

Dietary Influences on Arthritis



Increased synovial lipodystrophy induced by high fat diet aggravates synovitis in experimental osteoarthritis

Abstract

Background: Metabolic syndrome (MetS) may be associated with knee osteoarthritis (OA), but the association between the individual components and OA are not well-understood. We aimed to study the effect of hypercholesterolemia on synovial inflammation in knee OA.

Methods: OA was surgically induced in rabbits fed with standard diet (OA group, n = 10) or in rabbits fed with high fat diet (OA-HFD, n = 10). Healthy rabbits receiving standard diet (Control, n = 10) or fed with HFD (HFD, n = 6) were also monitored. Twelve weeks after OA induction, synovial membranes were isolated and processed for studies.

Results: Animals fed HFD showed higher levels of total serum cholesterol, triglycerides and C-reactive protein than control rabbits. Twelve weeks after OA induction, synovial membrane inflammation and macrophage infiltration were increased in rabbits with OA, particularly in the OA-HFD group. Extensive decrease of synovial adipose tissue area, adipocyte size and perilipin-1A synthesis were observed in the OA-HFD group in comparison to the OA and control groups. The HFD further increased the proinflammatory mediators IL-1 β , IL-6 and TNF in the OA synovium. However, the synovial gene expression of adipokines, such as leptin and adiponectin, were markedly decreased in the rabbits with OA, especially in the OA-HFD group, in correlation with adipose tissue loss. However, circulating leptin was upregulated in the HFD and OA-HFD groups.

Conclusion: Our results indicate that a HFD is an aggravating factor worsening synovial membrane inflammation during OA, guided by increased infiltration of macrophages and removal of the adipose tissue, together with a remarkable presence of proinflammatory factors. Synovial adipocytes and dyslipemia could probably play pivotal roles in OA joint deterioration in patients with MetS, supporting that the link between obesity and OA transcends mechanical loading.

Keywords: Osteoarthritis, Hypercholesterolemia, Synovial inflammation, Metabolic syndrome, Macrophages, Synovial adipose tissue, Adipokines

Background

Osteoarthritis (OA) is the most common joint disorder worldwide, characterized by joint pain, impaired mobility and structural changes in the joints. Although cartilage destruction is the main feature of the disease, every joint structure such as the synovium, bone, meniscus or muscle is affected, leading to the recognition of OA as a whole-organ disease [1]. Synovial inflammation is present in a substantial population of patients with OA and has been associated with different signs and symptoms of the disease, including increased pain and joint effusion, which could promote more rapid cartilage degeneration [2, 3]. Elevated thickness of the lining layer and greater presence and activation of synovial macrophages have been identified in cartilage degradation and osteophyte formation in both human and experimental OA [4–6].

OA is not merely a local disease, but there are different systemic processes that determine its progression. The concept of metabolic OA, coined in recent years, identifies a syndrome whereby the contribution of metabolic dysregulation and low-grade systemic inflammation to the progression of the disease has been firmly pointed out [7, 8]. The metabolic syndrome (MetS) comprises a cluster of conditions, including glucose intolerance, high blood pressure, hypercholesterolemia and hypertriglyceridemia, and obesity [9, 10]. The accumulation of the different components of MetS has been related to both the occurrence and progression of knee OA [11, 12]. However, little is known about the specific contribution of each of these metabolic alterations in OA progression, and specifically in the synovial damage associated with OA.

The contribution of obesity to OA progression is probably the most extensively studied association [7, 13]. In fact, obesity has been pointed out as the main contributing factor for the association between OA and MetS, in studies showing a markedly attenuated association after adjustment for body mass index [14]. However, OA is also common in non-weight bearing joints of obese persons, suggesting a systemic mechanism rather than a simply mechanic phenomenon [7].

The possible role of hyperlipidemia in mediating obesity-related effects on OA has been explored in different studies. Contradictory results have been published on the relationship between serum lipids and OA incidence in humans, probably due to the presence of obesity and being overweight as confounding factors [15]. In turn, different experimental studies have suggested that hypercholesterolemia could be mainly associated with osteophyte generation rather than to aggravation of cartilage lesions [16, 17]. Macrophages, endothelial cells and fibroblasts are dominant cells within the synovium, together with abundant adipose tissue that constitute the synovial stroma, and every component is sensitive to changes in lipid levels [17, 18].

Adipokines have been considered at least partially responsible for the link between systemic metabolic alterations and OA [19-21]. Adipokines are essentially released by adipocytes and exhibit pleiotropic functions both in central and peripheral systems, including blood pressure control, hemostasis, food intake, energy expenditure, cell metabolism and inflammation, among others [19–21]. They are also synthesized by joint cells during OA, mainly by the synovium, cartilage and intraarticular fat tissue, and have been demonstrated to play proinflammatory and catabolic or anabolic roles in OA pathophysiology. It has been hypothesized that the altered circulating patterns of adipokines induced by obesity could be responsible for the deleterious effect of this disease on OA. However, it is not known whether expression and release of adipokines in the joint could be modulated by metabolic factors during OA, thus contributing to disease progression.

Therefore, this work aimed to study the effect of hypercholesterolemia, without any other component of

the MetS, on synovial inflammation in an experimental model of knee OA. We have also determined the synovial expression and systemic concentration of adiponectin and leptin, two adipokines involved in joint deterioration associated with metabolic OA.

Methods

Animal model

Thirty-six New Zealand male white rabbits, 13-15 weeks of age, weighing 2.5–3.0 kg (Granja San Bernardo, Navarra, Spain) were housed individually in cages with transparent walls (0.5 m cage height and 0.6 m² floor space) exposed to a 12-hour light/dark cycle.

After 2 weeks of adaptation to our facilities, 16 rabbits started receiving a high fat diet (HFD) (0.5% cholesterol + 4% peanut oil; S9504-S010; 22% kJ from fat, 20% kJ from proteins and 58% kJ from carbohydrates; Ssniff, Soest, Germany) administered ad libitum (Fig. 1a, time point 0). At this time point, there were no significant differences between the group on HFD and the one that remained at standard diet (112; 10% kJ from fat, 17% kJ from proteins; 73% kJ from carbohydrates; Safe-Diets, Augy, France) regarding body weight or age, as can be observed in Fig. 1b. Six weeks later, bilateral osteoarthritis (OA) was surgically induced in 10 of these 16 animals (OA-HFD group, n = 10) by anterior cruciate ligament transection and partial medial meniscectomy [22] (week 6, Fig. 1a). At this time point, OA was also induced in 10 rabbits fed with standard diet (OA group, n = 10). The surgery was always performed in the morning after overnight fasting, under general anesthesia (intramuscular administration 20 mg/ml xylazine (Rompun, Bayer, Kiel, Germany) and 50 mg/ml ketamine (Ketolar, Pfizer, Hameln, Germany) in a 3:1 ratio), under aseptic conditions in an operating room. Besides, 10 rabbits fed with standard diet (control group, n = 10), and six rabbits fed with the HFD (HFD group, n=6) underwent no experimental intervention.

Two animals in the OA-HFD group died during the time of the study due to OA surgery complications. Weight gain was monitored every week. Systolic blood pressure (SBP) was measured before OA surgery and 1 week before euthanasia, using a High Definition Oscillometry unit (DVM Solutions Houston TX, USA) adapted to the hind paw of the rabbits. This non-invasive method for SBP measurement has been validated in cats, an animal physiologically and anatomically similar to rabbits [23].

Twelve weeks after OA induction (Fig. 1), overnightfasted rabbits were bled from their marginal ear vein in the morning and killed by an intracardiac injection of pentobarbital (50 mg/kg, Tiobarbital, Braun medical S.A. Barcelona, Spain). The articular cavity of each rabbit was



accessed by sectioning the patellar tendon and taking out the patella, thus the entire infrapatellar synovial membrane (SM) was collected by the same operator (AL-V), always taking the same specimen from each animal (Additional file 1: Figure S1). The SM was not separated from the adipose tissue [24, 25]. Half of the SM containing both stroma and lining was then fixed in 4% paraformaldehyde for 24 h and then was embedded in paraffin for histological studies; the other portion was immediately frozen and used for molecular biology studies. Femoral condyles were also removed and fixed in 4% buffered paraformaldehyde, decalcified for 4 weeks in a solution of 10% formic acid plus 5% paraformaldehyde, and embedded in paraffin [26]. The left and right SM and condyles were analyzed as independent samples.

Serum and synovial measurements

Glucose, total cholesterol, HDL cholesterol and triglyceride levels were assayed by automatic techniques as previously described [27, 28]. Adiponectin, leptin and plasma C-reactive protein (CRP) were measured by ELISA using commercial specific kits (SEA605Rb and SEA084Rb, respectively, USCN, Houston TX, USA and ab157726, Abcam, Cambridge, UK). Both adiponectin and leptin were measured in synovial tissue homogenates. For this purpose, total protein from the SM was extracted as described elsewhere [28, 29], and equal amounts of proteins diluted in the same volume for each knee were tested by specific ELISA for each adipokine.

Histological synovitis grading

The SM from both knees of each rabbit were sectioned 5- μ m thick and stained with hematoxylin and eosin, and Masson's Trichrome. Synovitis was evaluated according to the Krenn score [30] as previously described [28], assessing lining hyperplasia, activation of synovial stroma related to fibrosis, and tissue infiltration. Each item was evaluated by a blinded observer using a subscale of 0– 3 points, where 0 indicated absence, 1 mild, 2 intermediate and 3 strong evidence of synovitis. The total score was obtained from the sum of partial grades with a maximum total score of 9.

Histological cartilage grading

The decalcified femurs were cleaved in a sagittal plane along the central portion of the articular surface of each medial femoral condyle corresponding to the weightbearing area, and subsequently embedded in paraffin wax. Cartilage was sectioned $5-\mu$ m thick and stained with hematoxylin/eosin and alcian blue to evaluate cartilage abnormalities. These samples were evaluated using a modified version of Mankin's grading score system, which analyses four different parameters with a total score up to 21: structure (0–8), proteoglycan staining (0–6), loss of chondrocytes (0–4), and clone formation (0–3) [25, 31].

Immunohistochemical analysis

SM infiltrating macrophages were visualized using mouse anti-rabbit macrophage monoclonal antibodies

(mAb) (RAM11; Dako, Glostrup, Denmark) as previously described [27], whereas adipocytes were identified with anti-perilipin A1 (PLIN, Abcam, ab61682, 1/100 dilution) antibody. To evaluate RAM11-positive immunoreactivity, five photographs were obtained using a Leica DMD108 digital micro-imaging instrument (Leica, Microsystems, Inc. Buffalo Grove, IL, USA) at × 10 magnification ensuring constant light exposure. Each image was analyzed with ImageJ software (NIH, Bethesda, MD, USA), and the percentage of positive area was calculated with the Color Deconvolution plugin [32] in relation to the total tissue area. For each SM, the percentage of positive staining was calculated as the mean of these five images corresponding to the same SM [28].

Adipose tissue area (%ATA) and adipocyte size were analyzed in PLIN-stained slides using the Coreo Iscan Au Scanner (Ventana Medical Systems, USA) and ImageJ software. Five representative images at × 20 magnification were used to identify stained adipocyte boundaries. Every white area showing no immunoreactivity to PLIN was manually removed. Finally, the area of each adipocyte was measured and the average size was calculated for each SM sample.

Western blot

Briefly, total protein was extracted from the SM as described elsewhere [28, 29]. Protein extracts were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The following primary antibodies were applied overnight at 4 °C: anti-human collagen type I (Col I, Merck Millipore, Billerica, MA, USA); anti-human PLIN (Abcam), anti-rabbit IL-1, anti-rabbit IL-6, anti-rabbit TNF (Cloud-Clone Corp, Houston TX, USA), and antihuman cyclooxygenase-2 (COX-2) (Santa Cruz Biotechnology, Dallas TX, USA). Loading control was performed employing EZBlue gel staining reagent (Sigma-Aldrich). Results were normalized relative to total protein presence and expressed as arbitrary densitometric units [28] (AU).

Gene expression

Total RNA was extracted from SM using TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. RNA was

Table 1 Characterization of the rabbit model

reverse-transcribed and RNA expression was quantified using the StepOnePlus[™] detection system and StepOne[™] software v2.2 (Applied Biosystems) as previously described [27, 33]. TaqMan[®] primers and probes were used to measure adiponectin (Oc03823307_s1), leptin (Oc03395809_s1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH Oc03823402_g1) as endogenous control. Target genes were normalized relative to the expression of the endogenous control.

Statistical analysis

Histological analyses were carried out by two observers (AL-V and RL) in a blinded fashion. Scoring and quantitative analyses were averaged for the images and sections from the same SM to calculate the value per sample for statistical analyses. Each limb was analyzed as an independent sample for the studies of synovial tissue. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). We employed the non-parametric Kruskal-Wallis test with a post-hoc correction for (Dunn's procedure) for comparisons between multiple groups, and the Mann-Whitney U test for comparisons between two groups. P values less than 0.05 were considered significant. Data are expressed as the mean \pm 95% confidence interval (CI).

Results

Metabolic profile

We first studied the effect of the HFD in rabbits over an 18-week period in order to ensure the different characteristics that have been associated with MetS, such as being overweight, hypertension, basal glucose and dyslipidemia. There were no significant differences between the different groups in weight gain at week 6, the time point of surgery to induce OA (Fig. 1b). At the end of the study after 18 weeks of HFD feeding, rabbits fed a HFD gained less weight than controls (Table 1). Animals in the OA and OA-HFD groups also gained less weight than controls, probably due to discomfort associated with knee surgery. Rabbits in the HFD, OA and O-HFDA groups maintained similar SBP to control animals during the whole study period (Table 1). After 18 weeks of HFD, rabbits did not have any alteration in basal

Group	Weight gain (kg)	SBP (mmHg)	Basal glucose (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	CRP (µg/ml)
Control ($n = 10$)	1.9 (1.7–2.0)	100 (93–107)	109 (101–116)	32.2 (8.9–55.5)	48 (32–64)	11.1 (8.6–13.6)	15.95 (8.2–23.7)
HFD (<i>n</i> = 6)	1.5* (1.1–1.7)	103 (87–119)	105 (98–112)	1876* (1139–2613)	253* (4–502)	12.5 (6.9–18.1)	39.14 (1–77.3)
OA (<i>n</i> = 9)	1.5* (1.2–1.8)	108 (96–119)	105 (94–115)	28.3 (18.2–38.4)	67.3 (40–95)	13.8 (10.2–17.3)	6.9 (3.8–10.0)
OA-HFD (n = 8)	0.7* (-0.12–1.6)	106 (96–117)	104 (98–111)	2050* (1587–2514)	290* (99–480)	15.6 (11.0–20.3)	18.1 (5.5–30.7)

Measures were obtained from serum or plasma samples taken just before animals were killed. Values represent mean with 95% confidence interval *HFD* high-fat diet, *OA* osteoarthritis, *SBP* systolic blood pressure, *HDL* high-density lipoprotein, *CRP* C-reactive protein *P < 0.05 vs. Control

glucose levels or in oral glucose tolerance (data not shown) in comparison to control animals (Table 1). However, there was increased total serum cholesterol and triglycerides in the rabbits fed HFD in comparison to controls. Although no significant differences were observed in circulating CRP levels between either the HFD or OA-HFD groups and controls (Table 1), there was a significant increase in CRP in animals fed with HFD vs. those fed with the standard diet, as a result of grouping rabbits into HFD plus OA-HFD and control plus OA (27.1 \pm 7.3 vs 10.5 \pm 1.9, p = 0.026).

