The role of inflammation in sciatica: the contradictory effect of macrophages

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Chapter I

Introduction and general outline

"Don't count the days, make the days count"
(Muhammad Ali)

With an incidence of 1-3%, lumbar disc herniations cause a major burden for society worldwide [1]. Patients with lumbar disc herniations often suffer from disabling leg pain radiating down the dermatome, also known as sciatica. The origin of sciatica can partially be explained by mechanical compression of the nerve root by the herniated part of the disc, however, the presence of asymptomatic cases with clear nerve root compression, and vice versa symptomatic cases where clear compression is absent, illustrate that compression cannot be the only factor at play [2, 3]. In search for an additional cause for sciatica, an increasing amount of interest is being devoted to inflammation. Disc inflammation may occur if the nucleus pulposus herniates into the epidural space, where it is exposed to the systemic circulation, which creates an opportunity for macrophages to infiltrate [4]. This inflammation response is believed to irritate the nerve root, thereby exacerbating sciatica symptoms. In contrast, macrophages are also thought to play a vital role in recovery through resorption of the herniated disc material.

It follows that macrophages can be seen as a double-edged sword: on one hand, macrophage infiltration may result in an exacerbation of sciatica through expression of pro-inflammatory cytokines such as IL-6, IL8 and TNF-alpha [5-7]: the so-called 'painful inflammation response'. On the other hand, macrophages may induce a resorption process by excreting matrix metalloproteases [8], inducing apoptosis, degrading collagen fibers [9] and phagocytizing dead material [10]: the so-called 'functional inflammation response'. As of today, it remains unknown whether all patients unavoidably experience both effects or whether some mainly experience the beneficial resorption abilities while others mostly suffer from their nerve sensitizing features. Moreover, the extent of macrophage infiltration, and therefore the impact of their effects, may vary widely from patient to patient and, in many patients, macrophages are absent.

Whether macrophages will infiltrate the area with the lesioned disc and how they behave once they are there is believed to depend upon the local environment. One of these environmental factors is the type of disc herniation, which can be categorized in 2 groups: The first is called disc bulging, in which the nucleus pulposus (NP), the inner part of the disc, may bulge into the epidural space, but is maintained within the outer structure of the disc, the annulus fibrosus (AF). Therefore, the NP is not directly exposed to the epidural space. This is contrary to the extrusion, in which case the NP is extended in the epidural space due to a defect in the structural integrity of the outer layer. Because the extruded NP is more exposed to the systemic circulation in the epidural space, extrusion may result in a higher degree of neovascularization and consequently a higher degree of inflammation.

A different characteristic that could influence macrophage infiltration is the mechanism of disc extrusion: One mechanism is through an endplate avulsion (EPA), in which case the annulus fibrosus is detached from the endplate; the other is due to an annulus fibrosus tear (AFT). The important difference between these two mechanisms is the rate of vascularization between the AF and endplate. In contrast to the avascular AF and NP, the endplate is highly vascularized. In a physiological situation, the endplate supplies the NP and AF with nutrients through diffusion, which prevents inflammatory cells like macrophages from entering the NP and AF. However, when the endplate is avulsed, it is directly exposed to the ruptured blood vessels of the endplate, which may influence the rate of macrophage infiltration. This is in contrast to the AFT, in which case exposure to circulation is limited to the epidural space and no additional source of local neovascularization is present.

Another phenomenon that may be of significance for macrophage infiltration in the presence of Modic Changes (MC) in the endplate, also known as vertebral end-plate signal changes (VESC) [11]. MC are visible on the MRI and can be subdivided into 3 types: MC type 1 (MC1) is characterized by acute inflammation and bone marrow oedema, MC type 2 (MC2) by chronic inflammation and fatty marrow proliferation, and MC type 3 (MC3) by sclerosis [12, 13]. These inflammatory reactions of the endplate, visualized as MC have been proposed to be associated with slowing the recovery rate in patients that suffer from a herniated disc [14]. These characteristics of disc rupture and its local environment may not only matter for degree of macrophage infiltration, but also could be of influence on the macrophage differentiation process.

Macrophage differentiation depends on environmental factors. Each set of environmental cues will lead to distinct macrophage phenotypes, which show unique behavior and expression profiles [15]. Even though each phenotype is unique, many expression profiles can be polarized to pro- or anti-inflammatory, and their corresponding macrophage phenotypes are often referred to as M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophages [16]. The expression profile of M1 macrophage has been associated with exacerbation of pain symptoms [6], whereas the expression profile of M2 is involved in inflammation modulation, tissue repair and remodelling [5, 15, 17, 18]. Hence, polarization of macrophages may have a tremendous impact on the sciatic symptoms: through their impact on nerve irritation and sensitization, and through their impact on the resorption of the herniated disc. As of today, only limited evidence is available on what influences the extent of macrophage infiltration [15], and even less on what influences macrophage differentiation in the herniated disc. A better understanding of how the inflammation profile in response to the disc lesion affects sciatica symptoms and the rate of recovery could lead to new, more personalized treatment strategies.

Aims and outline of this study:

1. It is crucial to have an overview of what is currently known on the effects of macrophage infiltration and their pro/anti-inflammatory expression profile on sciatic symptoms. Therefore, the first aim of this study is to systematically review all known associations between (M1/M2) macrophage infiltration or their related inflammation factors, and clinical outcomes in patients suffering from a lumbar disc herniation (Chapter 2).

2. The beneficial effect of macrophage infiltration is supposedly through inducing a resorption process, which has never been verified in a clinical trial. Moreover, previous studies have only focused on the effects of macrophage infiltration in general and have failed to assess the interactions with the local environment. The second aim of this study is to associate macrophage infiltration with disc resorption on MRI (Chapter 3) and with clinical recovery during follow-up after surgery (Chapter 4). More interestingly, since an interaction effect with the environment is to be expected, these associations will be assessed for patients with and without MC separately, and the influence of bulging versus extruded discs on the effect of macrophage infiltration will also be considered.

3. In order to integrate macrophage infiltration and inflammation in the clinical decision process a non-invasive biomarker would be required. Gadolinium enhancement in MRI is explored to have potential as such a biomarker. Hence the third aim of this study is to evaluate gadolinium enhancement as tool to assess macrophage infiltration of the disc (Chapter 5).

4. Although some studies have reported on altered gene expression profiles of MC, the effect of EPA on the inflammation profile remains to be elucidated. Hence the fourth aim of this study is to elucidate the effect of endplate pathology on the inflammatory profile of the lesioned disc material, using a proteomic and bioinformatic approach (Chapter 6).

5. Since altered behavior of macrophages is believed to be caused by altered differentiation, the fifth aim of this study is to identify the type of macrophage (M1 vs M2) found in disc herniations, and whether it is influenced by the presence of MC (Chapter 7).

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Chapter 2

The contradictory effect of macrophage related cytokine expression in lumbar disc herniation: a systematic review

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"Luck is what happens when preparation meets opportunity." (Seneca)

Abstract

Purpose

Sciatic symptoms due to lumbar disc herniation are likely to be not solely caused by mechanical compression of the nerve root, but also by pain inducing elements from inflammatory processes. Key components in the inflammatory reaction are M1- and M2 macrophages, the M1 type being associated with pro-inflammatory processes and M2 with anti-inflammatory-processes.

Methods

The present systematic review summarizes all literature on associations between M1 and M2 macrophages and their related inflammation factors, and pain symptoms in lumbar disc herniation. Literature search was performed using an optimally sensitive search string. Studies were selected for inclusion by means of predefined in- and exclusion criteria and subsequently graded for risk of bias. A total of 14 studies were included. Overall risk of bias was moderate (8/14), three studies had a high- and three a low risk of bias.

Results

Regarding M1 related cytokines, high levels of TNF-α, TNFR1, IL-6, IL-8, IFN- γ, were all associated high VAS scores. In contrast, high levels of TNFR2 was associated with lower VAS scores. Moreover, no associations were found for IL-1a and IL-1β. Results regarding M2 related cytokines revealed the opposite: high levels of both IL-4 and IL-10 were associated with lower VAS scores. No associations were established for TGF-β. Moreover, presence of macrophages (CD68) was negatively associated with VAS scores.

Conclusion

while M1 related pro-inflammatory cytokines worsen pain symptoms, M2 related anti-inflammatory cytokines alleviate pain symptoms, Nevertheless, present evidence is limited and further research on the underlying pathophysiological mechanism in sciatica is required.

Introduction

At present, one of the most prevalent causes for physical disability is herniation of the intervertebral disc. When a disc herniates, it often causes compression of the nerve root, which leads to a radiating pain alongside the dermatome, often referred to as sciatica [1, 2]. At first sight, the cause for sciatica seems purely mechanical, but the observation of nerve root compression due to disc herniation in 20-76% of asymptomatic cases, suggests that mechanical compression is not the only factor at play [3-5].

Over the past two decades several researchers suggested that inflammation of the nerve root and/or disc plays a significant role in sciatica [4, 6-8]. It is hypothesized that nucleus pulposus (NP) material that herniates into the epidural space, induces a foreign-body reaction that involves macrophage infiltration [8]. These macrophages are not only suggested to play a role in resorption of herniated disc material [4, 5, 9] but also to, at least partially, play a role in inducing an inflammatory response. This could in turn cause pain. The different roles of macrophages are reflected in the contradictory views of experts and physicians. Any discrepancy in these roles may be dependent on the type of macrophage present in the disc material.

Present literature distinguishes M1 and M2 macrophages [10]. An M1 macrophage can differentiate from a monocyte if stimulated by Lipopolysaccharide (LPS) or Interferon-gamma (IFN-γ) or tumor necrosis factor (TNF), or granulocyte macrophage colony-stimulating factor (GM-CSF). M1 produces pro-inflammatory cytokines and products such as IL-1, IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18, IL-23 IL-27, TNF-α, and Bone morphogenic protein 2 (BMP-2) [10-12], the main focus of this type is microbicidal activity [11], and its expression profile is associated with exacerbation of pain symptoms [13]. On the contrary, if a monocyte is stimulated by IL-4, IL-10, IL-13, glucocorticoids or macrophage colony-stimulating factor (M-CSF), it differentiates into a M2 macrophage. This alternative type of macrophage excretes anti-inflammatory cytokines such as IL-1Ra, IL-10, and transforming growth factor-beta (TGF-β) [11], which are involved in multiple functions such as tissue repair and remodelling [10, 11, 14]. In contrast to the effect of M1 macrophages, M2 is believed to alleviate pain symptoms through resorption of herniated disc material [15]. During most inflammation processes, M1 or M2 macrophages occur sequentially [11]. However, depending on the disease and genetic predisposition, their ratios may vary widely [11]. Despite extensive research in the field of sciatica, the role of M1 and M2 macrophages remains to be elucidated. A better understanding of these processes could lead to improved prognostics and personalized treatment. The aim of the present study is therefore to systematically review all literature concerning the role of macrophages and their related pro- and anti-inflammatory cytokines and factors in lumbar disc herniation patients suffering from sciatic symptoms.

Materials and methods

Inclusion criteria

Studies with patients suffering from sciatica were to be included if the study analyzed the correlation between the presence of macrophages or their related cytokines and/or excretion factors, as verified by serum, CSF or disc material samples, and quantitatively measured clinical outcome parameters.

Search and selection

The electronic databases Medline (from 1960), EMBASE (from 1947) and Web of Science (from 1960) where searched up until February 2018. A search string in order to systematically explore all studies that included presence of macrophages or their related cytokines and factors was constructed and adapted per database. Eligible studies were selected on title and abstract by two independent review authors (ND and GL), with consensus meeting and referee (CVL) available, according to PRISMA guidelines. If the abstract alone did not provide sufficient information, the full paper was assessed. Afterwards, citation tracking was performed and further eligible studies were acquired.

