OA in mice contribute to the idea of a beneficial effect of statins in human OA. Although limited human data are available, it has been demonstrated that the use of statins is associated with a substantial reduction in knee, but not hip, OA progression (36). A plausible mode of action for the effect of statins is the inhibition of the formation of atherosclerotic plaques, as a consequence of high cholesterol exposure, and therewith improving the blood flow of subchondral bone. Furthermore, a direct effect on chondrocytes by targeting MMPs (37) is suggested. The only moderate correlations between cholesterol levels, features of atherosclerosis and the development of OA we found (chapter 6) do not contribute to these hypotheses. Based on our data, we propose that the main effect of statins can be subscribed to their anti-inflammatory properties, possibly by the inhibition of nuclear factor kappa B-driven processes.
Unraveling the pathways involved in obesity-associated low-grade systemic inflammation leading to OA may reveal new targets for intervention. Adipokines, which are secreted by adipose tissues as well as by several joint tissues, may be interesting targets. It has been demonstrated that leptin expression is higher in OA cartilage than in normal cartilage (38). Furthermore, visfatin, resistin and adiponectin levels are associated with features of OA (39). A study in leptin-deficient mice shows that these mice become very obese, but do not develop features of OA, indicating a possible role of leptin in the development of OA (40). With the knowledge that, until now, regular anti-cytokine therapies failed in the treatment of OA, these relatively newly discovered adipokines should be considered as promising targets for interference. We measured a significant increase in leptin and resistin levels in mice fed a HFD compared to mice receiving a control diet (chapter 5). However, these levels (measured at end point) were not correlated with the development of OA and were not affected by statins or rosiglitazone. This suggests that, if leptin is involved in HFD-induced OA, the inhibitory effect of rosuvastatin and rosiglitazone treatments is not mediated through direct acting on leptin, but possibly by influencing the downstream inflammatory processes that are regulated by leptin.

To link the local (chapters 2-4) and systemic (chapters 5 and 6) alterations on OA development, the following model can be proposed (figure 1): the elevated intake of, for example, fat or cholesterol in the diet leads to systemic alterations, giving rise to features of the metabolic syndrome and resulting in low-grade systemic inflammation. Due to the increased release of pro-inflammatory mediators in the blood, cells in the different joint tissues are activated and stimulated to secrete more pro-inflammatory mediators and/or matrix-degrading enzymes, therewith influencing cartilage homeostasis and contributing to OA development. The local inflammation and degradation products of cartilage can accordingly amplify the inflammatory response. In addition, different features of the metabolic syndrome may directly lead to modification of the different joint tissues, for instance by the accumulation of lipid droplets in the cells. This proposed paradigm strongly suggests considering OA as part of a bigger circle and deserves more investigation in patients sensitive to develop features of the metabolic syndrome (4).
Figure 1. Proposed paradigm of osteoarthritis (OA) development based on data in this thesis, including both systemic and local factors. An increased dietary intake of e.g. fat, cholesterol or sugar leads to the development of features of the metabolic syndrome, such as obesity and hypercholesterolemia, which concurrently cause a systemic inflammation. This state of the body will in turn induce several tissues in the joint to secrete inflammatory mediators and matrix-degrading enzymes, leading to the development of OA. Consequently, the inflammatory mediators and the degeneration products produced by the joint tissues will amplify the OA process. We cannot exclude that the elevated dietary intake of e.g. fat, cholesterol or sugar has a direct effect on the joint tissues and contributes to the progression of OA as well.
**Concluding remarks**

OA is a frequently occurring disease and multiple factors, such as genetic, environmental, mechanical, and diet contribute to the development, progression and severity. We identify obesity, high cholesterol and systemic inflammation associated with these conditions as major players in OA development, which may activate joint tissues to secrete inflammatory mediators and contribute to the initiation and progression of OA. Our work suggests that a stratification of OA patients with (features of) the metabolic syndrome as underlying mechanism is recommendable, to optimize the efficacy of clinical trials. Approaching OA as a disease induced by whole body metabolism, and integrating knowledge about different potentially active tissues in the OA process, will provide new insights for possible pharmacological interventions.
References