Histological synovial inflammation and cartilage damage

Rabbits fed HFD had mild lining hyperplasia, discrete presence of infiltrating cells and a slight increment in stromal fibrosis, and thus the synovitis score was significantly higher than that observed in healthy controls (Fig. 2b, 2i). The OA group had a higher synovitis score than the control and HFD groups, with similar lesions to those described in synovitis in humans with advanced OA: mild to moderate lining hyperplasia, discrete presence of inflammatory cells, and stromal activation. The OA-HFD group had mild lining thickening, a clear increment in stromal cellularity, presence of infiltrating cells and inflammatory foci. All samples had enlarged stroma with an intense cell density (Fig. 2d). The synovitis score in the OA-HFD group was significantly higher than in the other groups (Fig.2i).

The HFD administration did not modify the histological appearances of cartilage damage, with the HFD group having a similar score to control animals (HFD 2.8 ± 1.5 vs. control 2.9 ± 1.0; *p* not significant (NS)). In addition, HFD did not significantly modify the histopathological damage in the cartilage in the OA-HDF group in comparison to the damage observed in the OA group (OA 15.2 ± 2.1 vs. OA-HFD 14.0 ± 2.7; *p* NS).

Macrophage infiltration and presence of foam cells

There was moderate presence of macrophages in the SM of rabbits fed HFD, which were especially localized in the lining layer (Fig. 2f, j). Lipid droplets were identified in their cytoplasm and their morphological shape resembled to pro-atherosclerotic foam cells, as previously described [5, 28] (Fig. 2f). RAM11 staining was scarce in the SM in the OA group, whereas there was extensive infiltration of RAM11-positive cells in the OA-HFD group to a much greater extent than in the HFD and OA groups (Fig. 2g, h, j). They were both consistently localized in the lining and sub-lining layers in every sample, and had the characteristic phenotype of foam cells [5, 28] (Fig. 2h).

Characterization of synovial stroma

Whereas healthy SM was mainly composed of adipocytes with little surrounding matrix, we observed patchy distribution of some fibrotic areas in the HFD group (Fig. 3a, b). OA membranes had a highly vascularized fibrotic stroma with some lax and dense stents, greencolored on Masson's Trichrome staining (Fig. 3). OA-HFD samples also had highly vascularized fibrotic membranes. The quantification of col I protein revealed a clear increase in the fibrotic content of the SM in the OA and OA-HFD groups (Fig. 3e-f) in comparison to control and HFD groups.

Adipose tissue area and adipocyte size in the SM

We quantified the adipose tissue fraction using PLIN staining, a distinguishing marker of adipocytes [34, 35]. A clear diminution in the percentage of adipose tissue area (%ATA) in the SM in the OA and OA-HFD samples in comparison to control and OA groups was observed, which was even lower in the OA-HFD than in the OA group (Fig 4a-e). Furthermore, SM adipocytes were significantly smaller in both the OA and OA-HFD groups than in the controls (Fig. 4f). The shape of these cells in control tissues was regular (Fig. 4a), whereas we observed high heterogeneity in the appearance of these cells in the SM in the OA and OA-HFD groups (Fig. 4c, d). Adipocyte size further decreased in the SM in the OA-HFD group in comparison to the OA group (Fig. 4f). PLIN content in the SM was also evaluated by western blot. In correlation with the %ATA, there was diminution in the SM PLIN in the OA and OA-HFD groups in comparison to the controls (Fig. 4g, h). Of note, the synthesis of PLIN was also significantly diminished in the OA-HFD group in comparison to the OA group.

Adipokine gene expression and concentration in SM and serum

Rabbits in both the HFD and OA groups had a clear decrease in leptin and adiponectin gene expression in the SM in comparison to controls (Fig. 5a, b). We observed an additive effect of these interventions in the OA-HFD group, where the gene expression of both leptin and adiponectin was significantly lower to that observed in the HFD and OA groups (Fig. 5). Interestingly, there was significant correlation between adipokine gene expression and the %ATA (R = 0.746; p = 0.001 for leptin expression; R = 0.732; p = 0.002 for adiponectin expression). Leptin levels in the SM measured by ELISA were decreased in the HFD group in comparison to controls, and it was also significantly reduced in the SM in the OA-HFD group in comparison to controls, the HDF and the OA groups (Fig. 5c). Adiponectin concentration only significantly diminished in the HFD group in comparison to controls (Fig. 5d). There was no correlation between the presence of these proteins and the %ATA in the SM.

However, HFD increased the circulating concentration of both adipokines, and there were no significant



(See figure on previous page.)

Fig. 2 Histopathological and macrophage analysis in the synovial membrane (SM). **a-d** Representative sections of SM stained with hematoxylin-eosin or **e-h** stained with a monoclonal anti-rabbit macrophage antibody (RAM11) from Control rabbit (**a** and **e**); rabbit fed with a high-fat diet (HFD) (**b** and **f**); osteoarthritic (OA) rabbit (**c** and **g**); and OA rabbit fed with a HFD (OA-HFD) (**d** and **h**). **a-d** Scale bar = 100 μ m. **e-f** Scale bar = 100 μ m. **i** Synovitis score quantified as described in "Methods. **j** Quantification of RAM11-positive area represented as percentage of total area. Data from individual measurements and mean for each group are shown. *n* = 12–20 SM per group for histopathological analysis; *n* = 10–16 SM per group for RAM11 analysis

differences between the HFD and OA-HFD group in the serum concentration of these mediators (Fig. 5e, f).

Synovial proinflammatory mediators

We then explored whether the HFD was able to modify the presence of different proinflammatory cytokines, such as IL-1 β , IL-6, TNF and COX-2 in the SM of rabbits in the OA group. As expected, western blot studies that OA induced a marked increase in the presence of all the studied proinflammatory mediators in comparison to control animals. The presence of hyperlipidemia further increased the presence of IL-1 β , IL-6 and TNF in the SM in the OA-HFD group in comparison to the OA group (Fig. 6).





Discussion

In this study, we have shown that HFD aggravated OA synovitis, by inducing severe tissue architecture disorganization of the synovium, along with remarkable intensification of the proinflammatory cytokines IL-1 β , IL-6 and TNF, and extensive infiltration of macrophages. However, HFD did not have any effect

on the aggravation of the pathologic change in cartilage associated with OA. A relevant histological synovial alteration was the significant loss of synovial adipose tissue content, in correlation with decreased leptin and adiponectin gene expression.

In order to isolate the effect of hyperlipidemia, we employed an experimental model of HFD intake that



high-fat diet; OA, osteoarthritis

was not associated with weight gain [28]. The lack of significant weight gain in the HFD group has been previously reported and attributed to the animal selfregulation of caloric intake [36]. In fact, animals in both the HFD and OA-HFD groups had a significant decrease in weight gain, which was probably related to the increase in systemic inflammation induced by the diet [28]. Different studies using lipid-rich diets have not been able to adequately apportion the contribution of added mechanical load and hyperlipidemia in OA, a factor that was avoided in our experiments. Different patterns of synoviopathy have been described in patients with OA, both in late and early disease, such as those with an increased fibrotic component or those essentially characterized by augmented inflammatory parameters [37]. In our rabbits, OA synovitis was associated with a significant increment of fibrotic tissue and partial loss of adipose tissue, and scarce presence of macrophages. A HFD induced both qualitative and quantitative changes in the SM in the rabbits with OA. However, HFD did not significantly aggravate cartilage damage in either the HFD group or the OA-HFD group.



These data are in line with previously published results [38, 39], and suggest that the aggravation in synovial inflammation induced by HFD is not a secondary event induced by more severe pathological change in the cartilage.

The higher grade of synovial inflammation in the OA-HFD group was characterized by the remodeling of adipose tissue and by adipocyte loss. The remaining adipocytes had heterogeneous morphology and were significantly smaller in comparison to the OA and control groups, confirmed by the decreased presence of PLIN. HFD further increased synovial macrophages, most of them with the appearance of foam cells, whereas the fibrotic component was similar to that observed in the OA group. To our knowledge, this is the first report of correlation between synovial inflammation and loss of adipose tissue in this localization. Contradictory reports have been published on the contribution of the volume or area of the intra-articular fat tissue to joint deterioration and OA symptoms [40–42]. However, the alterations in adipocyte size, morphology, loss of adipose tissue with increased fibrosis and inflammatory content have been well-described in inflamed adipose tissue in other anatomic localizations, and described as lipody-strophy [43, 44]. The study of the synovial fat pad as an independent adipose intra-articular tissue has contributed to its identification as a crucial player in OA progression [45], although lack of recognition of the

synovium as a whole, integrated, functional and structural unit hampers the understanding of the mechanisms involved in the synovial alterations in OA. Histologically, synovial lining, adipose sub-lining and synovial fat pad represent a continuum. Sub-lining adipose tissue and the fat pad seem to share a common inflammatory state, both in cell content and cell phenotype, induced by the disease process more than by tissue-specific signals [24, 46].

The mechanisms by which adipose tissue can be replaced by fibrotic tissue in the OA synovium have not been fully elucidated. However, the increase in the hypoxia-associated mediators, induced by biomechanical alterations and proinflammatory cytokines, could be at least partially responsible for this phenomenon. An increase in hypoxia-induced factor-1 (HIF-1) α has been described in the OA synovium, in correlation with greater joint destruction [47, 48]. In adipose tissue, with a similar structure and cellular component to that observed in the stroma of the SM, HIF-1 α induces tissue fibrosis and inhibits pre-adipocyte differentiation [49]. Furthermore, in inflamed adipose tissue from mice fed a HFD, HIF-1 α -stimulated macrophages form highly hypoxic structures called crown-like structures (CLS), comprising macrophages encircling dead or dying adipocytes [50]. Indeed, we have previously identified CLS in the OA synovium in both human and hypercholesterolemic rabbits with synovial inflammation [5, 28].

Hyperlipidemia in rabbits in the OA group did not seem to enhance the presence of fibrosis-associated proteins, such as col I. However, it evoked a dramatic increase in macrophage infiltration in the synovium and greater decrease in adipose tissue content. Dyslipemia has been directly related to macrophage infiltration and inflammation in the synovium and adipose tissue [51]. In hyperlipidemic mice with OA, the synovial proinflammatory macrophage subset was identified as responsible for an increase in TNF synthesis and extracellular matrix remodeling in the synovial membrane [51]. In line with these data, we identified greater TNF expression in the synovium in the OA-HFD group that paralleled the increased macrophage density in this tissue. Although little is known about the metabolic regulation of synovial macrophages, prolonged lipid exposure could result in failure of the lipid-handling mechanisms, leading to different lipotoxic events, such as those described in obesity-associated insulin resistance, atherosclerosis and other inflammatory diseases related to MetS [52]. Thus, hyperlipidemia could drive M1 macrophage polarization in the OA synovium, resulting in a major presence of proinflammatory cytokines, as has been described in adipose tissue [52, 53]. Furthermore, adipocyte apoptosis and impaired adipogenesis have been also associated with the increased lipolysis induced by over-nutrition or HFD feeding [52]. Although hyperlipidemia could aggravate OA synovial inflammation, increasing macrophage density and adipose tissue destruction, the presence of hyperlipidemia per se could only have limited effects on SM alterations, as recently reported in HFD-fed mice [54].

Leptin and adiponectin gene expression diminished in the SM in the OA and OA-HFD group in comparison to control animals. These results appear to correlate with the amount of intra-articular adipose tissue rather than with the presence of a proinflammatory milieu. Furthermore, circulating leptin was significantly increased in HDF-fed animals, probably due to the effect of the diet on the extra-articular fat tissue [55]. Our data are in line with previous reports indicating that hyperlipidemia could be an aggravating factor for OA through the stimulation of systemic proinflammatory mediators [56]. In the OA group we also found increased circulating leptin as previously described in human and experimental OA, related to joint damage [19]. Therefore, our data do not support the hypothesis that hyperlipidemia could be an aggravating factor in metabolic OA, stimulating adipokine expression within the intra-articular adipose tissue. Different joint cells, such as chondrocytes or bone cells, could be responsible for adipokine synthesis in response to biomechanical or proinflammatory stimuli [19, 20].

Conclusions

In summary, these data show that HFD aggravates the inflammation in the SM of rabbits with OA by inducing an increase in the infiltrating macrophages in the synovium, together with macrophage and metabolic-mediated remodeling of adipose tissue, and further elevation of proinflammatory cytokines. The lipotoxic effects induced by dyslipemia in adipocytes and macrophages could play a decisive role in the joint deterioration of patients with OA and MetS, supporting the hypothesis of a plausible link between obesity and OA going beyond mechanical loading.

Vitamin D and juvenile idiopathic arthritis

Abstract

Background: Vitamin D has been implicated in the pathogenesis of autoimmune diseases. While the roles of vitamin D in other autoimmune diseases have been investigated, less is known about the role of vitamin D in chronic childhood arthritis.

Main body: This review summarizes and evaluates evidence relating to 25-hydroxyvitamin D (25(OH)D) and chronic childhood arthritis. A scoping literature review was conducted using Ovid Medline, Ovid Embase, Cumulative Index to Nursing and Allied Health Literature, Web of Science and Scopus. Further, we geo-mapped the results of the studies to identify the patterns of the association between vitamin D and chronic childhood arthritis across the globe. Of 38 studies reporting 25(OH)D concentrations in childhood chronic arthritis, 32 (84.2%) reported that a significant number of children had suboptimal (< 75 nmol/L) status.

Conclusion: The data indicate suboptimal vitamin D status in children with chronic arthritis. Further, the association between low vitamin D and increased arthritis activity follow a north-south geographical gradient.

Keywords: Vitamin D, Childhood arthritis, Juvenile idiopathic arthritis

Background

Arthritis is among the most common chronic diseases in children. Juvenile Idiopathic Arthritis (JIA) is the current nomenclature applied to denote a group of clinically distinguishable subsets that share chronic, childhood-onset arthritis of unknown cause as a unifying feature. The etiologies of JIA are unknown and the pathogeneses unclear but are likely multifactorial. Among epidemiologic studies there is substantial variability in the frequencies with which JIA and its respective subtypes are reported to occur; chronic arthritis prevalence rates range from 0.07 to 4.01/1000 children and annual incidences from 0.008 to 0.226/1000 children [1]. Putative explanations for the disparities in reported juvenile arthritis prevalence rates include, as examples, differences in diagnostic criteria applied (specifically, JIA or the earlier Juvenile Rheumatoid Arthritis (JRA) [2] or Juvenile Chronic Arthritis (JCA) [3] classification systems) and in case ascertainment methods.

While methodologic inconsistencies among JIA epidemiologic studies might account for perceived prevalence differences, actual differences might occur as a consequence of genetic, ethnic, environmental, and lifestyle influences. Vitamin D status is potentially governed by these same factors; vitamin D receptor genotype, ethnically related skin tone and clothing, environmental variations in exposure to ultraviolet B radiation relating to the latitude of residence and season, and vitamin D nutritional intake are factors that modulate vitamin D concentrations.