The search strategy comprised strings for sciatica and macrophages, granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumor necrosis factor-α (TNF-α), tumor necrosis factor-beta (TNF-b), bone morphogenic protein 2 (BMP-2), tumor growth factor beta TGF-β, IL-1, IL-1α, IL-1Ra, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18, IL-27. No restrictions on publication date were made and all articles were to be fully published in English. Conference proceedings were excluded. The included studies had to consist of a minimum of 10 patients suffering from acute, sub-acute or chronic pain in lumbar disc herniation. No restrictions were made on follow-up. Furthermore, studies were only included if clinical outcome was measured reporting a pain scale, the Straight Leg Raising test (SLR) or the Oswestry Disability Index (ODI). Pains scales are the Visual Analogue Scale (VAS) leg pain or a comparable scale like the Visual Rating Scale (VRS) for pain or the Numeric Rating Scale (NRS) for pain. The ODI scale evaluates functionality focussing on the leg and back. Additionally, studies were excluded if they failed to specify which cytokine or excretion factor was present, or if the cytokine or excretion factor was not measured in serum, CSF or disc material.

Quality assessment

Two authors (ND, GL) reviewed the methodological quality of all included articles individually, using an adjusted version of the scoring criteria by Cowley (supplementary Table 1) [16], in which a maximum of 10 points can be given. Risk of bias was deemed low if the Cowley score was ≥ 8, moderate between 5-7 and High risk of bias with a score of 0-4. Differences in quality assessment between the two reviewers were justified in a consensus meeting.

Data extraction

The primary outcome of the present study comprises associations between macrophage related parameters and pain symptoms. From each study, basic information was gathered concerning authors (sponsoring, affiliation), methods (study design, sample size and type of analysis), patients (source population, inclusion criteria, exclusion criteria, baseline characteristics, and diagnostic characteristics), treatments (interventions), outcome variables and results.

Results

Study selection

The search in the PubMed database yielded 305 results, EMBASE yielded 585 and Web of Science yielded 272 results. In total 1162 references were obtained. After removal of duplicates, 755 remained. After abstract and full text screening, fourteen articles met inclusion criteria. Subsequently, citation tracking was applied, which did not lead to any additional findings. Hence the final number of included articles was fourteen (Figure 1).

Risk of bias Assessment

Of the fourteen studies, three were scored to have low, eight to have moderate and three to have high risk of bias. Regarding individual categories: First, risk of population bias was generally moderate, all studies reported age and sex, whereas only five studies provided specific and explicit in- and exclusion criteria for lumbar disc herniation [13, 17-20]. Second, selection bias could be ruled out in six studies and was also regarded as generally moderate [13, 17, 18, 20-22]. Third, risk of outcome bias was generally considered moderate as well; most studies clearly defined outcome measures except for Schistad et al (2014). Here, the authors described IL-8 measurements in the method section but failed to elaborate on them in the results [18]. If studies failed to test parametric test assumptions for VAS scores, no points for statistical analyses were awarded [13, 18, 20, 21, 23-26]. None of the studies described clinical evaluation as independent of the treating physician. Fourth, the selected studies showed a low risk of attrition bias, as all of the fourteen selected articles were prospective studies. Eight studies had a follow-up period longer than six months in all described studies [13, 18, 20, 22-25, 27]. Finally, only five studies explicitly reported to have no conflict of interest [18-20, 23, 24]. An overview of the risk of bias scores is provided in Table 1.

Data extraction – macrophages and related cytokines and -factors

The reported methods of measuring macrophages, cytokines and excretion factors varied widely. Some authors histologically described their presence in nucleus pulposus material that was taken out during surgery, others looked at presence of macrophages and accompanying inflammatory factors in blood or cerebral spinal fluid. Moreover, the choice of parameter studied varied widely. Not all the parameters that are associated with M1 and M2 macrophages were reported in the studies that were eligible for this review. The histological parameter for macrophages, CD68 (surface marker), was reported in a few studies. M1 related factors that were encountered are: interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF-α), tumor necrosis factor receptor 1 / 2 (TNFR1 / TNFR2), and M1 related cytokines that were reported are: IL-1α, IL-1β, IL-6, IL-8, IL-12. M2 related factors that were reported in the articles are: tumor growth factor-beta, (TGF- β), and the M2 related cytokines that were reported are: IL-4 and IL-10.

Association between macrophage marker and pain

Two out of the four studies on CD68 [22, 27], found a negative association with pain scores during follow-up [22, 27-29] and one study found a negative association with Straight Leg Raising test [29], which means that patients with higher CD68 (macrophage) expression, had less pain and lower SLR scores.

Association between pro-inflammatory factors (M1) and clinical outcome (Table 2)
In studies examining the association of TNF-α with VAS pain or SLR or ODI, five out of six studies found a positive association [17, 19, 20, 24-26], which means that patients with higher TNF-α levels had higher pain scores. The only study that did not find such association had a high risk of bias [26]. In most studies, TNF-α association with clinical parameters was evaluated at baseline, but in follow-up data the association remained present [20, 25]. Both studies on TNFR1, a TNF-α receptor, found a positive association with pain scores, one at baseline [24], and both during follow-up [23, 25]. In contrast, the same studies found that TNFR2 had a negative association with pain scores, which means that patients with high levels of TNFR2 reported lower pain scores [23, 25]. Three out of five studies on IL-6 found a positive association with pain scores and ODI [13, 18, 19, 23, 26]. One of the studies that did not find an association had high risk of bias [26]. The other study that did not demonstrate an association between pain and IL-6 determined the IL-6 concentration in disc material, while the three studies that did find a positive correlation examined IL-6 in serum.

Two out of four studies on IL-8 found a positive association with pain scores and SLR [13, 17, 19, 26], ; one of these studies examined IL-8 in disc material [17], and the other in serum [13]. Two of four studies on IL-8 found no association with pain scores, SLR or ODI, one of these studies (high risk of bias [26]) examined IL-8 in CSF and the other study examined IL-8 in serum [19]. All three studies on IL-1β showed no association with pain scores and SLR [24, 26]. The IL-1beta expression was examined in disc material, CSF and serum. The study on IL-1a found no association with pain scores [17].

Two studies examined the association of IFN- γ with pain or SLR and did not find an association [21, 26]. However, one of these studies had a high risk of bias [26], and the other examined the association with several VAS cut-off scores, thereby inducing outcome bias [21].

Association between anti-inflammatory factors (M2) and clinical outcomes (Table 2)
Two studies examining IL-10 demonstrated different results [17, 19]. One study did not demonstrate an association with pain score or SLR [17]. The other study demonstrated a negative association: in patients with higher pain scores or ODI, the concentration of IL-10 in serum was lower as compared to patients with a low pain score or ODI [19].

One of the two studies on IL-4 found a negative association with pain scores at 12 months follow up [20]. The other study demonstrated no association with VAS or ODI [19]. The study on TGF-β found no association with pain or SLR [17].

Discussion

The present systematic review established associations between the presence of macrophages and their pro-, and anti-inflammatory cytokines with pain and/or disability in lumbar disc herniation. Because of the heterogeneity in outcome measures and data presentation, only a qualitative analysis was performed. Also, methodological quality of the studies varied widely. For the M1 related factors, literature presented moderate evidence for associations between high pain scores and high levels of TNF- α, TNFR1 and IL-6, limited evidence for associations between high pain scores and high levels of IL-8, no associations between pain related outcome measures and IL-1α , IL-1β or IFN-gamma, and moderate evidence for an association between low pain scores and high levels of TNFR2. In contrast, for the M2 related factors, evidence with moderate quality was found for an association between low pain scores and high levels of IL-4, limited evidence for an association between low pain scores and high levels of IL-10, no association was found with TGF-beta.

Associations between inflammation markers and clinical outcomes

Primary outcome measures were sciatic symptoms, and cytokines and other macrophage related parameters. The present review specifically included studies that measured pain symptoms expressed by VAS- and/or ODI scores and/or SLR. These clinical symptoms were subsequently correlated to inflammatory parameters. The tissue or fluid in which these parameters were examined varied among studies. Some studies looked at mRNA [17, 23-25] or protein expression patterns [21, 23-25] in the nucleus pulposus or annulus fibrosis, while others measured concentrations of cytokines in CSF [26] or blood [13, 18-20, 26]. Other studies examined macrophage infiltration histologically [22, 27-29] in herniated disc tissue. The comparability of the studies included for review is therefore rather limited and these differences could hence have confounded our results.

The most convincing positive association between pain-related outcome measures and M1 excretion factors was provided by studies on TNF-α. Five out of six studies examining TNF-α expression patterns indicate that higher pain scores associate with a higher protein- and mRNA expression intensity in the nucleus pulposus [17, 23, 25], and with higher serum concentrations [19, 20, 26]. Of these studies, one had low risk of bias [20] and four had moderate risk of bias [19]. Only the study with lowest quality could not establish an association between inflammation and clinical outcome [26]. These findings suggest that lowering TNF-α levels with drugs such as TNF-α blockers may alleviate sciatic symptoms. The efficacy of TNF-α inhibitors has recently been reviewed by Williams et al. (2013) and Wang et al. (2014) [30, 31]. Both studies concluded that evidence supporting anti-inflammatory agents, as a means of therapy in sciatica, is present but not yet convincing. Combining these results with the findings from the present systematic literature review, we suggest the lack of convincing evidence of the previous reviews may be explained by the fact that TNF-α levels vary among patients, which diminishes the overall efficacy of TNF-α inhibitors when given to all sciatica patients instead of only the subpopulation with actual high TNF-α levels. In order to properly evaluate the efficacy of these inhibitors, an RCT that only includes patients with high TNF-α levels is needed.

Previous systematic reviews

Our findings are in agreement with previous systematic literature reviews and meta-analyses. Goupille et al. (1998) were the first to identify inflammatory mediators in disc herniation, and to suggest that inflammation is involved in sciatic symptom development [32]. However, the literature of 1998 failed to provide evidence for the suggested involvement. The present literature review is the first to specifically outline all established associations between different cytokines and other macrophage related inflammatory factors involved in sciatica on clinical outcomes.

Clinical implications and recommendations

Our current treatment for radicular pain is conservative care for a period of 8-12 weeks, if symptoms persist however, a surgical intervention is offered [33]. Unfortunately, even after surgery, some patients do not recover satisfactorily. As of today, it remains unclear why nerve decompression does not lead to pain reduction. Recent evidence by Lama et al. showed that in some discs cartilage fragments were found, and that in these discs only little swelling and infiltration of immune cells was present [34]. The authors suggested that cartilage fragments could interfere with the resorption process, which could be an explanation for the abovementioned variety in recovery rate. Presence of cartilage fragments in the intervertebral disc may be caused by a defect in the endplate. Moreover, endplate defects are known to increase permeability of the intervertebral disc, thereby increasing the risk of infection in the endplate. Pre-clinical findings suggest that when an inflammatory response is induced in mice discs by bacteria, increasing nerve outgrowth from the dorsal root ganglion into the disc consequently occurs [35]. Nerve outgrowth could subsequently lead to sensitization of the disc, thereby facilitating pain symptoms. Currently, the evidence for this theory is still limited and further exploration of these concepts is required.

Nevertheless, recent clinical studies are in line with the abovementioned findings and show that discs of some lumbar disc herniation patients were infected with Propionibacterium acnes or Staphylococcus Epidermidis [36, 37]. In addition, Dudli (2017) induced herniated disc samples with P. Acnes and found that 6 out 10 discs responded with excretion of pro-inflammatory cytokines [38]. This inflammatory response was associated with endplate defects on MRI, more often described as Modic changes [39, 40]. Likewise, others have associated the presence of Modic changes with less recovery after surgery [41], and a chronic inflammatory response. However, other factors than bacterial infection may induce a shift of macrophage differentiation towards M1 macrophages and induce the excretion of pro-inflammatory cytokines, like for instance endplate pathology, or an innate defect in macrophage differentiation [42].

Even though the abovementioned findings are still inconclusive; it has inspired the following theory (Figure 2): Patients that suffer from disc inflammation without any complicating factors such as bacterial infection, endplate pathology or immune defects will show a response that is dominated by M2 macrophages, which excrete anti-inflammatory cytokines such as IL-4 and IL-10. This type of inflammation will likely induce a resorption process and may thus be beneficial to the patient. On the contrary, if patients show an inflammation reaction of the disc with one of the abovementioned complicating factors, the reaction is likely to be mediated by M1 macrophages and pro-inflammatory products such as IL-6, IL-8, TNF-α and IFN-γ, originating from the disc and/or endplate. This type of inflammation, will likely aggravate the symptoms of the patients and could be recognised by presence of Modic changes on MRI.

As mentioned earlier, current evidence for this theory is limited, and the underlying pathofysiological mechanism should be further explored before this theory can be confirmed.. Hence we recommend that future studies focus on exploring the possible causes of macrophage differentiation towards M1 and the excretion of pro-inflammatory cytokines, and how the different causes affect symptoms of sciatica.

Moreover, despite the fact that inflammation has shown to play a significant role in sciatica, the impact of mechanical compression should not be forgotten. Inflammation only occurs in a fraction of the patients [43], indicating that for many, the pain has a mechanical origin that can be alliviated through decompression [33]. Nonetheless, in many cases both the mechanical and the inflammatory component will attribute to the sciatic symptoms. For such caseses, it remains difficult to define how much each component contributes to the experienced pain, since excision of the hernia will not only releave the compression, but also a part of inflammation, which was present in the herniated part. Likewise, anti-inflammatory agents are usualy studied in a population that contains both patients with and without inflammation. This attenuates the reported effect of the anti-inflammatory agents and prevents us from finding the real effect size of inflammation. This stresses the importance of taking inflammation into consideration in a study population. By doing so, steps can be made in delivering a more personalized treatment that takes the heterogeneity of sciatica into account.

Limitations of our study

Because this review only assessed Embase, Pubmed and Web of Science, relevant studies that are hidden elsewhere might have been missed. Furthermore, the criteria used for risk of bias assessment included arbitrary cut of points, such as: duration of follow up, exclusion criteria and validity of statistical analysis (Supplementary Table 1). Therefore, the risk of bias scores given to the evaluated studies may alter in a different review, which could lead to different qualities of evidence for the found associations. At last, this review was only able to include fourteen papers, and thus only has a limited amount of evidence to draw conclusions from. This illustrates that more studies on this topic are needed in order to validate the results from previous trials and further explore the role of inflammation in sciatica.

Conclusion

Cytokines excreted during the process of disc herniation in sciatica seem to have a contradictionary effect on pain symptoms. Pro-inflammatory cytokines worsen pain symptoms, while anti-inflammatory cytokines alleviate pain symptoms.

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Conflict of interest

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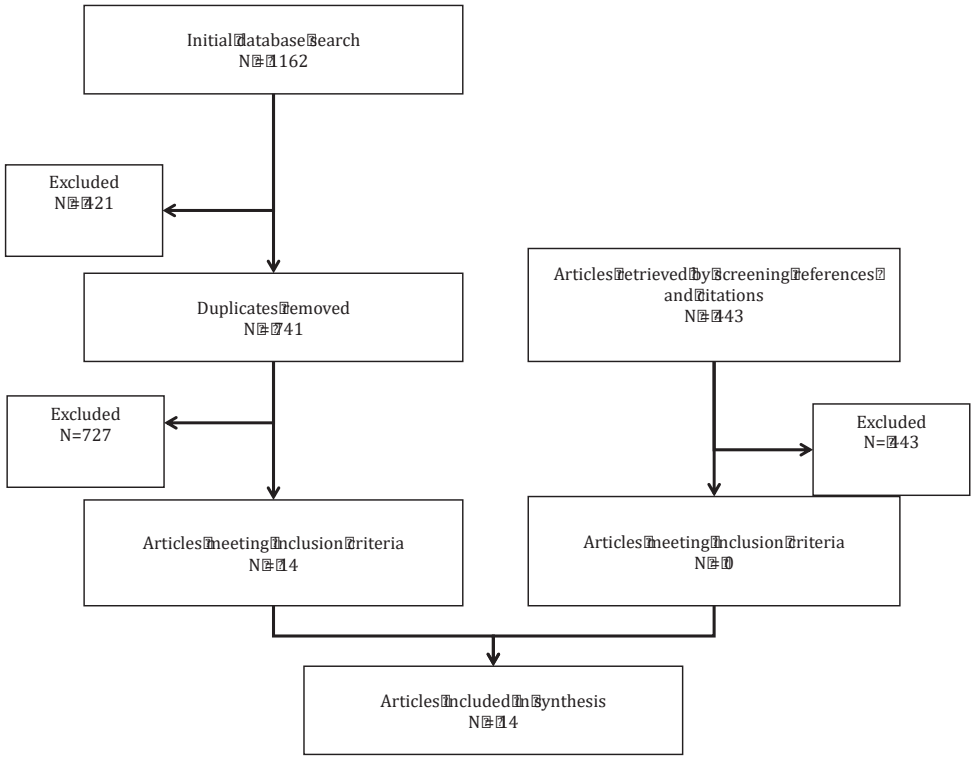
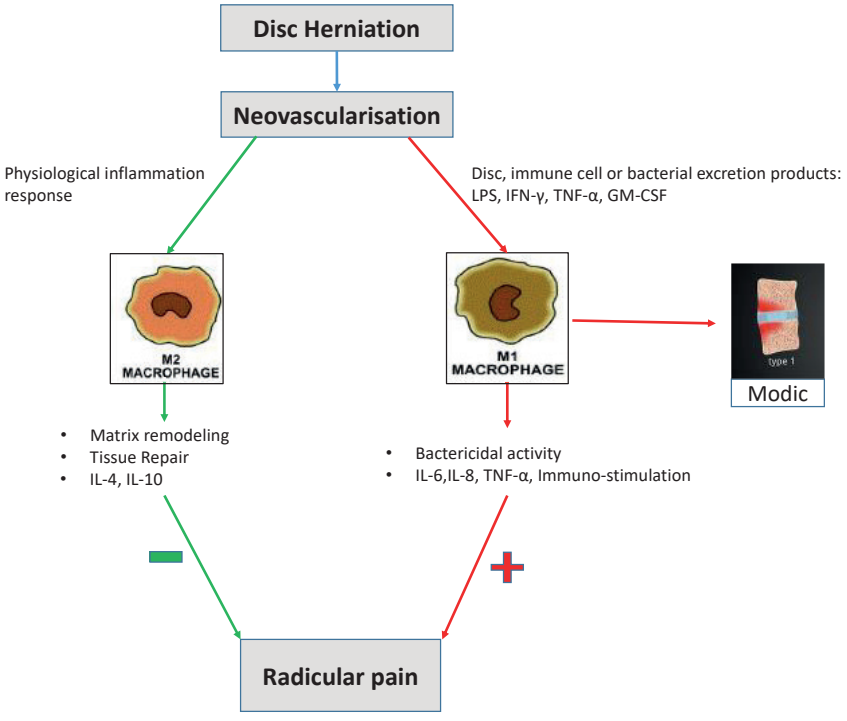
Appendix

Figure 1. Flow chart of the database search.



From the initial database search 1175 articles were obtained, of which 741 remained after removal of duplicates. 14 articles from the initial search met the inclusion criteria. After citation tracking, 443 articles were found. After the duplicates were removed and the inclusion criteria were applied, no articles were used from the citation tracking.

Figure 2. Proposed hypothesis



Disc inflammation with a M2 macrophage dominance is beneficial for the pain symptoms, whereas a dominance of M2 macrophages, likely induced by a bacterial infection and recognizable by Modic changes, will aggravate the pain symptoms.

Table 1. Overview of the Risk of Bias.
This table shows the overall risk of bias and the individual categories; Population bias (P), selection bias (S), outcome bias (o), attrition bias (A) and conflict of interest.

Study (year of publication)	Score on risk of bias scale	P	S	O	A	I
Ahn et al. 2002	6/10	3/3	1/1	1/3	1/2	0/1
Andrade et al. 2011	6/10	1/3	0/1	2/3	2/2	1/1
Andrade et al. 2013	6/10	1/3	0/1	2/3	2/2	1/1
Andrade et al. 2016	5/10	2/3	0/1	1/3	2/2	0/1
Brisby et al. 2002	4/10	1/3	0/1	2/3	1/2	0/1
Cuellar et al. 2000	5/10	2/3	1/1	1/3	1/2	0/1
Pedersen et al. 2015	8/10	3/3	1/1	2/3	2/2	0/1
Rothoerl et al. 1998 Acta Neurochirurgia	3/10	1/3	0/1	1/3	1/2	0/1
Rothoerl et al. 2002	5/10	1/3	0/1	2/3	2/2	0/1
Rothoerl et al. 1998 Spine	3/10	1/3	0/1	1/3	1/2	0/1
Schistad et al. 2014	8/10	3/3	1/1	1/3	2/2	1/1
Wang et al. 2016	6/10	3/3	0/1	1/3	1/2	1/1
Woertgen et al. 2000	5/10	1/3	1/1	1/3	2/2	0/1
Zu et al. 2016	8/10	3/3	1/1	2/3	2/2	1/1

Table 2 . An overview of the evidence on the associations between macrophage infiltration, M1-related or M2-related factors and the clinical outcomes.

Study	Cohort size (n)	Risk of bias	Specification	Clinical parameter	Association
CD68 macrophage marker					
Rothoerl et al. [28] (Acta Neurochirurgica)	179	3/10	Disc infiltration	Pre-op pain (VAS) Pre-op SLR	No No
Rothoerl et al. [27]	177	6/10	Disc infiltration	Pre-op pain (VAS) 7-month FU pain (VAS) Pre-op SLR	No Neg No
Rothoerl et al. [29] (Spine)	44	4/10	Disc infiltration	Pre-op pain (VAS) Pre-op SLR	No Neg
Woertgen et al. [22]	79	5/10	Disc infiltration	Pre-op SLR Pre-op pain (VAS) 6-month FU pain (VAS)	No No Neg
M1 expression profile					
TNF-α					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	Pos Pos
Andrade et al. [25]	15	8/10	Protein expression NP/AF mRNA expression NP/AF	Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS) Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS)	No/no Pos/no Pos/no No/no Pos/no Pos/no
Andrade et al. [24]	20	5/10	Protein expression mRNA expression	Pre-op pain (VAS) 6-month FU pain (VAS) VAS < 3.5 vs > 3.5	Pos Pos Pos
Brisby et al. [26]	39	3/10	CSF and serum concentration	Pre-op pain (VAS) Pre-op SLR	No No
Wang et al. [19]	138	6/10	Serum concentration	Pre-op pain (VAS) Pre-op ODI	Pos Pos
Zu et al. [20]	262	9/10	Serum concentration at baseline Serum concentration at 1-month FU Serum concentration at 12-month FU	12-month FU pain (VAS) 12-month FU pain (VAS) 12-month FU pain (VAS) 12-month FU ODI	Pos Pos Pos Pos

Study	Cohort size (n)	Risk of bias	Specification	Clinical parameter	Association
TNFR1					
Andrade et al. [25]	15	8/10	Protein expression NP/AF	Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS)	Pos/pos Pos/no Pos/no
Andrade et al. [23]	20	5/10	Protein expression mRNA expression	Pre-op pain (VAS) 6-month FU pain (VAS) Pre-op pain VAS	Pos Pos Pos
TNFR2					
Andrade et al. [25]	15	8/10	Protein expression NP/AF	Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS)	No/neg No/neg No/neg
Andrade et al. [23]	20	5/10	Protein expression mRNA expression	Pre-op pain (VAS) 6-month FU pain (VAS) Pre-op pain VAS	Neg Neg Neg
IL-6					
Andrade et al. [24]	15	8/10	mRNA expression NP/AF protein expression NP/AF	Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS) Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS)	No/no No/no No/no No/no No/no No/no
Brisby et al. [26]	39	3/10	CSF and serum concentration	Pre-op pain (VAS) Pre-op SLR	No No
Pedersen et al. [13]	127	8/10	Serum concentration	12-month FU pain (VAS)	Pos
Schistad et al. [18]	54	7/10	Serum concentration	ODI baseline—1 year FU 12-month FU back pain (VAS) 12-month FU leg pain (VAS)	Pos Pos Pos
Wang et al. [19]	138	6/10	Serum concentration	Pre-op pain (VAS) Pre-op ODI	Pos Pos
IL-8					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	Pos Pos
Brisby et al. [26]	39	3/10	CSF concentration	Pre-op pain (VAS) Pre-op SLR	No No