As an immune and inflammatory mediator, vitamin D is implicated in the pathogenesis of autoimmune diseases including, as examples, multiple sclerosis, type 1 diabetes, rheumatoid arthritis, Crohn's disease, and chronic childhood arthritis [4-6]. Cells involved in innate and adaptive immune responses such as macrophages, dendritic cells, T cells, and B cells express enzymes required to activate and respond to vitamin D [7-9]. Cytochrome p450 27B1 (CYP27B1) is the enzyme required to synthesize 1,25-dihydroxyvitamin D (1,25(OH)2D), the active form of vitamin D, from circulating 25-hydroxyvitamin D (25(OH)D). The actions of 1,25(OH)2D are mediated by its binding to the vitamin D receptor (VDR), a nuclear transcription factor. VDR then binds to the Vitamin D Response Element (VDRE), a genetic sequence located in the promotor region of genes regulated by vitamin D [7– 9]. Vitamin D tends to suppress the immune response [6]. Consequently, low vitamin D concentrations are associated with an increase in pro-inflammatory mediators and more active disease [6, 10] consistent, for example, with the observation that low serum 25(OH)D is associated with increased disease activity in rheumatoid arthritis [11].

Reports of relationships between vitamin D and chronic childhood arthritis are derived from studies having different methodologic approaches, originating from multiple geographic regions, and comprising demographically disparate populations. Since the last analysis of these reports in 2013 [12] the number of studies reporting 25(OH)D concentrations in children with chronic arthritis have increased from 14 to 38. An updated, systematic analysis of pertinent literature should help to further refine understanding of the relationships between vitamin D and juvenile arthritis, contribute to optimizing management of vitamin D status in children with arthritis, and clarify vitamin D's potential role in mediating disease pathogenesis.

Scoping reviews are methodologic approaches for thoroughly distilling and synthesizing information derived from different studies having varied designs. The purposes of scoping reviews are to not only capture key concepts that can guide care but also to recognize knowledge gaps that can inspire future research priorities [13].

Although nomenclature applied to chronic childhood arthritis classification systems has changed over the years, JIA is the current terminology. For clarity, this review will hereafter use the term JIA to encompass the forms of chronic childhood arthritis also included in the JCA and JRA classification systems. However, in this review, when quoting the literature, we use the terminology for chronic childhood arthritis (JCA, JRA, or JIA) that was applicable at the time the cited reference was published [14]. Here we report the results of a vitamin D- JIA scoping review that summarizes, synthesizes, evaluates, and interprets pertinent evidence from the literature to address the following research questions: 1) What is the relationship between vitamin D status and the occurrence of JIA? 2) What is the relationship between vitamin D status and childhood arthritis activity? 3) What is the relationship between vitamin D status in JIA and medication use? 4) What is the relationship between vitamin D status and geographic and demographic characteristics in children with JIA?

Methodology

To ensure a comprehensive literature scan, this scoping review applied the iterative methodological framework escribed by Arksey and O'Malley, with refinements by Levac et al. and Colquhoun et al. [14-16]. Five biomedical literature search engines were accessed, in the following sequence: Medline (using Ovid), Embase (using Ovid), Cumulative Index to Nursing and Allied Health Literature (CINHAL), Web of Science, and Scopus. Reference lists within each of the publications retrieved from the web-based searches were scanned to ensure that no relevant citations were missed. Medical Subject Heading (MeSH) terms used for retrieval were "vitamin D" and "juvenile arthritis". The search term "juvenile arthritis" was general enough to capture citations that referred to JCA, JRA and JIA classification terms (Table 1). Search results, which included published articles, letters, and abstracts

	American College of Rheumatology 1977 [2]	European League Against Rheumatism 1978 [3]	International League Against Rheumatism 1994 and 2001 [17]
Classification Title	Juvenile Rheumatoid Arthritis	Juvenile Chronic Arthritis	Juvenile Idiopathic Arthritis
Symptom duration	Minimum 6 weeks	Minimum 3 month	Minimum 6 weeks
Subtypes	Systemic	Systemic	Systemic
	Polyarticular	Polyarticular	Polyarthritis RF negative
		JRA (RF positive Polyarticular)	Polyarthritis RF positive
	Pauciarticular	Pauciarticular	Oligoarthritis
			Persistent
			Extended
		Juvenile psoriatic	Psoriatic arthritis
		Juvenile ankylosing spondylitis	Enthesitis-related arthritis
		Arthritis associated with inflammatory bowel disease	
			Undifferentiated arthritis

Table 1 Comparison of classification systems of chronic childhood arthritis^a

^aPrior to 1997, two chronic childhood arthritis classification systems were used. The American College of Rheumatology (ACR) [2] classification criteria referred to chronic childhood arthritis as Juvenile Rheumatoid Arthritis (JRA) and the European League Against Rheumatism (EULAR) applied the term Juvenile Chronic Arthritis (JCA) [3]. Differences between the two classification systems hindered exchange and comparison of data between the two systems [61]. To reconcile differences between ACR and EULAR criteria, the International League Against Rheumatism (ILAR) JIA criteria were introduced. This table provides a comparison of diagnostic criteria [17]. The ILAR classification system defines JIA as all forms of inflammation of one or more joints beginning in children younger than age16 years [17]. JIA is further classified into seven categories based on inclusion and exclusion criteria according to features present within the first six months of disease. The seventh category includes those who do not fit into one category, meet criteria for more than one category, or have exclusion criteria that preclude assigning a category

from conference proceedings, were collated and duplicates of articles removed. Publication titles and abstracts were then screened for relevance to the subject of vitamin D in children with idiopathic chronic arthritis as defined by JIA, JCA, or JRA classification criteria [2, 3, 17]. The full texts of relevant articles were then reviewed.

Inclusion criteria for the review were 1) study conducted in humans, 2) 25(OH)D concentrations reported, 3) participants having a diagnosis of JCA, JRA, or JIA, and 4) JCA, JRA, JIA diagnosis without an associated coexistent autoimmune disease. Exclusion criteria were 1) study conducted in animals, 2) the presence of an associated autoimmune disease 3) pregnant or lactating subjects, 4) 25(OH)D concentrations not reported), and 5) review articles. The process applied to identify eligible articles for the review is shown in Fig. 1 [18]. Articles in any language were eligible; however, no non-English language articles without at least an English abstract were found. From all relevant articles retrieved the following information was extracted: juvenile arthritis classification system (JCA, JRA, or JIA); sample size; sex ratio; patient age (at baseline or time of study if the study was cross-sectional); geographic location (country, city, and latitude and longitude), year of study; 25(OH)D concentration; study conclusion; and, if applicable, control group sample size, characteristics, and 25(OH)D concentration. The latitudes and longitudes reported were that of the city where the study was conducted; if unavailable, the province, state or region's center latitude was used and, as a last resort, the country's central latitude was used. 25(OH)D status by season of measurement was not reported in any of the articles found and therefore could not be considered.

Geographic Information Systems (GIS) mapping was performed using ArcGIS version 10.4 to visualize studies by juvenile arthritis classification, 25(OH)D status, location, and latitude. For all reported studies, an additional map comparing the difference in 25(OH)D concentration between those reporting active versus inactive disease was made.

Defining vitamin D status

Vitamin D deficiency is defined as a serum 25(OH)D concentration less than 30 nmol/L, a 25(OH)D concentration between 30 and 50 nmol/L is considered insufficient, greater than 50 nmol/L is considered sufficient and a 25(OH)D status greater than 125 nmol/L is considered at risk of adverse effects [19]. These values are based on the Institute of Medicine (IOM) review of published research focused on determining the optimal vitamin D concentration for maximal calcium absorption, prevention of rickets, reduction of fracture risk and prevention of osteomalacia in healthy populations [19]. In the most recent review of vitamin D requirements, the IOM concluded that there was inadequate information to make intake recommendations in relation to other biologic roles of vitamin D [19]. The current recommended dietary allowances (RAD) of vitamin D are 400 IU from 0 to 12 months of age and 600 IU per day from 1 to 18 years of age [19].

The Endocrine Society has published clinical practice guidelines for patients at risk of vitamin D deficiency [20]. The Society recommends that at-risk populations, including "obese children and adults and children and adults on anticonvulsant medications, glucocorticoids, antifungals such as ketoconazole, and medications for acquired immune deficiency syndrome be given at least two to three times more vitamin D for their age group to satisfy their body's vitamin D requirement" [20]. The optimal 25(OH)D concentration suggested by the Endocrine Society is 75 nmol/L. To meet this concentration it is recommended that 400-1000 IU be given between 0 to 12 months of age, 600-1000 IU per day from 1 to 8 years of age, and 1500-2000 IU for children between ages 9-18 years [20]. These recommendations, however, are not specific for children with chronic arthritis.

Results of scoping review and discussion

Considerations when evaluating the role of vitamin D in JIA include vitamin D requirements for this population and the role that vitamin D plays in disease activity. Using the specified MeSH search terms (vitamin D and childhood arthritis), 386 reports (full-text articles, conference abstracts, and letters to the editor) were identified. Thirty-eight studies met the inclusion criteria and are the subject of this review (Table 2). One meta-analysis reported cumulative 25(OH)D concentrations from fourteen studies comprising children with JIA, JCA, and JRA and other rheumatic conditions; this meta-analysis was not included in our scoping review but is referenced in the discussion [12]. This present review summarizes accumulated evidence on vitamin D and chronic childhood arthritis by disease activity and latitude. Additionally, this study provides new information about differences in 25(OH)D status between healthy controls and children with JIA.

Vitamin D status in relation to chronic childhood arthritis classification

Twenty-one of the 38 studies (55.3%) reported 25(OH)D status for patients with JIA [21–40], eight (21.1%) for patients with JRA [41–48] and five (13.2%) for JCA patients [49–53]. Additionally, there were four studies that included patients with juvenile arthritis and other rheumatic diseases [54–57]. As the JRA classification system tended to be applied in North America and the JCA classification in Europe, there was a corresponding hemispheric-specific division in the geographic region from which JRA and JCA articles originated. Studies originated from 17 countries at latitudes ranging from 3°S to 61°N (Table 2). There were no eligible studies found that reported data below a latitude of 39°N prior to the



introduction of the ILAR JIA disease classification systemic and no eligible JRA studies above 42°N.

The 2013, systematic literature review of 19 childhood arthritis studies reported vitamin D status (14 reporting 25(OH)D and 11 reporting 1,25(OH)D) suggested that at that time there was no clear link between vitamin D status and children with chronic arthritis [12]. The review also contained a meta-analysis comprising three studies that reported the prevalence of vitamin D insufficiency to be 82% in JIA [12]. Only three studies reported in the meta-analysis were conducted using ILAR JIA criteria [12].

Comparison of study design

Seventeen studies used a cross-sectional design (*n* = 17; 44.7%) [27–30, 32–35, 37, 42, 44, 51, 53, 57–59], 16 (42.1%) a case-control design [21–26, 31, 39, 41, 43, 47–

50, 60] and the remainder (5; 13.2%) were randomized controlled trials [40, 45, 46, 54, 55]. The primary objective of the majority of studies was to investigate the relationship between juvenile arthritis and bone health (n = 20; 52.6%). In all studies where sex distribution was reported, there were significantly more female participants than males, an observation consistent with the overall preponderance of females in JIA [61]. With the exception of one study [35], the age range of the participants was 0–21 years.

25(OH)D status

Of the 38 studies reviewed, six (15.8%) had mean 25(OH)D concentrations above 75 nmol/L [28, 42, 44, 45, 53, 54]. Seventeen studies (44.7%) had mean 25(OH)D concentrations between 50 and 75 nmol/L [22, 23, 25, 27,

Study Location and Reference	Disease	Sample size	Age (years) Mean + SD or	25(OH)D (nmol/L) Mean + SD or	Results relating to vitamin D	Control Group Results	Vitamin D Intake
		female)	range	range			
Study Design: Meta-Analysis							
Meta-Analysis Nisar et al. 2013 [12]	JRA JCA & JIA	n = 529	0-18	61.4	Mean of 14 studies 61.4 nmo//L (Range 28.7–139.8) prevalence reported from 3 studies 82% insufficient.		
Study Design: Randomized Contrc	olled Trial						
Cincinnati, Ohio USA Stark et al. 2006 40°N [45]	JRA	n = 49	4-10 y	79.9 ± 25.0 (39.9−142.3)	Behaviour intervention to increase calcium intake successful.		
loannina, Greece Siampoulou et al. 2001 39°N [40]	AIL	n = 10 (6F)	13.1 ± 2.5	53.9 ± 8.5	All patients were vitamin D replete 25(OH)D > 17.5 nmo//L, most were measured between February to May.		
Missouri, USA Hillman et al. 2008 37°N [54]	Children with arthritis	n = 18	3-15	82.1 ± 38.7	Supplemental vitamin D improved status, but supplemental vitamin D or calcium did not improve bone mass.		
Kansas, USA Warady et al. 1994 39°N [55]	Rheumatic disease (6 with JRA)	<i>n</i> = 10 (7F) 6 JRA	13.(10.9–18.0)	70.142 242	Children with rheumatic disease would benefit from receiving calcium and vitamin D supplements.		
lllinois, USA Reed et al. 1991 40°N [46]	JRA	<i>n</i> = 13 (12F)	5-18	70.0 ± 40	Vitamin D may help prevent bone loss in children with active disease.		
Study Design: Case-Control							
Istanbul, Turkey Dagdeviren- Cakir et al. 2016 41°N [21]	AIL	Active disease: n = 64 (41) Remission: 53(35)	Active disease: 9.7 ± 4.3 Remission: 9.8 ± 4.3	Active disease: 46.5 \pm 23.0 Remission: 47.3 \pm 27.5	Vitamin D concentrations in children with JIA were significantly lower than healthy children. Of those who were measured while in remission there was no difference in 25(OH)D concentrations.	Healthy control n = 100 66.8 ± 26.6 nmol/L.	
Mexico city, Mexico Hernandez Rosiles et al. 2015 19°N [39]	AIL	n = 37 (27)	12.5 ± 3.1	55.0 ± 13.9	No difference between children with JIA and controls.	Healthy controls n = 79 59.0 ± 7.7 nmol∕L.	
Fortaleza, Brazil De Sousa- Studart et al. 2015 3°S [22]	AIL	<i>n</i> = 51 (31 F)	13.4 ± 4	55.4 ± 25.0	25(OH)D similar for disease activity status, JIA category, and arthritis severity measure.	Age sex-matched controls 25(OH)D, 75.9 ± 14.0 nmol/L.	
	AIL	<i>n</i> = 53	Not reported	Median 42.6	A significant difference between JIA and control 25(OH)D p < 0.01.	Control <i>n</i> = 106 25(OH)D 49.9 nmol/L.	