Study	Cohort size (n)	Risk of bias	Specification	Clinical parameter	Association
Pedersen et al. [13]	127	8/10	Serum concentration	12-month FU pain (VAS)	Pos
Wang et al. [19]	138	6/10	Serum concentration	Pre-op pain (VAS) Pre-op ODI	No No
IL-1β					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	No No
Andrade et al. [24]	15	8/10	mRNA expression NP/AF protein expression NP/AF	Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS) Pre-op pain (VAS) 6-week FU pain (VAS) 12-month FU pain (VAS)	No/no No/no No/no No/no No/no No/no
Brisby et al. [26]	39	3/10	CSF and serum concentration	Pre-op pain (VAS) Pre-op SLR	No No
IL-1α					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	No No
IFN-γ					
Brisby et al. [26]	39	3/10	CSF and serum concentration	Pre-op pain (VAS) Pre-op SLR	No No
Cuellar et al. [21]	24	5/10	Protein expression NP	Pre-op VAS	Pos
M2 expression profile					
IL-10					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	No No
Wang et al. [19]	138	6/10	Serum concentration	Pre-op pain (VAS) ODI	Neg Neg
IL-4					
Wang et al. [19]	138	6/10	Serum concentration	Pre-op pain (VAS) ODI	No No
Zu et al. [20]	262	9/10	Serum concentration at baseline Serum concentration at 1-month FU Serum concentration at 12-month FU	12-month FU pain (VAS) 12-month FU pain (VAS) 12-month FU pain (VAS) 12-month FU ODI	Neg Neg Neg No

Study	Cohort size (n)	Risk of bias	Specification	Clinical parameter	Association
TGF-β					
Ahn et al. [17]	23	5/10	mRNA expression NP	Pre-op pain (VAS) Pre-op SLR	No No

'No' infers that no association was established. If an association is indicated as 'positive', the inflammatory factor positively association to the clinical outcome parameter (as an example: 'Andrade 2011': if the TNFR1 expression was higher, patients experienced more postoperative pain). 'Negative' infers a negative association between the inflammatory and clinical outcome parameter in question. As an example: in Andrade 2011, high TNFR2 expression associated with less postoperative pain. The negative associations indicate a protective effect of an inflammatory reaction on pain or disability. Of these fourteen studies, three used a correlation test instead of an association test: Brisby (2002), Cuellar (2010), Wang (2016). NP = nucleus pulposus, AF = annulus fibrosis, FU = follow-up, Pre-op = pre-operative, Pos = positive association, Neg = negative association

Chapter 3

Lumbar disc extrusions reduce faster than bulging disc due to an active role of macrophages in sciatica

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"Have no fear of perfection, you'll never reach it"
(Salvador Dali)

Abstract

Objective

This retrospective observational histological study aims to associate the size and type of disc herniation with the degree of macrophage infiltration in disc material retrieved during disc surgery in patients with sciatica.

Methods

Disc tissue of 119 sciatica patients was embedded in paraffin and stained with haematoxylin and CD68. Tissue samples were categorized as mild (0-10/cm^2), moderate (10-100/cm^2), and considerable (>100/cm^2) macrophage infiltration. All 119 patients received an MRI at baseline, and 108 received a follow-up MRI at one-year. MRIs were reviewed for the size and type of the disc herniations, and for Modic Changes in the vertebral end plates.

Results

Baseline characteristics and duration of symptoms before surgery were comparable in all macrophage infiltration groups. The degree of macrophage infiltration was not associated with herniation size at baseline, but significantly associated with reduction of size of the herniated disc at one-year post surgery. Moreover, the degree of macrophage infiltration was higher in extrusion in comparison to bulging (protrusion) of the disc. Results were comparable in patients with and without Modic changes.

Conclusion

macrophage infiltration was positively associated with an extruded type of disc herniation as well as the extent of reduction of the herniated disc during one-year follow-up in patients with sciatica. This is an indication that the macrophages play an active role in reducing herniated discs. An extruded disc herniation has a larger surface for the macrophages to adhere to, which leads to more size reduction.

Introduction

Herniation of the intervertebral disc is a highly prevalent disease [11], characterized by radiating pain symptoms due to a compression of the nerve root by the herniated disc. Since 1934 it has been widely accepted that a disc may herniate due to a disruption of the annulus fibrosus or due to its attachments to the adjacent endplate [16]. This disruption of the annulus fibrosus can result in a foreign body reaction aimed at the nucleus pulposus that induces neovascularization [13], which is accompanied by macrophage infiltration [15, 23, 26].

This macrophage infiltration is believed to exacerbate pain symptoms through secretion of pro-inflammatory cytokines such as IL-6, IL8 and TNF-alfa [1, 20, 29]. In contrast, macrophage infiltration may also have a positive effect on symptoms through inducing a phagocytic resorption process, mediated by anti-inflammatory cytokines such as IL-4 and IL-10 [27, 29]. This discrepancy in effect of macrophage infiltration is reflected in the inconsistent findings regarding the correlation between macrophage infiltration and clinical symptoms [14, 23-25, 28]

A possible factor that may reflect the type of macrophage is the presence of Modic Changes (MC), also known as vertebral end-plate signal changes (VESC) on MRI [17, 18]. Recent studies have shown that patients with MC recover more slowly from their herniated discs than those without MC [2, 26]. In addition, Dudli et al. showed that both Modic type 1 and type 2 changes are associated with inflammatory dysmyelopoiesis and fibrogenic changes [4], which infers that MC reflect a factor that plays a role in the inflammatory process in the disc. Hence, it is to be expected that this factor in the inflammatory process, reflected by presence of MC, interferes with the resorption process, possibly by changing macrophage differentiation away from the anti-inflammatory type and towards the pro-inflammatory type. Until now, the interaction between MC and macrophage infiltration on the resorption of the herniated disc (size reduction) remains unknown.

Furthermore, if a herniated disc extrudes instead of bulges (protrudes), it is likely to have a higher exposure to the systemic circulation through more neovascularization, which is in turn may result in more macrophage infiltration. However, the evidence supporting this theory is limited [15]. In addition, recent studies have found that MC are associated with less neovascularization [12, 26]. It is thus hypothesized that the presumed association between macrophage infiltration and disc extrusion will be less strong in patients with MC.

In order to test the previously mentioned theories, this study aims to associate macrophage infiltration with the type and size of disc herniation as well as the reduction in disc herniation at one-year follow-up in patients with sciatica. We will also explore this association in patient with and without MC separately. Exploring these associations will provide a more profound understanding of the roles of macrophage infiltration and MC in patients with sciatica due to a lumbar disc herniation.

Materials and Methods

Study population

This retrospective study was performed using participants from the Sciatica Trial [22],, a multicentre RCT with 283 patients who suffered from sciatica for 6-12 weeks and had a disc herniation as assessed by means of MRI. 141 patients were randomized to surgery and 125 patients actually underwent surgery (16 recovered before surgery could be performed). The other 142 patients were randomized to prolonged conservative care, of which 55 patients underwent surgery within 1 year, with a mean time to surgery of 15 weeks after randomization. Thus, in the first year after randomization a total of 180 patients underwent surgery for sciatica. Out of the 180 patients, 120 disc samples were available for analysis. Missing samples were due to multiple reasons: not collected during surgery, got lost after surgery, not preserved properly, or got lost after preservation. All surgeries were performed between November 2002 and Feb 2005. The protocol, which included analysis of the disc material, was approved by the medical ethics committees at all participating hospitals.

Histological analysis

Disc material of all operated patients was collected and fixed in 4% formaldehyde solution after surgery and were subsequently stored for future analysis. For the purpose of this retrospective study, samples were embedded in blocks of paraffin and stained with CD68 to evaluate macrophage infiltration. A detailed description of the protocol was published in our previous work [3].

The evaluation was done by two independent investigators, who were blinded to clinical information and MRI data. The training of these independent researchers was carried out by a senior pathologist. The number of macrophages on each sample was counted and estimated. Using this method, the tissue samples were categorized based on the extent of macrophage infiltration. The categories consisted of mild (0-10 /cm^2), moderate (10-100 /cm^2), and considerable (>100 /cm^2) macrophage infiltration. Subsequently, a consensus score between the independent researchers was determined. An acceptable consensus score was predefined as 60%. It was pre-defined that all samples would be re-assessed with a senior pathologist if consensus was less than 60%.

MRI protocol and Image evaluation of the sciatica trial

MRI scans were performed at baseline by a 1.5 Tesla scanner, and both sagittal T1- and T2-weighted images of the lumbar spine were obtained. Image evaluation of MC was according to the criteria of Modic et al[17, 18]. Image evaluation was done according to a predefined protocol (Supplementary Table S1)[9]**.** Definitions of imaging characteristics were based on the recommendations from the combined task forces of the North American Spine Society, the American Society of Spine Radiology, and the American Society of Neuroradiology for classification of lumbar disc pathology. MRIs were evaluated by 2 neuroradiologists and 1 neurosurgeon. All three were blinded to histological data and clinical information. The readers were not involved in the selection or treatment of the patients included. Inter-observer agreement analyses was published earlier by el Barzouhi et al.[6, 7]. For the statistical analyses, the majority opinion of the three independent researchers (answer by at least two of the three MRI assessors) was used.

Type of disc herniation as quantified by MRI was categorized as follows: bulging (or protrusion) ,extrusion, or not applicable, Based on characteristics described by Fardon and Milette [8]. The definition of protrusion as defined by the protocol is more commonly known as 'bulging' in daily clinical practice. Therefore, this term in used in this article. The size of the herniated disc was measured as the surface in mm^2. The decrease in size of the disc herniation between baseline and 1 year after randomization was measured as a percentage reduction in surface. MC were scored as Type 1, Type 2, or Type 3 according to Modic et al. [17, 18]. The inter-observer agreement was substantial for the MC (69-97%), as described by el Barzouhi (2014) [7], no kappa calculated because the prevalence of MC type 1 and 3 were too low. At last, inter-observer agreement was substantial regarding disc type (kappa = 0.62) [6].

Statistical analysis

The categorized histological findings were associated with the MRI variables (type and size of the herniated disc, and disc herniation reduction at one year follow-up). In addition, histological findings were split into a group with, and a group without MC at the herniated disc level and comparisons with all MRI variables were repeated for these subgroups. The association between the histological findings and disc type and was tested using X^2 tests for categorical data. The association between the histological findings and disc size was done using a one-way ANOVA, At last, the association between the histological findings and disc size reduction was assessed using a Kruskal Wallis test. P-values of < 0.05 were regarded as significant.

Results

Demographics

Of the 180 patients that underwent surgery, 119 patients disc samples were preserved and analyzed. Missing samples were due to multiple reasons: samples were either not collected during surgery, got lost after surgery, were not preserved properly, or got lost after preservation. 103 of the 119 patients received an MRI at one-year follow-up. The baseline characteristics age, gender, BMI and duration of sciatica prior to surgery of the three macrophage infiltration groups were comparable (Table 1).

The histological data analysis

CD68 staining to identify macrophages resulted in the following distribution: 47 (39.5%) patients had mild-, 45(37.8%) patients moderate, and 27 (22.7%) considerable macrophage infiltration (Table 2).The consensus score was excellent (0.96). (Table 2). Examples of the CD68 samples and their categories are shown in Figure 1.

The MRI data analysis

Forty-one patients demonstrated bulging (protrusion) of the disc, 74 an extrusion, and in 2 patients discs were scored as not evidently protruding; in 2 cases information was missing. The disc size was measured in 116 patients with a mean of 77.9 mm^2 ± 3.6 SE (2 samples were missing). The reduction in disc size was measured over 108 patients, which had a mean of 66.6% ± 4.1 SE, of which 46 disc herniations completely disappeared. Furthermore, a total of 2 patients with MC type 1, 32 patients with MC type 2, no patients with MC type 3, and 85 patients had no MC at the vertebrae adjacent to the herniated disc. Because only two patients showed MC type 1, these samples were excluded from the additional statistical analysis in which only patients with MC were assessed. Thus, in this additional analyses, only MC type 2 were taken into account.