Study Location and Reference	Disease	Sample size (number female)	Age (years) Mean ± SD or range	25(OH)D (nmol/L) Mean ± SD or range	Results relating to vitamin D	Control Group Results	Vitamin D Intake
Hangzhou, China (article in Chinese, English Abstract) Wang et al. 2015 30°N [60]					No correlation between 25(OH)D and JIA subtypes, ACR pediatric 30, CRP or ESR.		
Florence, Italy Stagi et al. (2014) 43°N [23]	AIL	n = 152 (115F)	16. ± 7.4	54.4 ± 20.5	JIA had reduced 25(OH)D and higher PTH compared to controls. Active disease or frequent flare-ups resulted in lower vitamin D than non-active and no frequent flare-ups.	Control group 25(OH)D 74.4 ± 28.0 nmol/L <i>p</i> < 0.005.	Intake JIA 164 \pm 84 IU/ day control 160 \pm 72 IU/ day.
New Delhi, India Dey et al. 2014 28°N [31]	AIL	n = 35	3–16	22.0 ± 18.0	Decreased dietary intake of vitamin D and calcium, decreased weight bearing physical activity and sunlight exposure were the major factors for low BMD. Duration of disease 2.30 ± 1.91 yrs.	Age sex-matched controls 25(OH)D 37.9 ± 10.0 nmol/L sig nificant difference.	Intake JIA 123 ± 53.6 (50-207) control 309 ± 62.38 (213- 387) IU/day.
Lodz, Poland Szyamanska-Kaluza et al. 2013 51°N [24]	AIL	<i>n</i> = 50 (40)	9.4 ± 5.52	43.4 ± 21.1	Vitamin D deficiency is common in this population. No correlation between disease activity, type of JIA or metabolites of vitamin D.	Control <i>n</i> = 28 Age, gender matched, hospitalized children 43.4±40.7 nmol/L.	
Sao Paulo, Brazil Munekata et al. 2013 23°S [25]	JIA- polyarticular	n = 30 (23F)	14 (4–20)	Participation of the second se	High frequency of 25(OH)D deficiency in both control and JIA groups; no difference between the two. No association of 25(OH)D with disease activity.	Control group age-sex matched ($n = 30$). 16 non-Caucasian; mean disease duration 5y (1–12) control 2 5(OH)D 67.2±19.0 nmo//L.	
Oslo, Norway Lien et al. 2005 59°N [26]	AIL	<i>n</i> = 108	6 to 18	497 ± 16.5	No difference in 25(OH)D between control and JIA groups.	Control n = 108 25(OH)D 50.4 ± 8.1 nmol/L D	Intake JIA 164 ± 84 IU control 160 ± 72 IU
London, UK Rooney et al. 2000 51°N [49]	JCA	n = 34 (23F)	9.2 (4.6–13.6)	Estimated from graph 45 (14.5–62.5)	Vitamin D status was significantly lower in JCA patients than age- matched controls before treatment, Steroid-treated children have low vitamin D. All but three children re ceived corticosteroids.	Control group 25(OH)D estimate 75 nmol/L.	
Florence, Italy Falcini et al. 1998 43°N [48]	JCA	n = 47 (34)	15 months- 12 years(7.13 ± 4.1)	61.4 ± 20.5	The lower serum concentrations of osteocalcin in active disease support the hypothesis that both bone formation and resorption are reduced in JRA	Controls <i>n</i> = 47 25(OH)D 56.7 ± 21.5 nmo//L.	
Missouri, USA Pepmueller et al. 1996 37°N [43]	JRA	n = 41	4–18.5	45.7 ± 23.5	Suggest an association between decreased bone mineralization in	Control $n = 62$ 65.5 ± 23.5 nmol/L significant difference.	Vitamin D intake in

Study Location and Reference	Disease	Sample size (number female)	Age (years) Mean ± SD or range	25(OH)D (nmol/L) Mean ± SD or range	Results relating to vitamin D	Control Group Results	Vitamin D Intake
					JRA and low bone formation that is related to disease severity.		JRA 464 ± 262 IU Intake of controls not reported.
loannina, Greece Tzoufi et al. 1994 39°N [50]	JCA	n = 35 (14)	88 ± 4.1	39.8 ± 20.5	Disease activity of JCA appears to be associated with lower vitamin D.	Mean disease duration 3.4 years Control $n = 15$ 25(OH)D 68.1 \pm 15.5 nmol/L control group taking corticosteroids $n = 4$ 25(OH)D 51.4 \pm 24.5 nmol/L.	
Missouri, USA Hillman et al. (1994) 37°N [47]	JRA	n = 44 (28)	11.8±3.8	66.6 ± 26.7	Lower bone mineral content and bone biomarkers in JRA patients that controls but higher vitamin D in JRA.	N = 37 controls 25(OH)D 53.2 ± 18.7 nmol/L.	
Milano, Italy Bianchi et al. 1990 45°N [41]	AIL	n = 36 (64%)	9.96 (5–17)	45.9 nmol/L Reported from Nissar et al. Review	Suggests severe JRA has an influence on bone mass possibly mediated by a decrease in active vitamin D metabolites.	Study duration one year, controls only measured at baseline 25(OH)D 92 ± 17.5 nmo/L N = 20	
Huddinge, Sweden Johansson et al. 1986 59°N [52] Study Design: Cross Sectional	JCA	26 (all female)	11–16	63.2 ±364	Statistically lower than controls, however no evidence of deficiency.	Healthy controls n = 28 76.2 ± 28.0 nmol/L	
Chongqing, China Tang & Mingyue Conference Abstract 2016 29°N [27]	AIL	<i>n</i> = 76 (36)	8.49 ± 3.09	52.8 ± 15.3 nmol/F	JJA patients have reduced serum 25(OH)D3, particularly those with active disease or/and using glucocorticoid.		
Riyahad, Saudi Arabia Alhomaidah et al. 2016 24°N [28]	AIL	n = 22 (13)	12.4	14 > 75 nmol/L 8 < 75 nmol/L	Vitamin D insufficiency is frequent in children with JIA.		
Bialystok, Poland (abstract only) Goralczyk et al. 2015 53°N [29]	AIL	n = 189 (113)	3-17.7	40.6 ± 23.5	67% 25(OH)D < 50 nmol/L. Obese children had significantly reduced 25(OH)D compared to normal weight peers. Negative relationship between MTX use and 25(OH)D.		
Oporto, Portugal Peixoto et al. 2013 Conference Abstract 41°N [30]	AIL	<i>n</i> = 40 (31)	22.3 (4–63)	10 > 75 nmol/L, 19 between 50 and 75 nmol/L, 11 < 20 nmol/L	Prevalence of vitamin D deficiency/ insufficiency among JIA patients is very high.		
Antalya, Turkey Comak et al. 2014 36°N [32]	AIL	n = 47 (29)	9.3 ± 3.9	44.2 ± 29.0	Only 27.7% patients had 25(OH)D > 50 mmol/L. There was a significant negative correlation between vitamin D concentration and disease activity ($p = 0.01$, $r = -0.37$).		

Table 2 Summary of current	literature of 25(C)H)D status and (chronic childho	od arthritis (Continue	(d)		
Study Location and Reference	Disease	Sample size (number female)	Age (years) Mean ± SD or range	25(OH)D (nmol/L) Mean ± SD or range	Results relating to vitamin D	Control Group Results	Vitamin D Intake
Salé, Morocco Bouaddi et al. (2014) 34°N [33]	AIL	n = 40 (18)	11±4.23	55.4±27.2	25(OH)D < 75 nmol/L in 75% of sample. Poly arthritis and oligoarthritis 25(OH)D status negatively associated with disease activity in univariate but not multivariate analysis.	Median disease duration two years.	
Helinski, Finland Miettinen et al. 2013 Letter to the Editor 60°N [34]	AIL	n = 136	1-18	M: 63.9 ± 18.0 F: 62.9 ± 20.0	Suggest that JIA subtype may be associated with 25(OHJ) concentration in female patients. Seasonal difference with female patients.		
CambridgeUK Nisar et al. 2013 Conference Abstract 52°N [35]	AIL	n = 37 (31)	0-10 (n = 13), 11-20 (n = 12) and > 21 years (n = 12)	49.6 nmol/L (range 13.2– 112.0 nmol/L).	Half of patients with JIA have low Vitamin D levels which are inversely related to disease activity and disease duration.		
Boston, USA Pelajo et al. 2012 42°N [36]	ЧГ	n = 154 (61%)	10.6	729±23.0	13% deficient, 42% insufficient. Age, ethnicity, season, BMI associated with 25(OH)D but not vitamin D deficiency.No association with whole sample: small negative association for new onset JIA; mean time since onset 28 months.		
Helinski, Finland Markula-Patjas et al. 2012 60°N [37]	ЧГ	n = 50 (41)	14.8 (7.0–18.7)	53 nmol/L and (20-95 nmol/L)	62% sufficient, 24% insufficient and 14% deficient.		52% taking vitamin D supplement % of DRI median and IQR 187 (57,331).
Saskatchewan, Canada McNally et al. 2009 52°N [59]	Pediatric arthralgia	<i>n</i> = 730 25(OH)D <i>n</i> = 73	~ 18	59.9	Significantly more reported fall and winter as season of onset – more referrals from northern SK 40% < 50 nmol/L 42% 50–75 nmol/L Association between psychological stress, school absenteeism vitamin D insufficiency and arthralgia.		
Helinski, Finland Valta et al. 2007 60°N	JIA Glucocorticoid treated	n = 62 (43)	Median 11.8 (4.6–17.9	Median 49 nmol/L 16 (23%) ≤ 37.5 nmol/L	Osteoporosis is a concern in glucocorticoid treated children with JIA.		32% prescribed 400–800 IU vitamin D daily Mean intake 316 IU

Study Location and Reference	Disease	Sample size (number female)	Age (years) Mean ± SD or range	25(OH)D (nmol/L) Mean ± SD or range	Results relating to vitamin D	Control Group Results	Vitamin D Intake
							(range 44–1204 I
Ohio, USA Henderson et al. 1997 40°N [44]	JRA	n = 48 (37)	8.1 ± 1.9	89.4 ± 28.7	Serum 1,25-dihydroxyvitamin D concentrations were able to accurately segregate 79.6% of the JRA subjects into either the low or normal BMD groups.		%RDA 87. ± 52.7.
Illinois, USA Reed et al. 1993 40°N [42]	JRA	n = 27 (23)	2.9–16	84.9 ± 11.0	No difference in vitamin D status between active and inactive groups. Children with JRA who have improvement in their disease activity have an improvement in BMD heralded by an increase in serum osteocalcin values 4–87 months from disease onset.		
Harrow, UK Reeve et al. 1993 51°N [53]	JCA- treated with glucocorticoids	Prednisone n = 17 Deflazacort <i>n</i> = 17	Prednisone 10.6 ± 3.7 Deflazacort 10.3 ± 3.9	Prednisone 140.8Deflazacort 115.6	25(OH)D was surprisingly high, there was no difference between the two groups $p = 0.8$.		
Chicago, Illinois Reed et al. 1990 40°N [57]	Chronic Rheumatic Disease	<i>n</i> = 113 (82) JRA <i>n</i> = 83	1.5 to 21	Range of groups 44.9 ± 15.0 to 54.9 ± 22.5	No difference between those with active and inactive disease.		
London, UK Elsasser et al. 1982 51°N [51]	JCA	n = 63 serum 25(OH)D n = 29	Not reported	24.5 nmol/L (9> 25 nmol/L, 20 <n 25 nmol/L, 20 <n< td=""><td>There was a marginally significant correlation between TBD and 25(OH)D concentrations ($r = 0.37$. $P < 0.05$). Only nine children had acceptable vitamin D status.</td><td></td><td></td></n<></n 	There was a marginally significant correlation between TBD and 25(OH)D concentrations ($r = 0.37$. $P < 0.05$). Only nine children had acceptable vitamin D status.		

30, 33, 34, 36, 37, 39, 40, 46-48, 55, 59] and 15 (39.5%) had values below 50 nmol/L [21, 24, 26, 29, 31, 32, 35, 38, 41, 43, 49–51, 57, 60]. Of those 15 studies, 10 reported the mean 25(OH)D value, the median value reported for one study and a range or cutoff was provided for four studies). Of the 14 studies (36.8%) that published mean 25(OH)D status of healthy control groups, nine (64.3%) had mean control values significantly greater than the population with childhood arthritis [21-23, 31, 41, 43, 49, 50], three (21.4%) had concentrations that were statistically similar [25, 39, 60], and two studies (14.3%) had mean 25(OH)D values significantly below the juvenile arthritis comparison groups [47, 48]. One study compared 25(OH)D concentrations in children with JIA to hospitalized children and found no statistically significant difference between the two groups [24]. Mean 25(OH)D status of most studies (32 of the 38; 84.2%) was below the optimal concentration of 75 nmol/L in children with arthritis [20].

Geography in relation to 25(OH)D status in chronic childhood arthritis

Vitamin D status in children with JIA appears to follow a north-south gradient (Fig. 2). While this could be due to the diagnostic resources of the countries reporting values, the gradient does appear to be present in Europe where access to care and diagnostic resources are similar. Interestingly, the relationship between reduced vitamin D status and increased disease activity also appears to be present and follow a north-south gradient (Fig. 3). More studies are required to confirm this relationship worldwide, especially in locations around the equator as well as in the southern hemisphere where thus far only two studies have taken place [22, 25].

The major source of vitamin D for most people is endogenous vitamin D synthesis induced by sunlight exposure [62]. Above 33° latitude UVB radiation is not intense enough for the cutaneous synthesis of vitamin D all year long [63, 64]. At latitudes 42° and 53° North, between October to April, UVB radiation is not intense enough to elicit endogenous vitamin D synthesis [65] thus potentiating the risk of vitamin D deficiency, [63].

The prevalence of JIA, as well as the dominating subtype, varies with latitude [1]. As illustrated in Fig. 2, seven reviewed studies (18.4%) were conducted in populations residing at latitudes at or below 33° [22, 25, 27, 28, 31, 39, 60], 19 studies (50.0%) were conducted between 33 and 50° [21, 23, 30, 32, 36, 40–48, 50, 54, 55, 57, 66], and 12 at a latitude above 50° [24, 26, 29, 34, 35, 37, 38, 49, 51, 53, 59]. For those below 33°, 1 study (14.3%) reported a mean 25(OH)D concentration > 75 nmol/L, 4 (57%) reported a concentration between 50 and 75 nmol/L, and two (29%) reported values less than 50 nmol/L. For the studies that took place between 33 and 45° latitude, four studies (21.1%) reported a 25(OH)D concentration > 75 nmol/L (21%), nine (47.4%) reported a concentration between 50 and 75 nmol/L(47%) and six (31.6%) reported values less than 50 nmol/L. From the studies that took place above 45° latitude, one study (8.3%) reported a 25(OH)D concentration > 75 nmol/L, four (21.1%) reported a concentration between 50 and 75 nmol/L (33%) and seven (36.8%) reported values less than 50 nmol/L (39%).

Current chronic childhood arthritis diagnostic criteria

Of the 21 studies that applied the current chronic childhood arthritis criteria used by ILAR to diagnose children with JIA, only one study reported mean 25(OH)D concentrations above 75 nmol/L (15%) [28]. The majority of the studies (n = 11; 52.4%) reported mean concentrations between 50 and 75 (52%) nmol/L [22, 23, 25, 27, 30, 33, 34, 36, 37, 40, 67], and the remaining studies (n = 9; 42.9%) reported a mean concentration below 50 nmol/L (43%) [21, 24, 26, 29, 31, 32, 35, 38, 60]. As latitude increased, the percentage of studies that reported mean 25(OH)D in the 50–75 nmol/L range decreased.

Vitamin D and disease activity

No single measure has been established as an accurate indicator of childhood arthritis disease activity. While G-reactive protein and Erythrocyte Sedimentation Rate are indicators of inflammation, they alone do not fully reflect overall disease activity. In the studies reviewed, a variety of validated composite scores were used to measure function or disease activity, including the Childhood Health Assessment Questionnaire (CHAQ), the Juvenile Arthritis Disease Activity Score – 27 (JADAS-27) and American College of Rheumatology Pediatric 30 Criteria (ARC Peds 30) [68–70].

Fifteen of the 38 studies comprising this present review (39.5%) evaluated the relationship between vitamin D and disease activity (Fig. 3). Seven studies (18.4%) reported that patients with active disease or those with elevated inflammatory biomarkers had lower 25(OH)D concentrations than those patients who were in remission or who had less disease activity [23, 27, 30, 32, 35, 41, 50]. One study (26.3%) showed the opposite relationship; those with active disease had higher vitamin D concentrations than those with inactive disease [34]. Of the seven studies (18.5%) that reported no relationship between 25(OH)D and disease activity [21, 22, 25, 33, 36, 42, 57] one found a relationship between 25(OH)D concentrations and disease activity in the univariate but not the multivariate analysis [33]. Except for one study conducted in Turkey [21], all other studies conducted in Europe that explored disease activity reported an inverse association between vitamin D and disease activity.

Long-term cohort studies can further clarify the relationship between vitamin D concentration and disease



duration or frequency of relapse. Such an association was explored in a cross-sectional study which found significantly reduced 25(OH)D status in JIA patients (n = 152) compared with 188 age-and-sex-matched controls [23]. Active disease or frequent relapse was associated with reduced vitamin D status compared to patients with no active disease or frequent flare-ups. The authors questioned whether JIA patients with more severe disease require higher supplementation of vitamin D to maintain normal 25(OH) D concentrations. As latitude increases more studies report a difference in vitamin D status between patients with active versus inactive disease in comparison to lower latitudes as illustrated in Fig. 3.

To date, the evidence to support a relationship between vitamin D and disease activity with autoimmune diseases in humans is correlative and not causative [71]. Long-term, adequately powered randomized studies which control for confounding variables (sun exposure, season, and vitamin D intake) are required to confirm a causative relationship between vitamin D and disease activity.

Potential requirements of vitamin D intake

Vitamin D intake was only measured in seven studies [23, 26, 31, 37, 38, 43, 44]. All of these studies reported a mean or median vitamin D intake that was less than the Estimated Average Requirement (EAR) of 400 IU per day set by the IOM [19]. This is the amount of vitamin D that is expected to be sufficient for 50% of the population [19]. This indicator is used to evaluate the prevalence of inadequacy at the population level. The recommendation at the individual level is the Recommended Dietary Allowance (RDA) which ranges from 400 to 600 IU based on age groups. Two studies reported intake of vitamin D supplements by study participants but neither had a mean 25(OH)D that reached the optimal concentration [38, 44]. Three studies reported intake of both children with JIA and healthy controls [23, 26, 31]. Vitamin D intake and status was similar for two JIA patient groups [23, 26], and lower intake resulted in lower vitamin D status in the third group [31]. In comparison to the control groups, Lien et al. found similar intake and 25(OH)D status between those with



JIA and controls [26]. Stagi et al. reported similar intake of vitamin D and higher 25(OH)D in controls, and in the study reported by Dey et al. the intake of control participants was two times higher than the participants with JIA and the control group had higher 25(OH)D concentrations [23, 31].

It has been theorized that there may be an increased utilization of vitamin D during active inflammation, possibly caused by \the presence of vitamin D receptor polymorphisms in patients with autoimmune diseases [6, 72]. Additional studies investigating vitamin D intake from all sources (both food and supplements) are required to determine if children with chronic arthritis require additional vitamin D to maintain serum concentrations in comparison to healthy children. Understanding this relationship will be important in the use of vitamin D as a potential adjunct therapy. Additionally, improved understanding of vitamin D needs in children with chronic arthritis will help to clarify the role of vitamin D in the underlying disease processes so that therapies that target specific vitamin D responsive immune pathways can be developed. By exploring the factors that influence vitamin D status (genetics, environment, and nutrition), we will be better able to discern an association between vitamin D and JIA.

Two articles have discussed vitamin D requirements for children with rheumatic conditions not in the context of corticosteroids. In 2011, von Scheven and Burnham suggested that in the absence of specific guidelines for children with rheumatic conditions that the American Academy of Pediatrics guidelines of 400 IU per day be used as a suggested minimum dosing regimen [4]. The authors cautioned that providing large doses of vitamin D can result in providing "too much of a good thing" and that studies comparing children with rheumatic diseases to healthy children are required. The second article was published by Vojinovic and Cimaz in 2015 and recommends that the guidelines set out by the Endocrinology Society for patients receiving corticosteroids be followed for all children with rheumatic diseases [73]. This would result in a dose of 2-3 times the current recommendation

and would be approximately 2000 IU/day. This dose is still below the IOM's tolerable upper limit for all children over the age of one (2500 IU) [19].

Vitamin D and medication interactions in JIA patients

All but one study [24], have been conducted with children already being treated for arthritis, many receiving corticosteroids, which could impact 25(OH)D concentration and inflammatory status. Corticosteroids promote the breakdown of both 25(OH)D and 1,25(OH)D and also counteract effects of vitamin D on bone formation [74, 75]. These patients also had varying disease duration. A study of newly diagnosed individuals, however, did not compare patients with JIA to healthy controls but to hospitalized children [24] . Comparing children with JIA to healthy controls allows for discerning biologic differences that could inform treatment targets.

The lowest mean 25(OH)D concentration, 22 nmol/L (n = 35) was reported from India. These patients were found to be consuming significantly less vitamin D and had less sun exposure compared to healthy controls [31]. The highest 25(OH)D, 140.8 nmol/L (n = 17), concentrations were reported in Finland, in a population receiving Prednisone [53]. The authors hypothesized that the reason their patients' 25(OH)D concentrations were so high was that their previous research had found low concentrations of 25(OH)D and they were encouraging their patients to consume vitamin D fortified foods and to spend time in the sun [53]. A survey of steroid-related osteoporosis, prevention and treatment practices of pediatric rheumatologists in North America was conducted by Soybilgic et al. in 2014 [76]. They found that the majority of pediatric rheumatologists are recommending vitamin D for patients who were on long-term corticosteroids [76]. The role of vitamin D in mediating bone health, especially in relation to corticosteroids, has been established [75]. Both short and long-term corticosteroid intake even at small doses impact bone health in patients with autoimmune diseases [74]. The role and an appropriate amount of vitamin D intake or 25(OH)D target for inflammation or disease activity have yet to be established.

Additional research directions

Considering ethnicity when comparing incidence and prevalence amongst populations would be useful in understanding if the regional differences observed are due to environmental or genetic factors or a combination of the two. Evidence from a multiethnic cohort study of 1082 children at The Hospital for Sick Children, Toronto, Canada investigated the influence of ethnicity on the risk of developing JIA [77]. When the diversity of the study population was compared to that of the general Toronto region population, there was an overrepresentation of patients of European and Indigenous descent and an underrepresentation of patients of Black, Asian, or Indian subcontinent ethnicity in their cohort. European descent was significantly associated with an increased risk of developing JIA, including all subtypes except RF-positive polyarticular JIA. Exploring the environmental and genetic factors that may contribute to JIA risk in the same individual will help to clarify these findings.

While this review focused on vitamin D status in children with JIA, vitamin D may be involved in both disease development and subsequent disease activity status. Exploring elements of vitamin D status that may have a role in disease development such as early life and gestational vitamin D status as well as genes in the vitamin D pathway will help to clarify the role of vitamin D.

Season of birth has been suggested to have an impact on the risk of developing a number of autoimmune diseases such as multiple sclerosis, type 1 diabetes and celiac disease [78]. A recent study investigating month of birth and risk of JIA found a difference in the pattern of the birth month for children with JIA compared to that of the general population [78]. Children with JIA were more likely to be born between November to March, with the birth month for the general population peaking in the summer months. The study by Carlens et al. also investigated the relationship between season of birth and the risk of developing JIA and found no increased risk [79]. Season of birth may be a marker for vitamin D status in utero with children born in the non-vitamin D synthesizing periods being exposed to less vitamin D during their time in utero than those who are born during the vitamin D synthesizing seasons.

The Childhood Arthritis Risk factor Identification sTudY (CLARITY) explored the use of nutritional supplements during pregnancy and the risk of developing JIA [80]. The use of vitamin D and fish oil during pregnancy in case mothers was not significantly different from controls following covariate adjustments [80]. A case-cohort investigation from Denmark, comparing 25(OH)D status in children diagnosed with either oligoarticular or polyarticular JIA using dried blood spot samples that were collected at birth did not find any association between 25(OH)D status at birth and risk of developing JIA [81]. Concentrations of 25(OH)D fluctuated significantly by season of birth and year of birth (calendar year). There was no follow up to determine if 25(OH)D status or season during the first few months of life impacted risk of JIA or whether other subtypes of JIA were impacted by season of birth of 25(OH)D status at birth.

Certain VDR gene polymorphisms may be associated with different biologic response to vitamin D. The Cdx2 polymorphism of the VDR gene specifically the GG genotype, have been suggested to be more represented in patients with JIA compared to healthy controls who more often have the GA genotype [82]. Recently the idea of investigating epistasis (gene-gene interactions) amongst genes in the inflammatory and vitamin D pathway and how their interactions contribute to JIA risk was explored by Ellis et al. [86]. This is the first study to explore this interaction, and the authors suspect that through exploring these interactions there is the opportunity to account for the missing heritability that has been observed with complex diseases with genetic components [86]. Their work found evidence of epistasis amongst tyrosine-protein phosphatase non-receptor type 2 (PTPN2) gene and the vitamin D binding protein gene in contributing to the risk of JIA [86]. The role of genes in the vitamin D pathway on both disease development and disease activity are still in the early stages of investigation. Also how they impact the biological response involving vitamin D and inflammation remains unclear. Investigating genetic, nutritional and environmental factors that influence vitamin D in JIA could help inform ways in which vitamin D status influences the occurrence and activity of JIA. Understanding if genetic variants increase the risk of disease development will help tailor vitamin D management in individual patients and contribute to improving control of disease activity and improve outcomes.

A north-south gradient of incidence and a mechanism for the suppression of inflammation in relation to 25(OH)D status has been suggested. However, no study has summarized the current evidence of chronic childhood arthritis diagnosis by 25(OH)D status in relation to latitude and disease activity. This first step is important for the development of future studies leading to the exploration of potential optimal target concentrations of vitamin D for the reduction of inflammation in children with chronic arthritis.

Limitations

This scoping review has limitations due to the limited amount of comparative data. Season of measurement and JIA subtype could not be considered due to a lack of reporting in most reviewed articles. With the exception of one study, all studies reviewed used various unreported types of medication in patients who had had JIA for varying durations. These variables can make it difficult to interpret the relationship between vitamin D and disease activity in relation to both inflammation status and risk of relapse. Of the studies that investigated the relationship between vitamin D and function or disease activity, various measures were used. The most common included the CHAQ, JADAS-27 and ARC Peds 30. The JADAS-27 and ACRS Peds 30 both include active joint counts in their scoring which confounds comparisons of disease activity between patients with different JIA that are defined by numbers of joints involved. Subtypes [17, **83**, **84**]. Many studies included multiple subtypes of JIA measured by the same disease activity score that included an active joint count. This review was unable to explore the relationship between vitamin D status and ethnicity, vitamin D receptor genes or other genes that influence vitamin D metabolism.

Conclusion

This is the first scoping review to summarize research relating to vitamin D and JIA in the context of vitamin D status, latitude, disease activity. It is also the first to map the results according to geography. Thirty-two studies (84.2%) reported a mean 25(OH)D concentration below 75 nmol/L or the optimal value. This suggests that whether due to inadequate intake or increased utilization the majority of children with juvenile arthritis do not have optimal 25(OH)D status as defined by the Endocrine Society [20]. The optimal concentration of 25(OH)D and the corresponding dietary requirements for patients with chronic childhood arthritis has yet to be determined. Further, the relationship between vitamin D status and disease activity in children with JIA is still unclear. Studying newly diagnosed patients who are treatment naive for longer periods of time would help characterize this relationship as there would be fewer confounders associated with patients who have had the disease for varying durations (medication, lifestyle modifications, and disease duration). Thus far, we know that there is a role for vitamin D in the inflammatory pathways, a high prevalence of 25(OH)D insufficiency among children with JIA, and an established link of vitamin D with other autoimmune diseases. We do not, however, know the optimal vitamin D status for children with JIA, whether reduced vitamin D is caused by increased utilization or reduced vitamin D status in children with JIA, the impact of vitamin D in disease activity or the role of VDR polymorphisms with JIA. Larger, long-term studies of new-onset JIA are required to explore the association. The relationship between vitamin D status and JIA over time in newly diagnosed individuals has yet to be investigated. Investigating the genetic and environmental role that vitamin D plays in the prevention and control of JIA in the same children will help to tease out the multifaceted role played by vitamin D in this disease. Being able to suggest specific targets for vitamin D status as a potential adjunct therapy in the treatment of JIA and understanding how genetic variants increase the risk of disease development will enhance the quality of life of patients and their families.

Policosanol composition, antioxidant and anti-arthritic activities of milk thistle (*Silybium marianum* L.) oil at different seed maturity stages

Abstract

Background: Several anti-arthritic drugs and synthetic antioxidants have wide pharmaceutical uses and are often associated with various side effects on the human health. Dietary seed oils and their minor components like policosanol may offer an effective alternative treatment for arthritic and oxidative-stress related diseases. The biological effects of seed oils were affected by different parameters such as the stage of seed maturity. Hence, this study seeks to determine the policosanol content, antioxidant and anti-arthritic activities of milk thistle (*Silybium marianum* L) oil extracted at various stages of seed maturation.

Methods: Milk thistle oil samples were extracted from seeds collected at three maturation stages (immature, intermediate, and mature). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assays were used to determine the antioxidant activity of the extracted oils. The antiarthritic activity of oil samples was evaluated with bovine serum protein denaturation and egg albumin denaturation methods. Gas chromatography coupled to mass spectrometry (GC-MS) was employed to determine the policosanol profile.

Results: Policosanol profile, antioxidant and anti-arthritic activities of milk thistle oil were influenced by the seed maturity stages. The oil extracted from the immature seeds had the highest total policosanol content (987.68 mg/kg of oil) and displayed the maximum antiradical activity (96.42% and 90.35% for DPPH test and ABTS assay, respectively). Nine aliphatic alcohols were identified in the milk thistle oil. The dominant poliosanol in the mature seed oil was octacosanol (75.44%), while triacontanol was the major compound (40.25%) in the immature seed oil. Additionally, the maximum inhibition of bovine serum protein denaturation (92.53%) and egg albumin denaturation (86.36%) were observed in immature seed oil as compared to mature seed oil. A high correlation was found between the total policosanol content, anti-arthritic activity and antioxidant capacity of oil.