Associations between number of macrophages and type and size of disc herniation

The type of disc herniation at baseline showed a significant association with the degree of macrophage infiltration, suggesting that patients with more macrophage infiltration are more likely to have an extruded disc as compared to a bulging (protruded) disc (p = 0.05). The presence of MC type 2 was not of significant influence on this association (no MC: p = 0.07, MC type 2: p = 0.68) (Figure 2). In addition, MC2 was not associated with either macrophage infiltration (p=0,53) or type of disc herniation (p=0,32) (Supplementary Figure S1). This verifies that the association between extrusion and macrophage infiltration was not confounded by MC2.

Disc herniation size at baseline was not associated with the degree of macrophages infiltration (p = 0.43). Again, the presence or absence of MC type 2 changes did not significantly influence this finding (no MC: p = 0.91, MC type 2: p =0.11 (Figure 3).

Associations between number of macrophages and reduction at one-year follow-up

The timing of surgery was not equal for all 119 patients. In the sciatica RCT about 40% of patients that were randomized to conservative treatment, crossed over to surgical treatment because of unbearable symptoms. These patients had a longer timespan between start of the complaints and surgical intervention, varying from approximately the same waiting time as the surgical group (mean = 15 days after randomization) to 18 months after randomization. In our sample group, 34 of 119 patients were originally randomized to conservative care and crossed over. Because the follow-up MRI was made at one-year after randomization and not after surgery, not all of the crossed over patients were comparable with the patients randomized for surgery. Therefore, crossed over patients with waiting time for surgery longer than 6 months were excluded from this analyses, which resulted in exclusion of 7 of the 34 patients.

Reduction of disc herniation, one year after surgery, was positively associated with the degree of macrophage infiltration at baseline (p=0.01) (Figure 4). When testing for the presence and absence of MC Type 2 separately, no significant associations were found (no MC: p = 0.06, MC type 2: p = 0.23, Figure 4).

In addition, we found that disc extrusion were larger than bulging discs and we found that extruded discs were significantly associated with a larger reduction in size at one year ($p = 0.001$). Since macrophage infiltration, disc extrusion and relative reduction in size at one-year follow-up showed multicollinearity, the association between macrophage infiltration and relative reduction at one year could be confounded by extrusion. For example, because extruded discs could be easier to resect during surgery. In order to falsify this possibility, an additional analysis was performed with the data from the conservative group of the Sciatica trial [5]. If this was due to surgery, extrusion would not associate with reduction in the conservative group: The additional analysis showed that extrusion in the conservative group also significantly associated with size reduction at follow-up in the quantitative analyses (T-test: p-value = 0.03). Both additional analyses are shown in Supplementary Figure S2.

Discussion

The most important finding of this study is the association between the number of macrophages present at baseline and the decrease in size of the disc herniation at one-year follow-up in patients with sciatica due to a lumbar disc herniation. This is accompanied by an association between a higher number of macrophages, in extruded as compared to bulging disc herniations. Since extrusion of disc material was demonstrated to resorb faster, patients with a disc extrusion rather than a bulging disc are more likely to benefit from prolonged conservative treatment.

The association between macrophage infiltration and disc extrusion was in line with previous findings of Lohr et al. [14]. This could be explained by the likelihood that disc extrusion as compared to protrusion leads to a larger exposure to systemic circulation, which makes it easier for macrophages to infiltrate the disc during the foreign-body reaction [13]. When performing a separate analysis for patients with and without MC, an MRI feature that has been shown to interact with disc inflammation in recent literature [18], no significant differences were found. However, only in patients without MC an almost significant association between macrophage infiltration and disc extrusion ($p=0.07$) was demonstrated, while in patients with MC no such trend was seen ($p = 0.68$). This lack of significance could be explained by the small sample sizes. Hence, this finding could indicate that MC type 2 interfere with the normal course of macrophage infiltration, possibly through a disruption in neovascularization [12, 26]. Nevertheless, due to the small sample sizes, these findings should be interpreted with caution.

Furthermore, even though the size of the disc herniation was not associated with the degree of macrophage infiltration at baseline, we did find that the number of infiltrated macrophages was associated with a larger regression of herniated disc material at one-year follow-up. This indicates the importance of a resorption process induced by macrophages in cleaning up the herniated disc material [13, 21]. Since this study was the first that analyzed this association, no comparison with previous literature could be made.

The additional analyses performed with the data from the conservative group of the Sciatica trial showed that the association between macrophage infiltration and size reduction of the herniated disc was not confounded by surgery. Hence we conclude that the reduction was likely to be induced by the infiltrated macrophages.

A different possible confounder on the rate reduction of disc herniation, could be the duration of symptoms before surgery. For example, a longer chronic inflammation process could possibly lead to a different result as compared to a short lived inflammation process [19]. However, since the treatment duration was equal for all macrophage groups, this possibility was excluded.

This study has several strong points. Both the histological and MRI analyses were performed by multiple evaluators and demonstrated substantial inter agreement or consensus scores. Moreover, this study was the first to compare macrophage infiltration to disc parameters on MRI between patients with and without MC type 2. This study also has some limitations. The samples that were evaluated for the histological analysis were old, which might have reduced their quality. Nevertheless, the CD68 staining showed clearly identifiable macrophages and the quality was validated by a senior pathologist. Hence, we assume that the age of the samples did not alter our result. Also, macrophage infiltration does not cover the entire inflammatory picture including the different effects of other inflammatory- cells, cytokines and proteins that also infiltrate the disc as a consequence of neovascularization. However, previous findings have identified macrophages as the main type of inflammatory cells found in discus samples [10, 14]. Thus, it is likely that they contribute the most to the overall inflammation response. Lastly, because all patients already received surgery, it remains unclear whether the reduction of the herniated disc at one year is also associated with macrophage infiltration in a population without surgical intervention. Due to ethical considerations, this could not be studied.

In conclusion, macrophage infiltration was positively associated with an extruded type of disc herniation as well as the extent of reduction of the herniated disc during one-year follow-up in patients with sciatica. In order to evaluate our findings, our results should be repeated by a study with a larger sample size.

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Conflict of interest
None of the authors has any conflict of interest. No funding was received for the conductance of this study.

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Table 1. Baseline characteristics of the three histologically defined inflammation groups

	Mild (N=48)	moderate (N=45)	considerable (N=27)
Age	40.4±9.6	42.1±10.3	43.7±6.6
Male gender	36 (75.0%)	28 (62.2%)	22 (81%)
Body-mass index	26.0±4.1	25.8±3.5	25.9±3.2
Duration of sciatica in weeks	9.6±1.9	9.7±2.3	8.7±2.1

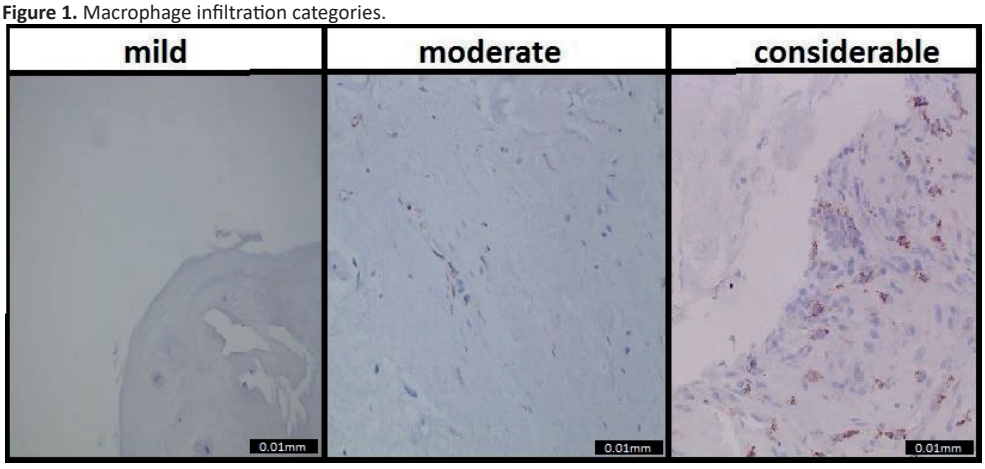
Values for gender are n and (%) or means ± SD.
No significant baseline differences were observed between the three inflammation groups.

Table 2. Consensus score of the pathological findings

	A vs. B % agreement
Inflammation at baseline (3 categories)	95.8
Mild	96.8
Moderate	94.6
considerable	96.2

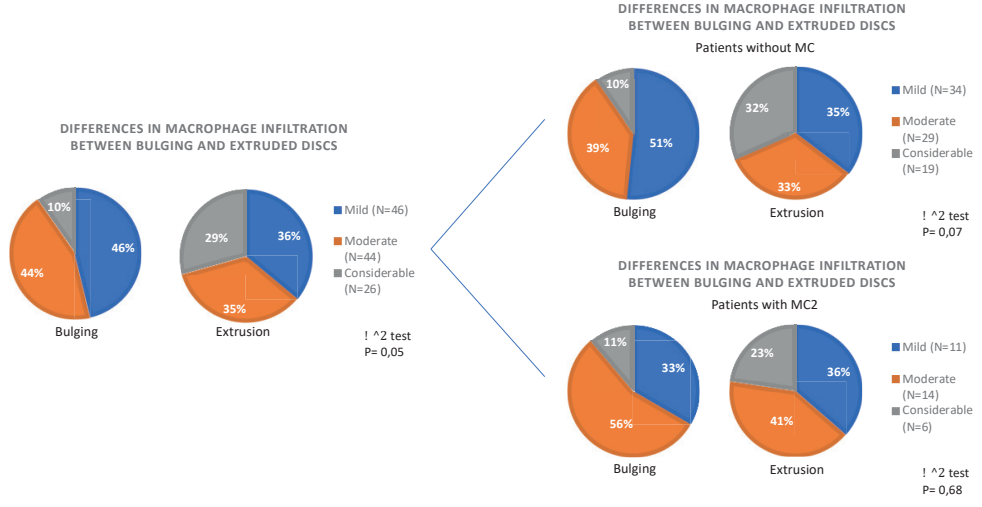
A and B both represent independent observers. The agreement was assessed after the consensus reading.

Figure 1. Macrophage infiltration categories.



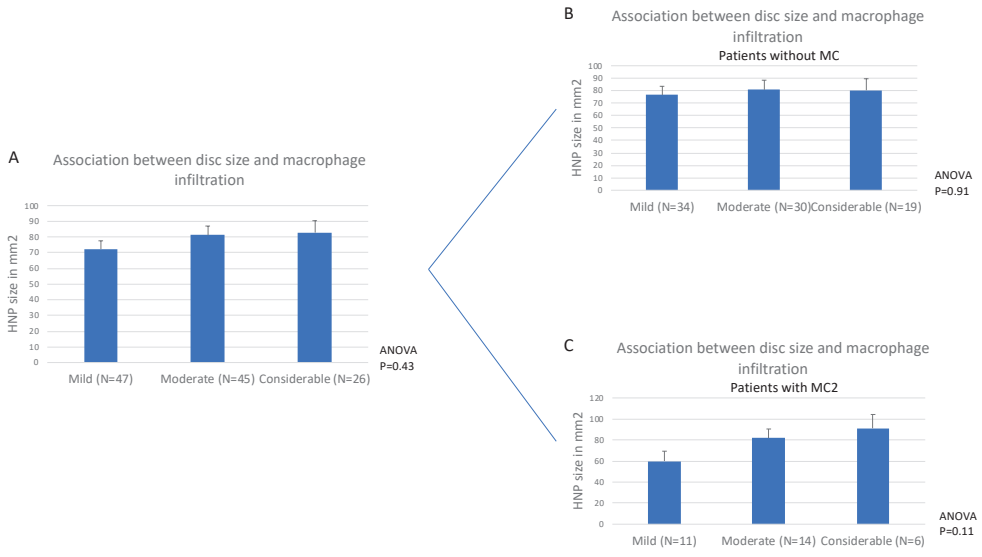
Examples of mild (0-10 macrophages per cm^2), moderate (10-100 macrophages per cm^2), and considerable (>100 macrophages per cm^2) inflammation

Figure 2. Association between the type of disc herniation and macrophage infiltration at baseline.