Conclusions: The milk thistle oil exhibited a potential anti-arthritic and antioxidant activities and that it might contribute to the protection of humans from a variety of diseases like rheumatoid arthritis. Also, it could serve as natural antioxidant and anti-arthritic agents for application in the food industries and pharmaceutic. Policosanol level in the seed oils might contribute to their anti-arthritic and antioxidant activities.

Keywords: Milk thistle, Oil, Anti-arthritic activity, Antioxidant capacity, Policosanol, Maturity stage

Background

Oxidative stress in the human tissues leads to several diseases like cancer, diabetes, arthritis, atherosclerosis and chronic inflammatory disorders. Nearly one-fifth of the world's population is affected by rheumatoid arthritis [1]. The anti-arthritic and anti-inflammatory drugs presently used are characterized by their possible adverse effects on the body such as ulcers and cardiovascular problems [2, 3]. Also, the use of synthetic antioxidants like butylated hydroxyanisole and butylated hydroxytoluene in the pharmaceutical and food industries has various side effects [4, 5]. Therefore, much attention has been focused on the development of alternative anti- arthritic agents and antioxidants from natural resources. It was reported that the intake of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders and other infections [6]. Medicinal plant may offer an alternative source for the anti-arthritic, antiinflammatory and antioxidant drugs. The antioxidant capacity of plant extracts was influenced by different parameters such as environmental conditions, genotype and stage of maturity [7-10].

Policosanol is a mixture of long-chain (C20 to C36) aliphatic primary alcohols exhibiting various beneficial effects on the human health. It was originally isolated from sugar cane wax and is also found in a number of other natural sources such as beeswax and vegetable oils [11–13]. Policosanol may be effective in the treatment of hypercholesterolemia by inhibiting hepatic cholesterol biosynthesis, enhancing LDL catabolism and increasing high density lipoprotein levels in serum [14, 15]. These aliphatic alcohols have also been shown to possess antiinflammatory and antioxidant effects [13, 16]. Octacosanol (C28-OH), the main active policosanol, has gained attention due to its health benefits including antiparkinsonian, antinociceptive and anti-inflammatory effects [17, 18]. Triacontanol (C30-OH) is able to induce anti-inflammatory responses in animals, prevent oxidative stress and inhibit lipid peroxidation [19, 20]. Millán et al. [21] revealed that the nutraceutical combination containing policosanol, berberine, and red yeast rice induced significant improvements in plasma lipids. Therefore, there is growing interest in the identification of natural sources of policosanol for the functional foods and nutraceutical applications [22]. Contents and compositions of policosanol in different plant sources such as rice bran, wheat bran, sugar cane wax, corn kernel, green tea leaves, grain sorghum, perilla seeds and grape seed have been reported [12, 22-25].

Milk thistle (*Silybum marianum* L.) is an important medicinal plant from the family *Asteraceae*. The bioactive compounds are mainly concentrated in its seeds which have been used for more than 2000 years to treat liver diseases. Milk thistle seeds contain 17.5–30.5% of

lipids rich in unsaturated fatty acids and 1-3% of silymarin [10, 26, 27].. Recently, certain Silybum marianum accessions have begun to be cultivated in several countries and the specie is undergoing domestication for making the supply of silymarin sustainable [28]. Previously published studies examined the triacylglycerol, fatty acid, tocopherol, sterol and polyhpenol composition of milk thistle seeds [10, 26, 29-32]. Significant differences were observed between milk thistle cultivars for the content of bioactive compounds [10]. Also, the antioxidant properties of ethanolic extracts of milk thistle seeds and methanolic extracts of cold-pressed milk thistle seed oil have been reported [10, 31]. To the best of our knowledge, however, no data has been published on the policosanol composition and the anti-arthritic activity of milk thistle oil. Therefore, this study aimed to examine the policosanol profile, anti-arthritic and antioxidant activities of milk thistle oil at different stages of seed maturity. Such data could serve for the evaluation of nutritional and health impact of milk thistle oil and for the development of new source of natural bioactive compounds.

Methods

Materials and reagents

The milk thistle (*Silybium marianum* L.) seeds were collected from plants growing in region of Sousse (Centre of Tunisia), during April and June, 2012. The seeds were authenticated at the National Botanical Research Institute Tunisia (INRAT). Seeds were selected according to external color; green seeds were chosen as immature stage, mahogany brown seeds as the intermediate stage and dark brown seeds as the last stage of maturity (mature stage).

Chloroform, methanol and petroleum ether were purchased from Lab-Scan analytical Sciences (Poland). Ethanol, diethyl ether and n-hexane were obtained from Scientific Limited (Northampton, UK). 2, 7-Dichlorofluorescein, DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) and the standard 1-eicosanol were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Potassium hydroxide pellets and anhydrous sodium sulfate were obtained from Appli-Chem (Darmstadt, Germany). ABTS, 2,29-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt, and potassium persulfate (di-potassium peroxdisulfate) were obtained from Sigma-Aldrich (Poole, Dorset, UK).

Seed oil extraction, saponification and thin layer chromatography

The oils were extracted by the method of Folch et al. [33]. Seeds (2.5 g) were washed with boiling water for 5 min and then crushed in a mortar with chloroform/ methanol (2:1, ν/ν). The mixture was centrifuged at 3000 g for 15 min and the lower chloroformic phase

containing the total lipids was kept and dried in a rotary evaporator at 40 °C.

The saponification and the Thin layer chromatography analysis of unsaponoifiables were done according to the previous reports [12]. The unsaponifiable fraction and the authentic 1-eicosanol were spotted on preparative silica gel thin-layer plates (silica gel 60G F254) and developed with hexane–diethyl ether (65:35, ν /v). After development, the plate was sprayed with 2,7-dichlorofluorescein and viewed under UV light. The band corresponding to aliphatic alcohols was scraped, extracted three times with chloroform–diethyl ether (1:1, ν /v), filtered to remove the residual silica, dried in a rotary evaporator and stored at – 10 °C.

Analysis of policosanol by GC-MS

GC-MS analyses were performed using a capillary HP-5MS column (30 m × 0.25 mm I.D., 0.25 µm film thickness; Agilent Technologies) with gas chromatography (Agilent Technologies 7820A) coupled directly to the mass detector (Agilent Technologies 5975 series MSD). Helium was used as carrier gas, with a constant flow rate of 1 ml/ min. The injector and detector temperatures were 230 °C. The oven temperature was programmed from 150 to 320 °C at 10 °C·min – 1 from 150 to 250 °C and at 5 °C·min – 1 from 250 to 320 °C. Manual injection of 1 µL of the aliphatic alcohol solution was performed in the split mode at a 10:1 split ratio. The policosanol compounds were identified by comparing their mass spectra with the Wiley 275.L Mass Spectral Library.

Measurement of in vitro antioxidant activity DPPH test

The antioxidant activity of seed oil samples was determined using DPPH radicals as described by Kozłowska et al. [34]. Fifty mg of oil was dissolved in 3 mL of ethyl acetate and then 1 mL of oil solution was diluted with 2.75 mL ethyl acetate. 0.25 mL of DPPH solution (1 mM) was added and the mixture was shaken vigorously for 10s in a vortex apparatus. After 20 min, the absorbance was measured at 515 nm using UV/Vis scanning spectrophotometer (Model 2650, Labomed, Inc. U.S.A) and the percent of inhibition was calculated using this formula:

Inhibition (%) = {(Absorbance of the control – Absorbance of sample)/Absorbance of the control}/ × 100.

ABTS antioxidant assay

The ABTS radical scavenging capacity of the oil was measured using the method described by Rubalya and Neelamegam [35]. The ABTS⁺ radical was generated by oxidation of 2.5 ml of ABTS solution (7 mM) with potassium persulfate (14.7 mM). The mixture is kept in the

dark for 16 h at room temperature (25 °C). Before usage, the mixture was diluted with water to obtain an absorbance of 0.70 ± 0.05 at 734 nm. The radical scavenging activity is assessed by mixing 2 ml of this diluted ABTS+ solution with different oil samples dissolved in benzene. After 30 min, the percentage inhibition at 734 nm was calculated for each sample relative to blank absorbance. The percentage inhibition of ABTS radical by the oils was calculated using the equation described in the DPPH assay.

Measurement of in vitro anti-arthritic activity

In vitro anti-arthritic activity was evaluated using bovine serum protein denaturation method and egg albumin denaturation method [36] with some modifications [37].

Bovine serum albumin (BSA) denaturation method

Test solution (0.5 ml) consisted of 0.45 ml bovine serum albumin (0.5% w/v aqueous solution) and 0.05 ml of oil samples. Then the samples were incubated at 37 °C for 20 min followed by incubation at 57 °C for 3 min. After cooling the samples, 2.5 ml phosphate buffer (pH 6.3) was added to each tube. UV-Visible spectrophotometer was used to measure the absorbance at 660 nm. The control represents 100% protein denaturation. For test control solution (0.5 ml) 0.05 ml distilled water was used instead of oil sample while for product control (0.5 ml) 0.45 ml distilled water and test solution (0.05 ml) were used. The percentage inhibition of protein denaturation was calculated by the following formula:

Percentage inhibition = {(Absorbance of test solution- Absorbance of the control)/Absorbance of the control}/ $\times 100$.

Egg albumin denaturation method

The reaction mixture (5 ml) was comprised of 0.2 ml of fresh hen's egg albumin, 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2mlof oil samples. Similar volume of double distilled water served as control. Then the mixture was incubated at 37 °C in the incubator for 15 min and then heated at 7°0 C for 5 min. After cooling, their absorbance was measured at 660 nm by using pure blank. The percentage inhibition of protein denaturation was calculated as mentioned in Bovine serum albumin (BSA) denaturation method.

Statistical analysis

The analysis of the studied samples was performed in triplicate and the results were expressed as means \pm standard deviation (SD). Statistical analysis was performed by using the Proc ANOVA in SAS (Software version 8). Duncan's Multiple Range Test was applied. The Pearson correlation coefficient (r) was used to examine the relation between the main parameters.

Results and discussion Total policosanol content

To the best of our knowledge, the policosanol composition of milk thistle oil has never been previously reported. As seen in Table 1, the total policosanol content of the mature milk thistle seeds was 574.4 mg/kg of oil (equivalent to about 175.2 mg/kg of dry weight) and was higher than those of grape seed and rice bran oils (171.17–245.15 mg/kg of oil) [23]. However, it was lower than those of other plant sources such as sorghum kernel (800 mg/kg dry weight) and green tea leaves (726.2– 1363.6 mg/kg dry weight) [22, 24]. Milk thistle seed oil had high content of policosanols which are considered important from a nutritional and functional point of view. Policosanol contents in vegetables are shown to vary due to many factors like species, ripening grade of fruits and type of tissue [12, 23, 38].

The content of policosanol in milk thistle oil was affected by seed maturity stages (Table 1). It was higher in the immature seeds (987.68 mg/kg oil) and then decreased during seed maturation. Also, a decreasing trend was observed for the policosanol content during the early stages of corn kernel development [12]. In contrast, during *Tilia tomentosa* leaf development, total primary alcohol content was found to increase from 0.031% to 0.197% of dry weight [38]. These quantitative differences observed in the policosanol content during seed development could be linked to change in the activity of fatty acyl-coA reductase which converts fatty acyl-coA into fatty alcohol.

Policosanol composition

The aliphatic alcohols fraction of the milk thistle oil was submitted to the GC-MS analysis. Nine aliphatic alcohols were identified in the oil samples and they range from C_{22} to C_{32} (Table 2). The dominant policosanols in the mature seed oil were Octacosanol (75.44%) and triacontanol (8.61%), however the other aliphatic alcohols detected including C_{22} , C_{23} , C_{24} , C_{26} , C_{27} , C_{29} , and C_{32} were present in amounts ranging from 1.52 to 3.34%. Milk thistle oil extracted from mature seeds could serve as natural source of octacosanol, which has been shown to exhibit various beneficial effects such as antiparkinsonian, antinociceptive and anti-inflammatory effects [17, 18]. Octacosanol is also the single most

abundant policosanol in sugar cane wax (60–70%) and perilla seeds (55.93%) with a minor quantity of many other policosanols [23, 25]. The policosanol composition in vegetable oils was greatly source dependent [23]. In the commercial green tea leaves, octacosanol (29.9– 42.7%) and triacontanol (27.4–41.5%) were the main policosanol components [22]. Olive oil was characterized by the predominance of hexacosanol, tetracosanol and octacosanol [23]. Harrabi et al. [12] reported that dotriacontanol (30.1–35.5%) was the major policosanol in whole corn kernel, followed by triacontanol (17.7– 24.8%) and tetracosanol (15.2–25.7%). However in wheat, tetracosanol was the most abundant compound, followed by docosanol and hexacosanol [39].

Figure 1 shows that the percentages of octacosanol, triacontanol and dotriacontanol were largely influenced by seed maturity stages. In immature seeds, the dominant alcohol was C₃₀ (40.25%), followed by C₂₈ (30.42%) and C_{32} (16.36%). During seed maturation, the percentage of octacosanol increased rapidly, while those of triacontanol and dotriacontanol decreased. The levels of the other detected compounds were relatively constant, as the seed developed. In agreement to our present trends, the level of triacontanol was also found to decrease during corn kernel development [12]. Immature seeds are rich in triacontanol which is essential for their development. In fact, triacontanol has been reported to stimulate plant growth, increase dry weight, prevent oxidative stress and act as an inhibitor of lipid peroxidation [20, 40]. The variation in the policosanol composition might be due to the physiological changes that accompany ripening of seeds. Triacontanol exhibited anti-inflammatory action in animals and has been suggested to be an effective anti-inflammatory drug [19]. Thus, immature milk thistle seeds could be exploited as a natural source of this bioactive compound.

Antioxidant activity

The antioxidant activity of the milk thistle oil samples was evaluated using the two most common radical scavenging assays DPPH and ABTS. The DPPH test has been widely used for the estimation of the antioxidant capacity of plant and health food extracts due to its simplicity, stability and reproducibility [8]. All the tested oil

Table 1 Total policosanol content and antioxidant capacity of milk thistle oil, at three seed maturity stages

Maturation stage	Policosanol content (mg/kg oil)	DPPH ^a scaveninig ability	ATBS ^b scaveninig ability
Immature	987.68 ± 16.41	96.42 ± 2.28	90.35 ± 2.6
Intermediate Mature	612.24 ± 5.89	84.46 ± 1.52	77.48 ± 1.8
	574.49 ± 6.34	76.52 ± 1.76	70.25 ± 1.34

Values were expressed as means \pm SD of triplicate experiments

^aDPPH 1,1-diphenyl-2-picrylhydrazyl radical and results are expressed as percentage of inhibition of DPPH by the oil

^bABTS : 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) and results are expressed as percentage of inhibition of ABTS by the oil

Table 2 Policosanol composition of milk thistle oil extracted from mature seeds

Policosanol (Chemical formula)	Percent
Docosan-1-ol (C ₂₂ -OH)	1.52 ± 0.24
Tricosan-1-ol (C ₂₃ -OH)	2.12 ± 0.39
Tetracosan-1-ol (C ₂₄ -OH)	2.02 ± 0.50
Hexacosan-1-ol (C ₂₆ -OH)	2.50 ± 0.16
Heptacosan-1-ol (C ₂₇₋ OH)	1.90 ± 0.25
Octacosan-1-ol (C ₂₈ -OH)	75.44 ± 2.41
Nonacosan-1-ol (C ₂₉ -OH)	2.55 ± 0.62
Triacontan-1-ol (C ₃₀ -OH)	8.61 ± 1.20
Dotriacontan-1-ol (C ₃₂ -OH)	3.34 ± 0.56

Values were expressed as means \pm SD of triplicate experiments

samples showed strong radical scavenging activity which varied from 70.25 to 96.42% (Table 1). The difference between DPPH and ABTS values obtained for the same sample might be due to the fact that ABTS assay is applicable to both hydropholic and lipopholic antioxidants systems, however DDPH assay is applicable to hydrophobic system [31]. Milk thistle seed oil had a higher DPPH value compared with other vegetables oils such as unheated sesame oil which had DPPH value of 69.2% [35]. This result is in agreement with previous studies that showed a high free radical scavenging capacity for cold-pressed milk thistle oil [31] and ethanolic extracts of milk thistle seeds [10]. Kiralan et al. [41] reported that olive oil was able to guench 52.31-94.91% of DPPH radicals, depending in olive cultivar. Using toluene to dissolve the DPPH and the oil samples, the order of effectiveness of some vegetable oils in inhibiting free radicals was as follows: coriander >black cumin> cottonseed> peanut> sunflower> walnut> hemp seed> linseed >olive >niger seed [42]. Solvent may influence the hydrogen-donating capacity of the antioxidant and affect the antioxidant activity of samples [34, 42].

Our results showed that the antiradical action of oil samples was affected by the seed maturity stage (Table 1). The



immature seed oil that contained the highest policosanol amount showed the maximum antiradical activity (96.42% and 90.35% for DPPH test and ABTS assay, respectively). A decline in the antioxidant activity was also observed for carob, pepper and guava throughout fruit ripening [7–9]. The stronger antioxidant capacity of immature seed oil compared to mature seed oil may be due to the differences in their content and composition of unsaponifiable matter. In our previous study, we found that the amount of total unsaponifiable matter decreased during milk thistle seed maturation, while the total lipid content increased [29]. Additionally, Ramadan and Mörsel [42] reported a positive correlation between the radical scavenging activity of vegetable oils and their levels of unsaponifiables.

During seed maturation, a high positive correlation was observed between total policosanol content and antiradical activity of seed oil samples through DPPH (r =0.952) and ABTS (r = 0.899) assays (Table 4). This result suggested that policosanol content may contribute to the antioxidant potential of oil sample. The antioxidant activity was correlated not only with the total amount of antioxidants, but also with the presence of selected compounds [42]. Dabbour et al. [31] reported that the high free radical scavenging capacity of cold-pressed milk thistle seed oil may be due to the presence of phenolic compounds and alpha-tocopherol. For the ethanolic extract of milk thistle seeds, the antioxidant potential was mainly correlated to the total content of flavonoids and phenolics rather than the content of individual compounds including silvbin [10]. Other reports indicated a positive correlation between radical scavenging capacity of seed oils and their levels of unsaponifiables and phytosterols, while a negative relation was noted with the amounts of phenolics and tocopherols [42]. Conversely, Kozłowska et al. [34] reported a positive correlation between total phenolics content and antioxidant activity of seed oils and a negative correlation with total sterols. The general consensus within the literature is that the antioxidant capacity appears to be related to the complementary role of different synergic compounds rather than being ascribed to one or a few compounds [10, 42].

In vitro anti-arthritic activity

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure. It is one of the causes of arthritic disease and inflammation [37]. Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [36]. In vitro anti-arthritic activity of studied oil samples was evaluated with bovine serum albumin (BSA) denaturation and egg albumin denaturation methods. Our study reveals that the milk thistle oil is capable to inhibit the denaturation of protein (Table 3). The oil extracted from mature seeds showed inhibition

Table 3 Effect of milk thistle oil on egg albumin and BSA denaturation, during seed maturation

Maturation stage	% of inhibition ^a	% of inhibition ^b
Immature	59.89 ± 1.4	72.49 ± 1.3
Intermediate	78.46 ± 1.2	85.32 ± 1.0
Mature	86.36 ± 1.5	92.53 ± 1.2

Values were expressed as means \pm SD of triplicate experiments

^a% of inhibition of egg albumin denaturation

^b% of inhibition of bovine serum albumin (BSA) denaturation

of denaturation of BSA and egg albumin by 72.49% and 59.89%, respectively. This result suggested that milk thistle oil might prevent denaturation of protein in rheumatoid arthritis and could be used as a potential anti- arthritic agent. Previously published studies examined the anti-arthritic activity of various plant extracts. The ethanolic extract of *Oryza sativa* inhibited the egg albumin and bovin serum denaturation by 84.15% 60.47%, respectively [36]. The methanolic stem extract of *Cuscuta pedicellata* also exhibited strong inhibition of protein denaturation [43]. Kamble et al. [44] indicated that ethanolic extract of leaves of *Vitex negundo* and Punica granatum showed potential anti-arthritic activity as compared to aqueous extract.

Our results showed that the anti-arthritic activity of oil samples was affected by the seed maturity stage, as shown in Table 3. The maximum inhibition of BSA denaturation (92.53%) and egg albumin denaturation (86.36%) were exhibited by the oil extracted from the immature seeds. Throughout seed maturation, the total policosanol content of milk thistle oil was highly correlated with the inhibition of BSA denaturation (0.774) and egg albumin denaturation (0.901) (Table 4).Therefore, the anti-arthritic activity of oil can be attributed, in part, to the policosanol content. Kumari et al. [37] reported that the anti-arthritic activity of methanolic extract of *Rhizophora mucronata* might be due to the presence of active principles such as polyphenolic content, triterpenoids, alkaloids and flavanoids. Though present in small amounts,

 Table 4
 Correlation coefficients (r) between policoanol content, antioxidant and anti-arthritic activities of milk thistle oil, during seed maturation

	Correlation coefficient
TPC-DPPH effect	0.952
TPC-ABTS effect	0.899
TPC- % of inhibition of EAD	-0.901
TPC- % of inhibition of BSA	0.774

TPC Total Policosanol content

DPPH 1,1-diphenyl-2-picrylhydrazyl radical

ABTS 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid)

EAD egg albumin denaturation BSA bovine serum albumin denaturation the minor constituents of dietary oils may supplement the dietary therapies for rheumatoid arthritis [45].

Conclusions

The results of the present study demonstrated that milk thistle oil exhibited potential antioxidant and antiarthritic activities. Thus, this oil might prevent denaturation of protein in rheumatoid arthritis and could serve as natural antioxidant and anti-arthritic agents for application in the food industries and pharmaceutic. The immature seed oil that contained the highest policosanol amount showed the maximum anti-arthritic and antioxidant activities as compared to mature seed oil. Policosanol contents in the seed oils may have a great impact on their biological effects. Further studies are needed to explore the medicinal value of milk thistle oil and to elucidate the mechanism of the In-vitro anti-arthritic activity of the seed oil. A randomized controlled cross-over trial investigating the effect of antiinflammatory diet on disease activity and quality of life in rheumatoid arthritis: the Anti-inflammatory Diet In Rheumatoid Arthritis (ADIRA) study protocol

Abstract

Background: Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects 0.5–1.0% of the population, and where many patients in spite of modern pharmacological treatment fail to reach remission. This affects physical as well as mental wellbeing and leads to severely reduced quality of life and reduced work capacity, thus yielding high individual as well as societal costs. As a complement to modern pharmacological treatment, lifestyle intervention should be evaluated as a treatment option. Scientific evidence exists for anti-inflammatory effects by single foods on RA, but no study exists where these foods have been combined to obtain maximum effect and thus offer a substantial improvement in patient life quality. The main goal of the randomized cross-over trial ADIRA (Anti-inflammatory Diet In Rheumatoid Arthritis) is to test the hypothesis that an anti-inflammatory diet intervention, compared to a regular diet, will decrease disease activity and improve quality of life in patients with stable established RA.

Methods: In total, 50 RA patients with moderate disease activity are randomized to receive initially either a portfolio diet based on several food items with suggested anti-inflammatory effects or a control diet during 2 × 10 weeks with 3 months wash-out between diets. Food bags are delivered weekly by a home food delivery chain and referred to as the fiber bag and the protein bag, respectively, to partially blind participants. Both groups continue with regular pharmacological treatment. Known food biomarkers will be analyzed to measure intervention compliance. Impact on disease severity (measured by DAS28, a composite score which predicts disability and progression of RA), risk markers for cardiovascular disease and quality of life are evaluated after each diet regimen. Metabolomics will be used to evaluate the potential to predict responders to dietary treatment. A health economic evaluation is also included.

Discussion: The nutritional status of patients with RA often is poor and many ask their physician for diet advice. No evidence-based dietary guidelines for patients with RA exist because of the paucity of well-conducted sufficiently large diet intervention trials. ADIRA is an efficacy study and will provide evidence as to whether dietary treatment of RA can reduce disease activity and improve quality of life as well as reduce individual and societal costs.

Trial registration: ClinicalTrials.gov Registration Number: NCT02941055.

Keywords: Rheumatoid arthritis, Diet, Anti-inflammatory, Quality of life, Sweden

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterized by systemic inflammation and joint damage. In spite of modern expensive pharmacological treatment, much pain and suffering as well as reduced work capacity remain. Hence, additional treatment options such as diet interventions are requested by patients and treating physicians.

RA affects 0.5–1% of the population globally [1]. The inflammation leads to joint destruction and fatigue as well as to impaired physical functioning, work productivity and activities of daily living, and compromise overall emotional well-being. A negative impact on relationships with friends and family has been reported by one-fifth of RA patients. Further, suboptimal mental health is often reported [2]. The inflammation also leads to increased risk for cardiovascular diseases (CVD) and together with the immune-suppressive treatment also to increased risk for infections-the two leading causes of death among patients with RA [3]. Patients with RA face approximately 50% increased risk of myocardial infarction and stroke compared to the general population and their risk of CVD is comparable to that of patients with diabetes [4, 5]. Consequently, life expectancy among patients with RA is reduced by 5-10 years, compared to non-diseased individuals.

Modern RA therapy includes immunosuppression by disease modifying anti rheumatic drugs (DMARD), steroids and the more recent line of biological therapies such as TNF- α -inhibitors. The treatment aims at long-term remission i.e. no pain, tenderness of joints or functional impairment. This is achieved in 10–50% of patients with early RA [6]. However, a recent systematic review highlighted that, in spite of these modern therapies, a substantial proportion of patients with RA still experience severely impaired health [2].

Thus, in spite of modern therapy, RA continues to present a considerable human and economic burden. An estimated one-third of patients with RA terminate employment prematurely, and 5 years after diagnosis 30–40% of patients experience work disability [2]. The authors of the systematic review conclude that "Despite advances in treatment that have helped to improve outcomes for patients with RA, treatment goals, aspirations, and expectations are seldom met for both patients and physicians. Novel treatment approaches for RA need to be tested for their ability to ameliorate contemporary unmet need." [2]

Associations between diet and chronic diseases such as CVD, cancer and diabetes have long been established [7, 8]. Hence, for these diseases evidence-based dietary treatment guidelines are available. In contrast, for inflammatory diseases such as RA no dietary guidelines exist, reflecting the ambiguous evidencebase. Currently, patients with RA in Sweden are encouraged to follow the same recommendations as the general population. These aim at increasing intake of fruit and vegetables to 500 g/day, fish and shellfish to 2–3 times per week, choosing whole grain over refined grain products and low-fat dairy products over full-fat varieties. Also, intake of red meat and refined sugars should be reduced [9].

The potential for diet to improve RA is obvious; diet could influence symptoms of RA by influencing the inflammatory activity, changing the lipid profile, increasing antioxidant levels, and altering the microflora of the intestine. Observational studies show lower incidences of RA among people with a healthy diet as captured by the Healthy Eating Index [10], larger consumption of LC n-3 PUFA [11] and fish [12]. Intervention studies using complete diets show that the Mediterranean diet (characterized by high intakes of olive oil and fish and low intake of red meat and dairy products), low-fat vegan diet and diets rich in unsaturated fat or probiotics have positive effects at alleviating pain and on inflammation markers [13]. Fish oil or dietary LC n-3 PUFA, i.e. reflecting fish intake, in addition to treatment with DMARD have been reported to increase the number of successful and continued DMARD treatments, indicating that pharmacological and dietary treatment complement each other [14, 15]. So far, no study has combined all components with indicative effects on RA, thus limiting the possibility to evaluate the full potential of dietary treatment of the disease. In sum, high-quality studies are needed that evaluate the combined effect of foods rich in anti-inflammatory and immune strengthening substances that could interact synergistically on RA.

Whether patients with RA respond to a dietary intervention or not might relate to genetics, habitual diet, the microbiome and/or the diversity and intensity of disease activity. Metabolomics, ie the analysis of low molecular weight metabolites, investigates overall metabolomic activity, taking genetic and environmental variation into account. It is a powerful tool to measure global and dynamic metabolic responses in disease and clinical intervention and some research also exist on RA. Different baseline metabolic profiles have been identified among patients with active RA compared to those in remission [16]. Thus, it may be possible to identify metabolic biomarkers to predict response also to dietary treatment among patients with RA. The metabolomics methods are relatively new and therefore few dietary interventions have used this approach to look at metabolic effects. Further, since the metabolism might be pertubated in patients with RA due to the metabolic syndrome or cachexia, metabolomics opens new possibilities to look at previously unknown metabolic changes as a consequence of a specific diet.

Research objective and specific questions

We hypothesize that an anti-inflammatory diet, in addition to pharmacological treatment, can improve health and quality of life in patients with rheumatoid arthritis. Specific questions are if an anti-inflammatory diet, compared to a usual Swedish diet, can reduce inflammation, pain and disease activity; reduce risk markers of cardiovascular disease; improve body composition; improve quality of life; reduce patient and societal costs related to the disease, and if metabolomics can be used to predict responders.

Methods/Design

The randomized controlled cross-over trial ADIRA (Anti-inflammatory Diet In Rheumatoid Arthritis) is carried out among patients with established RA with moderate disease activity to evaluate response to a portfolio diet treatment, compared to control diet (i.e. western diet). Both groups continue with pharmacological treatment as usual but herbal medicines or diet supplements are not allowed. In Sweden, patients with established RA are treated with DMARD and biologics at out-patient clinics according to the Swedish Society for Rheumatology Guidelines [17]. The portfolio diet treatment builds on a combination of individual food items with indicative effects on different RA symptoms. The trial thus evaluates the treatment potential of a diet based on a combination of functional concepts that likely potentiate each other in their anti-inflammatory effects. Although it is not possible to fully blind a diet intervention, the two groups are treated as similarly as possible except for diet content. Assessors of outcome are blinded. With respect to blinding of participants, see below.

The schedule of enrolment, intervention and measurements according to Standard Protocol Items: Recommendations for Intervention Trials (SPIRIT) requirements is shown in Fig. 1, and the SPIRIT Checklist is available as Additional file 1. The cross-over study is presented in Fig. 2.