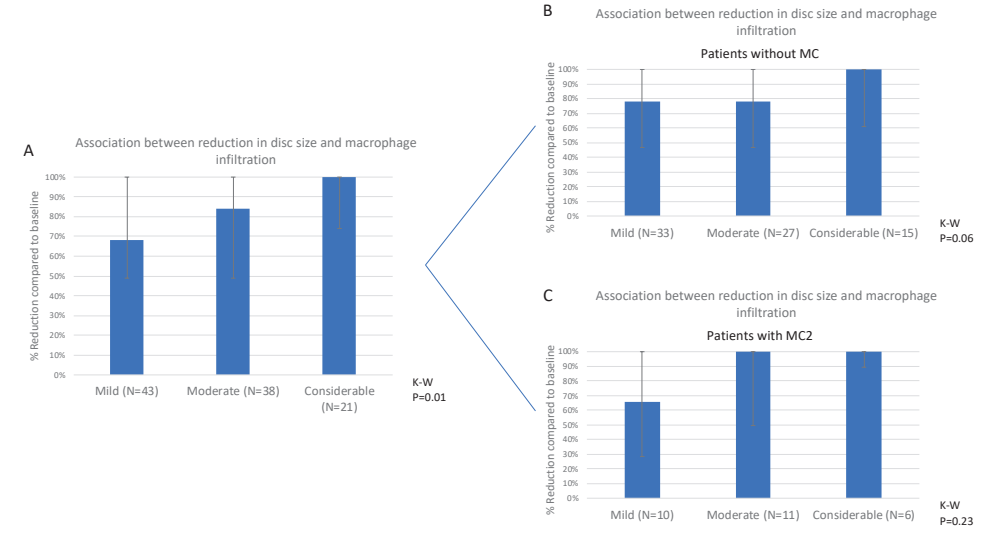
Pie charts display the distribution of the macrophage infiltration groups in percentages, a χ 2 test was performed to assess the significance in distribution between bulging and extruded discs, and p values are given. Comparison a for the whole population, 2B for patients without MC, and 2C for patients with MC

Figure 3. Association between the size of disc herniation and macrophage infiltration at baseline.



Macrophage infiltration groups are shown on the X axis, values on the Y axis are mean HNP surface size in squared. 3B for patients without MC, and 3C for patients with MC

Figure 4. Association between the degree of macrophage infiltration and the percentage surface reduction of the HNP between baseline and 1-year follow-up on MRI.



Macrophage infiltration groups are shown on the X axis, values on the Y axis are median percentages of axial surface millimeter, error bars are SEs and p values for the one-way ANOVA test are provided. Comparison a for the whole population, 4B for patients without MC, and 4C for patients with MC

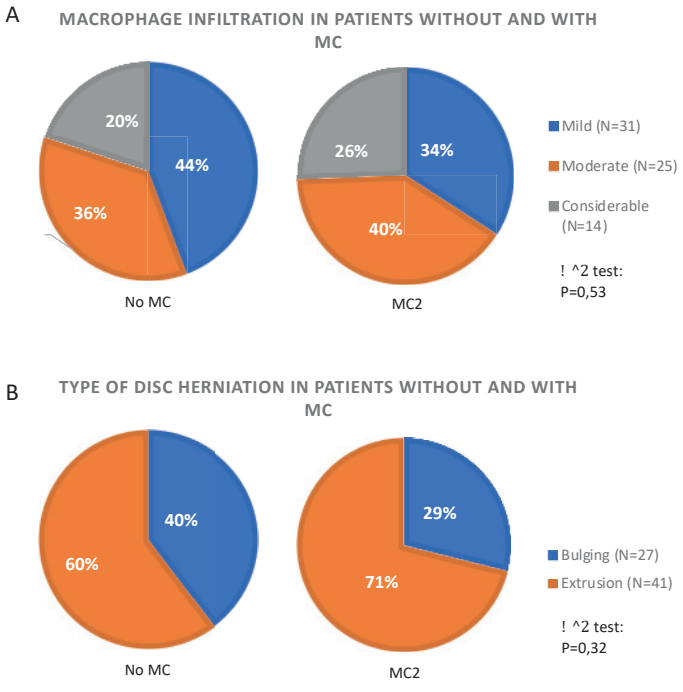
Supplementary Appendix

Supplementary Table S1. MRI study variables

Disc level	Variable	Category
Disc level with the most severe nerve root compression	Disc level	1. Not applicable: no nerve root compression 2. L2L3 3. L3L4 4. L4L5 5. L5S1
	Disc contour at this level	1. Bulging: presence of disc tissue circumferentially (50-100%) beyond the edges of the ring apophyses 2. Herniation: localized displacement of disc material beyond the normal margins of the intervertebral disc space
	Certainty about the presence of disc herniation	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but probability > 50% 3. Possible about the presence: reason to consider but probability < 50% 4. Definite about the absence: no doubt about the absence
	Loss of disc height at this level	1. Yes 2. No
	Signal intensity of nucleus pulposus on T2 images at this level	1. Hypointensity 2. Normal 3. Hyperintensity
	Certainty about the presence of nerve root compression	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but probability > 50% 3. Possible about the presence: reason to consider but probability < 50% 4. Definite about the absence: no doubt about the absence
	Spinal canal stenosis	1. Yes 2. No
	Disappearance of epidural fat	1. Completely disappeared 2. Partly disappeared 3. No disappearance
	Presence of vertebral end plate changes and its extent	1. No VESC (Vertebral Endplate Signal Changes) 2. VESC type 1: hypointense on T1-weighted sequences and hyperintense on T2-weighted sequences 3. VESC type 2: increased signal on T1 weighted sequences and isointense or slightly hyperintense signal on T2 weighted sequences 4. VESC type 3: hypointense both on T1- and T2-weighted sequences 5. VESC type 1 and 2
		1. Yes 2. No

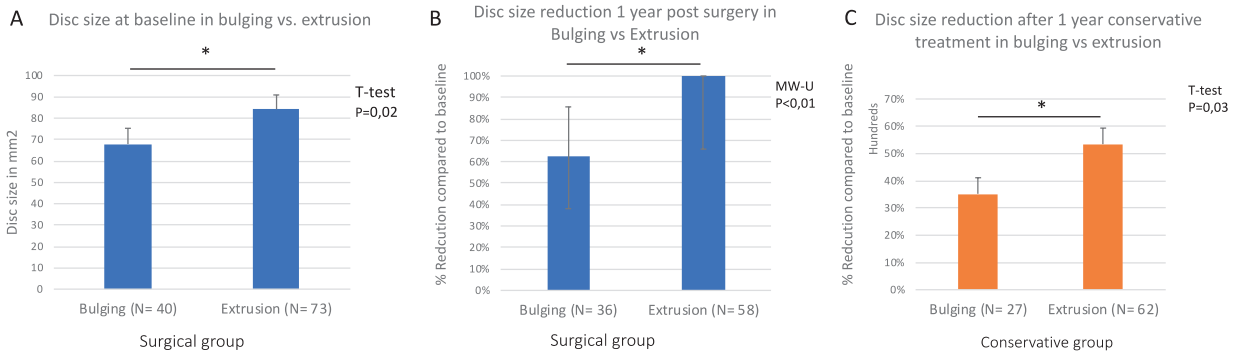
Disc level	Variable	Category
If a disc herniation is considered	Presence of impaired discs at more than one level	1. Central zone: zone within the vertebral canal between sagittal planes through the medial edges of each facet 2. Sub-articular zone: zone, within the vertebral canal, sagittally between the plane of the medial edges of the pedicles and the plane of the medial edges of the facets, and coronally between the planes of the posterior surfaces of the vertebral bodies and the under anterior surfaces of the superior facets. 3. Foraminal zone: zone between planes passing through the medial and lateral edges of the pedicles 4. Extra-foraminal zone: the zone beyond the sagittal plane of the lateral edges of the pedicles, having no well-defined lateral border
	Location	1. Right 2. Left 3. Right and left
	Size disc herniation in relation to spinal canal	1. Large stenosing, size >75% of the spinal canal 2. Large, size 50-75% of the spinal canal 3. Average, size 25-50% of the spinal canal 4. Small, size <25% of the spinal canal
	Form disc herniation	1. Protrusion: localized displacement of disc material beyond the intervertebral disc space, with the base against the disc of origin broader than any other dimension of the protrusion. 2. Extrusion: localized displacement of disc material beyond the intervertebral disc space, with the base against the disc of origin narrower than any one distance between the edges of the disc material beyond the disc space measured in the same plane, or when no continuity exists between the disc material beyond the disc space and that within the disc space.

Figure S1. Associations between MC and macrophage infiltration, and between MC and the type of disc herniation at baseline.



S1A: Pie charts display the distribution of the macrophage infiltration groups in percentages. **S1B:** Pie charts display the distribution of the bulging and extruded discs in percentages, X^2 tests were performed to assess the significance in distribution between patients without and with MC, p values are given.

Figure S2. Association between disc type and percentage reduction in axial surface at one year.



S2A compares disc size in the surgical group: disc type is shown on the X axis and values on the Y axis are mean size at baseline, error bars are SE. **S2B** compares axial surface reduction in the surgical group: disc type is shown on the X axis and values on the Y axis are median percentages of axial surface reduction compared to baseline, error bars are interquartile ranges. **S2C** compares axial surface reduction in the conservative group: disc type is shown on the X axis and values on the Y axis are percentages of axial surface reduction compared to baseline error bars are SE's. p values for T-tests or Mann Whitney-U tests are provided accordingly


4

Chapter 4

Disc inflammation and Modic changes show an interaction effect on recovery after surgery for lumbar disc herniation

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"I have no special talent, I am only passionately curious" (Albert Einstein)

Abstract

Purpose

To study the interaction between Modic Changes (MC) and inflammation by macrophages in the disc, in relation to clinical symptoms before and after discectomy for lumbar disc herniation.

Methods

Disc tissue was embedded in paraffin and stained with hematoxylin and CD68. Subsequently tissue samples were categorized for degree of inflammation. Type of MC was scored on MRI at baseline. Roland Disability Questionnaire (RDQ)score, and visual analogue scale (VAS) for back pain and leg pain separately were considered at baseline, and one-year follow-up post-surgery. Main and interaction effects of MC and inflammation were tested against clinical outcome questionnaires. In addition, this analysis was repeated in bulging and extruded discs separately.

Results

Disc material and MRI's of 119 patients were retrieved and analyzed. 48 patients demonstrated mild-, 45 moderate- and 26 considerable inflammation. 49/119 patients demonstrated MC. Grade of disc inflammation did not associate with presence of MC. At baseline, no main or interaction effects of MC and inflammation were found on the clinical scores. However, during follow-up after discectomy, significant interaction effects were found for RDQ score: Only in patients with MC at baseline, patients remained significantly more disabled (3.2 points $p = 0.006$) if they showed considerable disc inflammation compared to patients with mild inflammation. The additional analysis showed similar results in extruded discs, but no significant effects in bulging discs.

Conclusion

An interaction effect of MC and disc inflammation by macrophages is present. Only in patients with MC, those with considerable inflammation recover less satisfactory during follow-up after surgery.

Introduction

Patients with lumbar disc herniation often suffer from radicular pain symptoms, the origin of which is not fully understood. Even though a part can be explained by mechanical compression of the nerve root, inflammation also seems to play a major role. Disc inflammation may occur if the nucleus pulposus herniates into the epidural space. This may induce a foreign-body reaction accompanied by neovascularization and macrophage infiltration [1].

Macrophages may subsequently induce a resorption process by excreting matrix metalloproteases [2], inducing apoptosis and degrading collagen fibers [3]. This so called 'functional inflammation response' can explain spontaneous regression in herniation size [4]. On the other hand, they can also excrete pro-inflammatory cytokines such as IL-6, IL8 and TNF-alpha, which have been associated with exacerbation of the pain symptoms, the so called 'painful inflammation response' [5-7]. This discrepancy in inflammation response is reflected in the inconsistent findings regarding the correlation between presence of macrophages in herniated disc material and clinical symptoms [8, 9]. As of today, it remains unknown what factors are of influence on the type of inflammation response that patients experience. Nevertheless, the degree of inflammation can be influenced by specific characteristics of the disc. For example, extruded discs, which are more exposed to the systemic circulation, tend to have a higher degree of inflammation as compared to bulging discs[4]. Therefore, the type of disc herniation should not be neglected when the characteristics of disc inflammation are studied.