Participant selection

Invitation letters to the study were sent to patients with RA residing in the Västra Götaland region, Sweden, identified through the Swedish Rheumatology Quality Register, SRQ (http://srq.nu/). Responders to the invitation letters or to posters at the Sahlgrenska University Hospital were invited to screening visits. Inclusion criteria was disease duration >2 years, active disease i.e. $DAS28 \ge 2.6$ that was clinically stable and under adequate control and medication at the screening visit. DAS28 stands for Disease Activity Score, where swelling and tenderness in 28 different joints are evaluated in combination with information on erythrocyte sedimentation rate (ESR) as well as patient-reported global assessment of health [18]. Exclusion criteria included other condition demanding active medical attention, changes in DMARD during last 8 weeks, intolerant or allergic to food items included in the intervention diets or not willing to eat omnivore diet. Between February and September 2017, 13 men and 53 women, 18-70 years of age, were screened for eligibility. At the screening visit, study details were presented, informed consent obtained and habitual diet was assessed. All ADIRA study visits take place at the research ward of the Clinical Rheumatology

			STU	JDY PE	RIOD			
		Enrolment	Allocation		Pos	st-allocat	ion	
Time point		Pre- intervention	Time 0	Time 1	Time 2	Wash out	Time 3	Time 4
ENROLMENT	` :							
Eligibility s	creen	Х						
Informed co	nsent	Х						
Allo	cation		Х					
INTERVENTI SEQUENCE:	ON							
A ti	nen B				1		+	
B tl	nen A			+	1		+	
ASSESSMENT	'S:							
D	AS28	Х			Х		Х	Х
Blood and	urine			Х	Х		Х	X
Blood pre	essure			Х	Х		Х	X
Anthropor	netric isures			х	х		Х	X
Dietary asses	sment	Х		Х	Х		Х	Х
Question	naires	Х		Х	Х		Х	X
Content for the schedule of enrolmer	nt. inte	rvention and m	easurements	accord	ina to S	PIRIT reg	auireme	ents



Research Centre, Sahlgrenska University Hospital, Gothenburg, Sweden, and measurements are carried out by two registered nurses experienced in rheumatology assessments along with the project team.

Patients agreeing to participate and fulfilling all inclusion criteria were thereafter invited to baseline measurements within a few weeks. These included clinical phenotype, body composition (bioelectric impedance spectroscopy) and health assessment, 3 day food record, serum samples for blood lipids, urine sample, metabolomics and inflammation markers (e.g., high-sensitive Creactive protein (hs-CRP), cytokines and intercellular adhesion molecules), Health Assessment Questionnaire [19] (HAQ), Quality of Life (QoL) and a questionnaire on socio-demographics, lifestyle, acute health care visits and non-steroidal anti-inflammatory drugs (NSAID) and corticosteroid consumption.

Thereafter, patients were randomized to either diet regimen for 10 weeks. Randomization scheme was computer-generated and revealed to participants and study team at the baseline visit. At the end of the 10 wk. period, the same measurements are repeated. Individual pharmacological treatment is reported at baseline and any changes or additions in pharmacological treatment are noted during the study period. After a 2-3-mo wash-out period, the alternative diet regimen is followed for another 10 weeks. Compliance to the diets is monitored by a telephone administered interview at wk. 5 in both diet periods and with analyses of plasma phospholipid LC n-3 PUFA at the end of each diet period as well as with 3 d food record at the end of each diet period. Support for participants is provided at this telephone interview to promote trial retention; it is also possible for participants to contact the study team at any time during the intervention. Participants are asked to report any side effects. Should a participant decide to guit the trial, no further data are collected.

Diet intervention

For both diets, food bags are delivered weekly by a home food delivery chain and make up approximately 50% of the daily intake during 5 weekdays. All meals are easily prepared with minimal peeling and cutting that could be challenging during active phases of RA. The meals are varied in style and ingredients to promote compliance and to ensure satisfactory nutrient intake. Participants in both groups are provided with recipes and information on the diet they are to follow. All participants are encouraged to maintain weight stability. Macronutrient composition of both diets is presented in Table 1.

The anti-inflammatory diet bag contains 5 main meals, 5 breakfasts and 5 in-between-meal snacks per week: main meal dishes includes fish 3-4 times weekly, mainly oily fish, and 1-2 vegetarian meals rich in prebiotics (dietary fiber). Breakfast meals consist of low-fat dairy products, whole grains, nuts, berries and probiotic fruit juice is included with each breakfast meal. In-betweenmeal snacks comprise of 2 fruits per day. Participants are also provided with olive/rapeseed oil for cooking. Wholegrain, legumes and vegetables ensure an adequate intake of fiber and prebiotics, and fish, oils and nuts provide unsaturated and LC n-3 PUFA and probiotics are added to the diet. Participants are not provided with any meat and are encouraged to restrict intake of red meat to 3 servings per week. This diet therefore combines components of a vegetarian and Mediterranean diet with LC n-3 PUFA and probiotics, which all have shown promising effects on clinical outcomes of RA. Total content from animal sources (fish and low fat dairy products) is 28% and from vegetable sources 72%. In an effort to blind the intervention to participants, this bag is referred to as the fiber bag instead of antiinflammatory bag.

The control diet bag contains 5 main meals, 5 breakfasts and 5 in-between-meal snacks per week: five dishes including meat or chicken. The 5 servings of breakfast meals consist of full-fat dairy products, corn flakes, white bread and fruit juice without probiotics. Inbetween-meal snacks comprise of curd and protein bars. Participants will also be provided with a mix of butter and margarine for spread, together with recipes and instructions for food preparation. To blind the intervention to participants, this bag is referred to as the protein bag. Protein content is not unusually high; it is actually similar to the reported intakes of Swedish men and women between the ages of 45-65 years in the national dietary survey Riksmaten 2010 in terms of macronutrients and guality of fat and carbohydrate intake [20]. Thus, the diet is representative of the dietary habits of the average Swedish person. Total content from animal sources (beef and chicken) is 48% and from vegetable sources 52%. In Sweden, high protein and full fat products rich in saturated fat (e.g, butter) have been trendy during the last years and it is not obvious to most participants which diet is the healthy one. Also, they are informed that the effects of two different diets are evaluated in the study.

Participants expressing dislike of a certain food item have the possibility to exchange this for a food item of similar nutrient composition.

Analysis of outcome

Outcomes are measured after each diet regimen. Primary outcome is DAS28, where we expect a difference between diet regimens > 0.6 units (considered clinically relevant) in the intention-to-treat analysis. Secondary outcomes include DAS28-CRP (where hs-CRP is used instead of ESR in the DAS28 calculation), inflammation markers, anthropometry, QoL, HAQ, blood lipids, blood pressure, acute health care visits and NSAID and corticosteroid consumption. Differences in outcomes will be compared for the two diet regimens in ANCOVA analyses, adjusting for baseline disease activity scores and potential confounders (e.g., age, other lifestyle factors). Mixed models also will be applied to fully handle missing data. Interaction by sex and socio-economic position will be evaluated.

Table 1 Macronutrient content of the two intervention diets in ADIRA

Macronutrient	Fiber diet		Protein diet	
	Content	% of total energy	Content	% of total energy
Energy (kJ)	4623		4596	
Energy (kcal)	1105		1098	
Carbohydrate (g)	119	48	129	49
Protein (g)	46	17	62	23
Fat (g)	44	35	34	28
SFA (g)	12	9	16	13
MUFA (g)	14	11	11	9
PUFA (g)	14	11	11	3
Omega 3 (g)	4	3	0.8	0.6

SFA saturated fatty acids, MUFA mono saturated fatty acids, PUFA poly-unsaturated fatty acids

Drop-outs will be included in secondary analyses of changes in DAS28 within one diet period if they have completed at least one diet period. Drop-out analyses will be carried out in that baseline information will be compared between drop-outs and those who complete the study.

Data will be entered and stored at the Dept Internal Medicine and Clinical Nutrition, Sahlgrenska Academy, University of Gothenburg, Sweden. Stored data files will not contain any personal identifying information; only the PI will have the code list and this will be kept in a locked drawer. Quality control procedures of data will be carried out by the PI. All members of the research team have access to final trial dataset. Results will be published in international peer-reviewed journals. They will also be shared with professional societies in rheumatology and nutrition. Participants will receive information about their own results at the end of the trial.

Cost-effectiveness

ADIRA is an efficacy study and hence not optimal for evaluation of cost-effectiveness in clinical use. Like in all lifestyle interventions, the long lasting compliance to lifestyle changes is essential for such evaluations and this will be evaluated in the following effectiveness ADIRA trial. However, it will still be possible to evaluate if the ADIRA efficacy trial has *potential* to be cost-effective in clinical praxis. The main cost is incremental costs of anti-inflammatory diet compared to control diet and these exist as long as the new diet is consumed, but also participants' time spent will be recorded and valued. Expected benefits include gain in QoL and reduced use of acute health care and medication as well as sick leave. Gain in QoL (mainly pain relief, measured with SF-36/ SF-6D [21-23], EQ-5D and EQ VAS [24]) likely occurs in short time and is thus possible to evaluate 10 weeks from start. In contrast, changes in health care use and sick leave may take longer to accrue. Therefore, the main economic analysis will be a comparison between incremental food costs and incremental QoL while the dietary change is in use. In practice, the food costs and QoL gain per week diet is used, presented as costs per quality-adjusted life-years (QALY). Best evidence of the effect of pain relief for RA patients on use of acute health care, medication and sick leave will be obtained from literature and used in evaluations of the costeffectiveness of anti-inflammatory diet compared to control diet.

Sample size

To detect a DAS28 difference of 0.6 units (clinically relevant) with 90% power and $\alpha = 0.05$, 38 patients are needed in cross-over design. Skoldstam [13] detected a difference of 0.6 units with 30 patients per group in a

diet parallel trial. We have carried out a pilot cross-over diet study that detected a difference of 0.34 units with 23 patients, confirming that cross-over design reduces the sample size (unpublished results). To account for drop-out or non-compliance, ADIRA cross-over study included 50 women and men.

Metabolomics

For metabolomics, serum samples will be analyzed with nuclear magnetic resonance (NMR) at the Swedish NMR Centre at University of Gothenburg, Sweden, as well as with mass spectrometry (MS) at Chalmers Technical Institute, Gothenburg, Sweden. We will use an untargeted metabolomics approach for a global description of the metabolites that can be found in participants' serum using unsupervised statistical analyses, ie principal component analysis (PCA). Subsequently supervised clustering, ie orthogonal projection to latent structuresdiscriminant analyses (OPLS-DA [25]) and OPLS-EP [26] (for paired data) will be performed to compare metabolites between patients who respond and who don't respond to the inflammatory diet. Finally, in a targeted metabolomics approach, metabolites providing the greatest discrimination between groups of responders vs. non-responders will be identified using publicly and commercially available as well as in-house developed databases.

Ethical considerations

Ethical approval has been received from the Gothenburg Regional Ethical Review Board, Gothenburg, Sweden (agreement No: 976–16 of November 21, 2016).

Discussion

The nutritional status of patients with RA often is poor, many are overweight or obese and many ask their physician for diet advice. However, no evidence based dietary guidelines for patients with RA exist because of the paucity of well-conducted sufficiently large diet intervention trials. Most existing studies are observational or focusing on few dietary items. ADIRA will contribute highquality scientific evidence, as it is based on state-of-the art methodology in terms of diet composition and evaluation of compliance using biomarkers. It is original and innovative in that it applies a portfolio diet and homedelivery services. The portfolio diet treatment builds on a combination of individual food items with suggested effects on different RA symptoms that likely potentiate each other in their anti-inflammatory effects. The treatment likely has high cost benefit due to its low adverse event rate and low cost, in comparison with new and potent medications. It is an efficacy trial; hence internal validity is most crucial and great care is taken to achieve high internal validity. External validity, of secondary

importance in this efficacy trial, can be evaluated because patients are recruited from the Swedish Rheumatology Quality Register. Our previous pilot trial involving patients with RA indicated that high external validity was possible to achieve.

Strengths

ADIRA trial has several strengths. It has a cross-over design that minimizes individual variation between diet groups. The trial is population-based in that all eligible patients with RA in the region are identified through the Swedish Rheumatology Quality Register and invited to participate. Patients are randomized to a specific diet at the beginning of the study, meaning that effects of season, holidays and carry-over from previous test period should be evenly spread between the two diets. The intervention diets are developed by registered dieticians and have a similar content of macronutrients and energy. The food bags are delivered to participants' homes by a home delivery food chain every week, which is convenient for participants. Also the meals are easy to prepare or ready meals, with a high nutritional quality. The food bags are referred to as the fiber bag and the protein bag to partially blind participants, and assessors of outcome are fully blinded.

The primary outcome measure, DAS28, is the most clinically relevant outcome and often used in doubleblinded pharmacological interventions. The trial is carried out in collaboration with rheumatologists and, hence, results can easily be shared with treating physicians. Compliance to the diet is measured with objective food biomarkers. In addition, metabolomics will be used to identify biomarkers of response to the intervention, and an evaluation of the cost effectiveness of the trial also will be performed.

Limitations

The trial also has some limitations. The disease is more common among women than men and this is mirrored in our study sample where men make up a minority. Further, the recruitment area is limited to Västra Götaland Region so that patients can visit the research clinic at Sahlgrenska University Hospital in Gothenburg and receive the home delivered food bags. To obtain the planned sample size within this area, broad inclusion criteria are applied. Hence, age varies between 27 and 74 years, disease duration is minimally 2 years and type of pharmacological drugs used by participants varies tremendously between patients and over time. Potential participants were invited by postal mail to patients in SRQ residing in Västra Götaland Region and posters at the Sahlgrenska University Hospital. Among the 66 patients booked for screening, 75% qualified to start the trial. Hence, ADIRA participants represent a selected group of patients with RA and potential selection bias has to be evaluated. Socio-demographic information on ADIRA participants will later be compared with similar information on patients in Sweden with rheumatoid arthritis as described in the Swedish National Patient Register [27]. The main outcome DAS28 includes both objective components such as erythrocyte sedimentation rate and number of swollen and tender joints, and subjective patient-relevant components such as the patient-reported global assessment of health. It is the most clinically relevant outcome but, it is partially subjective, which must be considered when interpreting results.

Also, participants only receive about 50% of their total dietary intake during 5 days of the week during each 10-wk diet period. They are instructed to add foods in line with the intervention diet, but we cannot fully know to what extent this is happening. Objective biomarkers of compliance are used together with a telephone interview half way through each diet period and a 3-d food record at the end of each diet period. This will only partially capture compliance. However, because ADIRA is a cross-over study each participant acts as his/her control. Likely, the home diet will be similar for each individual in both diet periods except for what is guided by the intervention and this will minimize the effect of the home diet on the intervention. In addition, it is possible that the 10 week long period of each diet may be too short to provide a measurable effect on disease severity. Finally, RA is a disease where activity varies over time and pharmacological treatment changes periodically. This will likely dilute any true effects on disease activity of one diet over the other. Still, evaluations of additional treatment for patients with RA are needed and should be performed while accounting for these methodological challenges.

Conclusion

ADIRA is an efficacy study and will provide evidence whether dietary treatment of rheumatoid arthritis can reduce disease activity and improve quality of life as well as reduce individual and societal costs in addition to pharmacological treatment.



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