Modic Changes (MC), also known as vertebral end-plate signal changes (VESC) on MRI, are often seen in patients with lumbar disc herniation [10], and have been proposed to associate with slowing the recovery rate in patients that suffer from a herniated disc [11]. In addition, Dudli (2017) introduced the suggestion that MC represent the effect of cross-talk between bone marrow and the intervertebral disc [12]. Hence changes in the vertebral endplate, like inflammatory dysmyelopoiesis and upregulation in neurotrophic factors, may induce the presence of different types of macrophages, which may interact with the inflammation response in the disc. By doing so, possibly, MC represent a shift in the abovementioned inflammation reaction from 'functional' towards 'painful', thereby lowering the rate of recovery [11]. Until now, no studies have sought to enlighten this interaction. Therefore, the aim of the present study is to explore the interactions between disc inflammation and MC on the rate of recovery after surgery.

Materials and Methods

Hypothesis

A significant interaction effect is expected between disc inflammation and MC on the clinical symptoms: Patients with MC will suffer from a high degree of inflammation while patients without MC will benefit from inflammation. In addition, we expect this effect to be clearer in extruded discs as compared to bulging discs.

Study population

This retrospective study was performed using participants from the Sciatica Trial [13], a multicenter RCT with 283 patients who suffered from sciatica for 6-12 weeks and had a disc herniation as assessed by means of MRI. 141 patients were randomized to surgery and 125 patients actually underwent surgery (16 recovered before surgery could be performed). The other 142 patients were randomized to prolonged conservative care, of which 55 patients underwent surgery within 1 year, with a mean time to surgery of 15 weeks after randomization. Thus, in the first year after randomization a total of 180 patients underwent surgery for sciatica. Out of the 180 patients, 120 disc samples were available for analysis. Missing samples were due to multiple reasons: not collected during surgery, got lost after surgery, not preserved properly, or got lost after preservation. All surgeries were performed between November 2002 and Feb 2005. The protocol, which included analysis of the disc material, was approved by the medical ethics committees at all participating hospitals.

Histological analysis

Disc material of all operated patients was collected and fixed in 4% formaldehyde solution after surgery and were subsequently stored for future analysis. For the purpose of this retrospective study, samples were embedded in blocks of paraffin and stained with CD68 to evaluate for inflammation by macrophages. A detailed description of the protocol was published in our previous work [14].

The evaluation was done by two independent investigators, who were trained by a senior pathologist and were blinded to clinical information. The number of macrophages in each sample was counted semi quantitatively and categorized according to their inflammation grade. The categories consisted of mild (0-10 macrophages per cm^2), moderate (10-100 macrophages per cm^2), and considerable (>100 macrophages per cm^2) inflammation. Subsequently, the inter-observer agreement between the independent researchers was determined. The inter-observer agreement value was predefined to be over 60%. Therefore, it was scheduled to reevaluate the tissue sample if the agreement was less than 60%, involving evaluation of the senior pathologist. After the consensus reading, a consensus score was calculated.

MRI analysis for MC

MRI scans were performed at baseline by a 1.5 Tesla scanner, and both sagittal T1- and T2-weighted images of the lumbar spine were evaluated. according to the criteria of Modic et al. [15, 16]; Modic changes were scored as Type 1, Type 2, or Type 3. Type of disc herniation as quantified by MRI was categorized as follows: bulging, extrusion, sequestration or not applicable. The definition of protrusion as defined by the protocol is more commonly known as 'bulging' in daily clinical practice. Therefore, this term is used in this article. MRIs were evaluated by 2 neuroradiologists and 1 neurosurgeon. All three were blinded to histological data and clinical information. The readers were not involved in the selection or treatment of the patients included. Inter-observer agreement analysis was published earlier by el Barzouhi et al [17]. Because in earlier findings no MC were observed at level L1-L2, only images from L2-L3 through L5-S1 were evaluated. For the statistical analyses, the majority opinion of the three independent researchers (answer by at least two of the three MRI assessors) was used.

Clinical Outcome

The clinical outcome parameters from the sciatica trial that we used to associate the histological data with, were the Roland Disability Questionnaire (RDQ): the scores ranging from 0 to 23, with higher scores indicating worse functional status [18], the 100-mm visual-analogue scale (VAS) for leg and back pain: with 0 representing no pain and 100 the worst pain ever experienced [19]. These outcome measures were considered at baseline, and at 2, 4, 8, 12, 26, 38, 52 weeks post-surgery. For the RDQ score a difference of 3 points was regarded as the minimal clinically important change [20]. For the VAS score, this threshold was set at 19 points [21].

Statistical analysis

First, presence of MC was tested against the dichotomized histological findings and against the type of disc herniation using X^2 tests for categorical data. The association between disc type and inflammation was previously reported [4]. For the clinical analysis at both baseline and follow up, patients were first grouped based on the type of herniation, Subsequently, the main effects of inflammation and MC, and their interaction effect were tested against the clinical baseline scores (RDQ, VAS-Leg pain and VAS-back pain). For this analysis, a two-way ANOVA was used.

In order to analyze the predictive value of inflammation and MC at baseline, these were tested for associations with clinical outcomes (VAS-back, VAS-leg and RDQ) over the course of one-year follow-up using a mixed model analysis. The clinical outcomes were measured at 2, 4, 8 12, 26, 36 and 52 weeks post-surgery. For both baseline and follow-up clinical outcome analyses, alpha was initially set at 0.05 and corrected for multiple testing according to Bonferroni.

Results

The histological data analysis

CD68 staining to identify macrophages resulted in the following distribution: 48 (40%) patients were scored as mild inflammation, 45 (37.5%) patients as moderate inflammation, and 27 (22.5%) patients as considerable inflammation). The consensus score was excellent (0.96). Examples of the CD68 samples and their categories are shown in Figure 1.

MRI analysis for MC and disc type

Of the 120 patients included, one patient's MRI was lost, two MRI's showed MC type 1 changes (MC1) and 47 showed MC type 2 (MC2), of which 42 at the level of the disc herniation, and 70 showed no MC (inter-observer agreement 69-97%). Because only two patients demonstrated MC1, this subgroup was not suitable for statistical analysis. Hence, they were excluded from the analyses. Regarding disc type, 40 discs were characterized as bulging disc and 73 as extruded disc, no sequestrations were seen (kappa = 0.62).

Clinical outcome

Baseline characteristics age, gender, BMI and duration of sciatica symptoms prior to surgery in the three inflammation groups were comparable (Table 1). After surgery, VAS leg and back pain, and RDQ scores decreased significantly in all patients.

Association between inflammation grade, and clinical outcome at baseline

At baseline, no significant association was found between the grade of inflammation and presence of MC2 ($p = 0.525$), nor between MC2 and disc type ($p = 0.239$) (Table 2). As described earlier, a positive association was found between bulging discs and the degree of inflammation [4].

No significant main or interaction effects were found between MC2 and inflammation by macrophages with either RDQ, nor with VAS leg pain nor with VAS back pain scores at baseline (Table 3). These results did not change when bulging and extruded discs were considered separately (Table S1).

Association between inflammation grade and clinical outcome at follow-up
Clinical outcome during follow up was measured at the time points 2, 4, 8, 12, 26, 38 and 52 weeks after surgery. However, the timing of surgery was not equal for all 117 patients. In the sciatica RCT, about 40% of patients randomized to conservative treatment, crossed over to surgical treatment because of unbearable symptoms. These patients were clinically evaluated at the same time points after randomization. However, surgical intervention was performed with a mean of delay 15 weeks, ranging from 15 days to 18 months after randomization. Mean waiting time prior to randomization was 9.4 weeks. In our sample group, 34 of 120 patients were originally randomized to conservative care and crossed over. The measurements at time points 2, 4, 8, 12, 26, 38 and 52 after randomization were likely influenced by surgical intervention effects. Therefore, patients with a deviation of more than 3 SD's 'delay to surgery time' were considered outliers and excluded from the analysis. 21 of the 34 patients were therefore excluded, the 13 patients that remained had a mean waiting time for surgery of 25 days with a range of 2-51. The clinical data of the time points before the surgical intervention were ignored.

Over the course of one-year follow-up after surgery, no significant main effects were found of inflammation or MC2 on the RDQ score. However, a significant interaction effect was present ($p<0.001$), indicating that the association between inflammation and the RDQ score depended on the presence or absence of MC2. Post hoc tests revealed in the patients-subgroup without MC, no significant associations between inflammation and RDQ score were found. On the contrary, in patients with MC, those with a considerable inflammation had significantly and clinically relevant higher RDQ scores (mean = 6.8) compared to those with moderate- (mean = 3.8, $p = 0.001$) or mild inflammation (mean = 3.6, $p < 0.001$) (Table 4, 5, Figure 2A&B).

Furthermore, a significant association was found between inflammation and the VAS leg pain score during follow-up ($p=0.001$) (Table 4, 5). However, when assessing the interaction effect between MC2 and inflammation, this association between inflammation on the VAS leg score was only seen the patient-subgroup without MC, in which the patients with mild inflammation experienced significantly higher VAS leg score (mean = 16.7) compared to both the moderate (mean = 8.2, $p < 0.001$) and considerable inflammation (mean = 6.4, $p < 0.001$). In contrast, no significant associations between inflammation and VAS leg scores were found in the subgroup of patients with MC2 (Table 5, Figure 3A&B). Even though these associations were significant, they did not exceed the clinically relevant threshold of 19 points [21]. Moreover, no significant main effect of MC2 was found on VAS leg score.

At last, no significant associations were found between inflammation and VAS back scores or between MC2 and VAS back scores. Also, no interaction effects were found, indicating that the no associations between inflammation and VAS back score were seen in both patients with and without MC2 (Table 4).

When bulging discs and extruded discs were considered separately, extruded discs illustrated similar significant interaction effects between MC2 and inflammation on the RDQ ($p < 0,001$) and VAS leg score ($p = 0.006$) (Table S1), Post Hoc tests also revealed similar mean differences (Table S2). In contrast, no significant effects or interactions were seen in bulging discs.

Discussion

The present study demonstrates that the association between inflammation, as identified through macrophage infiltration, and clinical outcome after lumbar discectomy depends on the presence or absence of MC2. Patients with MC2 and considerable inflammation recovered less satisfactory after surgery in terms of disability compared to MC2 patients with mild inflammation. These results were not seen in patients without MC. In addition, in patients without MC, leg pain decreased slightly more in those with considerable inflammation as compared to those with mild inflammation. This association was not seen in patients with MC2. Interestingly, when these bulging and extruded discs were considered separately, the abovementioned results were only seen in extruded discs; in bulging discs, no significant effects were found. These results were in line with our hypothesis.

Here, inflammation was characterized by presence of macrophages identified in disc tissue. As mentioned in the introduction, the type of macrophage responses can be characterized as either a 'painful inflammation response' or a 'functional inflammation response'. This contradictory effect is likely due to the difference in macrophage differentiation; M1 macrophages will act pro-inflammatory while M2 macrophages are involved in resorption [5]. Based on our results, we expect a high percentage of M2 macrophages in patients without MC. In contrast, a high percentage of M1 macrophages can be expected in patients with MC2. The alternative differentiation of macrophages could possibly be explained due to the exposure to outside factors. In patients without MC, this will be limited to macrophage infiltration from the epidural space, but in patients with MC, the disc will likely also be exposed to inflammatory factors excreted from the endplate [12]. Markers such as CSF1, CCL2, IL-1β and IL6 could subsequently lead to the macrophage differentiation towards M1 [5, 12]. In addition, since more macrophages are present in extruded discs, any interaction with the endplate excretion products will be more noticeable, which is illustrated by the fact that the studied effects were most prominent in discs with considerable inflammation. This may also explain the absence of significant effects in bulging discs, where considerable inflammation was very scarce and could thus not be analyzed. Taken together this insinuates that a threshold value of inflammation has to be reached before the interaction with MC2 becomes clinically relevant, which is unlikely occur in bulging discs, since they are less exposed to the epidural space. Nevertheless, it should be noted that the number of bulging discs in this study was low, hence the absence of effects in bulging discs could also be due to a limited number of samples in this subgroup.

An additional factor that may lead to macrophage differentiation toward M1 might be a bacterial infection of Propionibacterium Acnes, which has been associated with MC in disc herniation patients [22]. Inferences have been made that this underlying infection may cause the difference in inflammation profile [23], and thus for macrophage differentiation towards M1. Moreover, when P. Acnes is phagocytized by a macrophage, the bacteria can disrupt its lysosomal activity and remain latent for an extended period of time [24]. P. Acnes has also been known for creating a biofilm, which does not only increase the difficulty of detecting the bacteria, but also protects them from both host immune defenses and antibiotics treatments [25]. This increased tolerance to antibiotics could also explains how P. acnes can survive perioperative antibiotic prophylaxis.

Taken together, an underlying infection of P. acnes could explain both the chronic inflammation process seen on MRI as MC, and the pathological effect that macrophage infiltration can have on recovery after surgery [26]. Unfortunately, studying the different types of macrophages or bacterial presence was beyond the scope of this study.

This study has some limitations: Samples were preserved in formaldehyde for many years, which may have reduced the quality. Nevertheless, the CD68 staining showed clearly identifiable macrophages, indicating that it was not a significant issue. Furthermore, only the number of macrophages and not T or B cells were used to study inflammation. This can be justified by previous findings, which identified macrophages as the main type of inflammatory cells in discus samples [9, 27], which makes them a reliable indicator for inflammation. At last, this study did not perform MRI scans with fat-saturated T2-sequences, which are the most sensitive for detecting bone marrow edema and thus MC1 [28]. Hence, the found percentage of MC1 may be underestimated.

More importantly, in future studies the dynamics of M1 and M2 macrophages in relation to bacterial infection of the disc and MC should be explored. Better understanding these may lead to a more accurate diagnosis, prognosis and treatment.

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Conflict of interest
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Appendix

Table 1. Baseline characteristics of the three histologically defined inflammation groups for patients with and without Modic type 2 changes.

	No/mild (without MC) (N=31)	No/mild (with MC2) (N=16)	Moderate (without MC) (N=25)	Moderate (with MC2) (N=19)	Strong (without MC) (N=14)	Strong (With MC2) (N=12)
Age	39.0 ±8.9	43.1 ±10.7	39.8 ±9.4	45.1 ±11.1	40.7 ±6.5	47.8 ±4.4
Male (%)	83.9%	62.5%	52%	73.7%	71.4%	100%
Body-mass index	26.0 ±4.3	26.3 ±3.7	25.2 ±3.8	26.0 ±2.7	24.2 ±2.3	27.6 ±3.0
Duration of sciatica in weeks	9.4 ±1.8	10.0 ±2.2	9.5 ±2.5	9.8 ±2.3	8.6 ±2.3	8.5 ±1.9
Roland Disability score	16.5 ±4.8	16.2 ±3.6	17.0 ±4.2	17.3 ±3.4	16.6 ±4.3	16.5 ±5.1
VAS leg pain	64.6 ±22.5	63.0 ±20.6	69.9 ±16.7	61.1 ±25.2	76.4 ±10.4	67.3 ±22.1
VAS back pain	27.8±31.7	46.6 ±32.8	40.5 ±27.4	28.3 ±27.8	32.8 ±28.0	21.3 ±30.8

Table 2. Associations between MC and inflammation at baseline.

X^2 test	No MC	MC2	P value
Inflammation			0.525
• Mild (n=48)	31	16	
• Moderate (n=45)	25	19	
• Considerable (n=26)	14	12	
Type of herniation			0.324
• Not applicable (3)	1	2	
• Bulging (40)	27	13	
• Extrusion (73)	41	32	
• Sequestration	0	0	

Table 3. Baseline main and interaction effects of MC2 and inflammation on clinical outcomes.

Two-way ANOVA	RDQ score F value (p-value)	VAS leg score F value (p-value)	VAS back score F value (p-value)
• MC2 effect	0.01 (p=0.946)	2.61 (p=0.109)	0.08 (p=0.775)
• Inflammation effect	0.34 (p=0.714)	1.25 (p=0.292)	0.93 (p=0.398)
• Interaction effect MC2* inflammation	0.07 (p=0.937)	0.415 (p=0.661)	3.45 (p=0.035)

Table 3 shows the results of a two-way ANOVA. Values are F values with p values, n total = 117. P-values are corrected for multiple testing according to Bonferroni (9 tests, α=0.006), significance is indicated with '*'.

Table 4. Follow-up main and interaction effects of MC2 and inflammation on clinical outcomes.

Mixed model test	RDQ score F value (p-value)	VAS leg score F value (p-value)	VAS back score F value (p-value)
• MC2 effect	2.47 (p=0.117)	0.02 (p=0.901)	1.52 (p=0.219)
• Inflammation effect	2.25 (p=0.107)	6.97 (p=0.001*)	2.32 (p=0.099)
• Interaction effect MC2* inflammation	7.10 (p<0.001*)	4.69 (p=0.010)	0.75 (p=0.474)

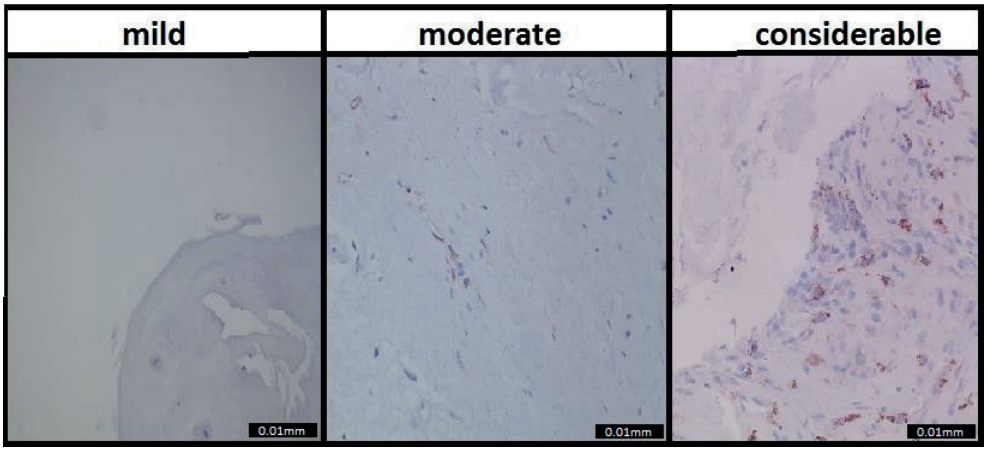
Table 4 displays the results of a mixed model analysis, values are F values with p values, n total = 117. P-values are corrected for multiple testing according to Bonferroni (9 tests, α=0.006), significance is indicated with '*'.

Table 5. Post-hoc test for the main effects of inflammation on the VAS leg score and interaction effects on the RDQ and VAS leg score during the one-year follow-up.

Inflammation in the entire population	**Mild (N = 46)**	**Moderate (N =44)**	**Considerable (N=27)**	**mild vs moderate**	**mild vs considerable**	**moderate vs considerable**
• VAS leg	14.1 (11.7; 16.4)	7.8 (5.5;10.2)	9.7 (6.7; 12.7)	p<0.001*	p=0.026	p=0.326
Inflammation in patients without MC:	Mild (N = 31)	Moderate (N =25)	Considerable (N=14)	mild vs moderate	mild vs considerable	moderate vs considerable
• RDQ	4.5 (3.7; 5.4)	4.0 (3.2; 4.9)	3.4 (2.3; 4.6)	p=0.454	p=0.142	p=0.410
• VAS leg	16.7 (13.9; 19.5)	8.2 (5.1;11.1)	6.4 (2.6;10.3)	p<0.001*	p<0.001*	p=0.472
Inflammation in patients with MC2:	Mild (N = 15)	Moderate (N =19)	Considerable (N=12)	mild vs moderate	mild vs considerable	moderate vs considerable
• RDQ	3.6 (2.4; 4.8)	3.8 (2.7; 5.0)	6.8 (5.4; 8.1)	p=0.785	p<0.001*	p=0.001
• VAS leg	11.4 (7.6; 15.2)	7.5 (3.7; 11.2)	13.1 (8.4; 17.7)	p=0.146	p=0.594	p=0.065

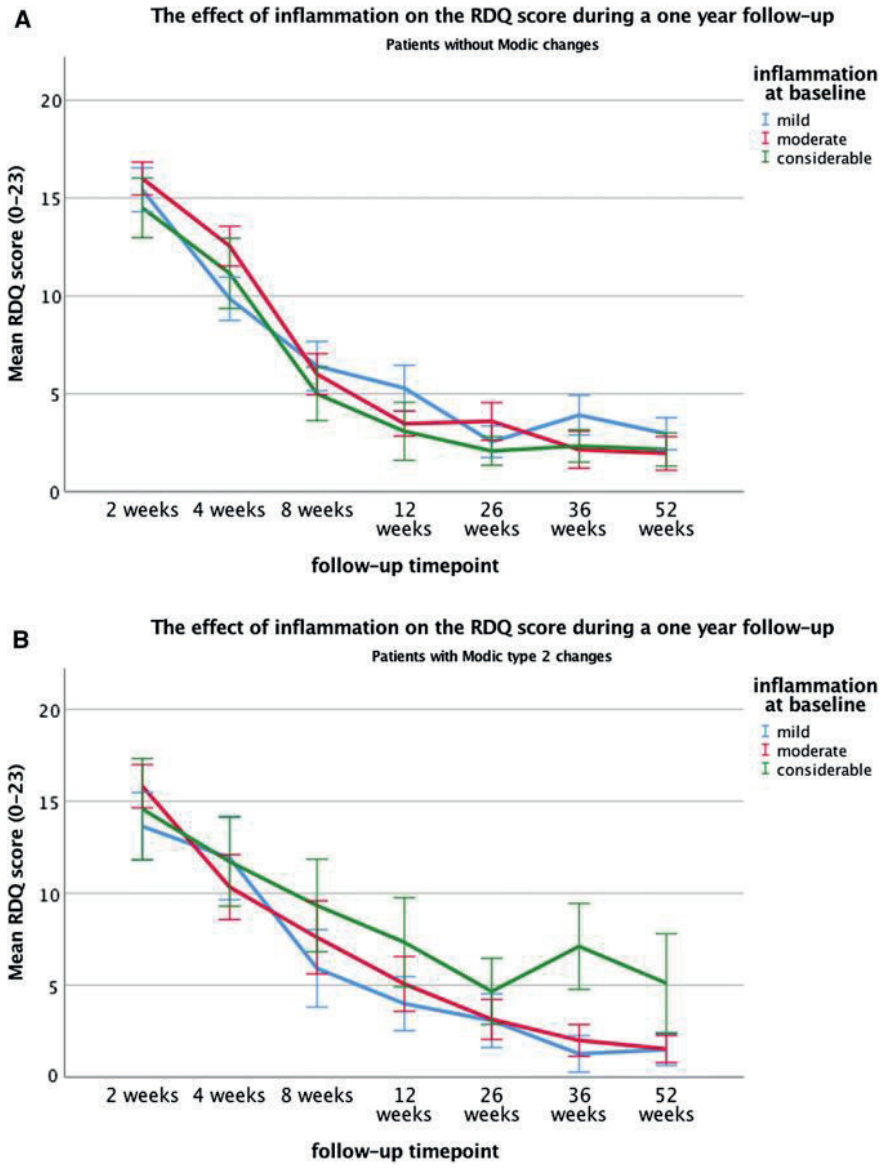
Table 5 shows which subgroups explain the main / interaction effect found in Table 4. mean RDQ and VAS leg scores are compared between different degrees of inflammation, separately for patients with and without MC2. Values are means (confidence interval), and p values, which are corrected for multiple testing according to Bonferroni (15 tests, α=0.003), significance is indicated with '*'.

Figure 1. Macrophage infiltration categories



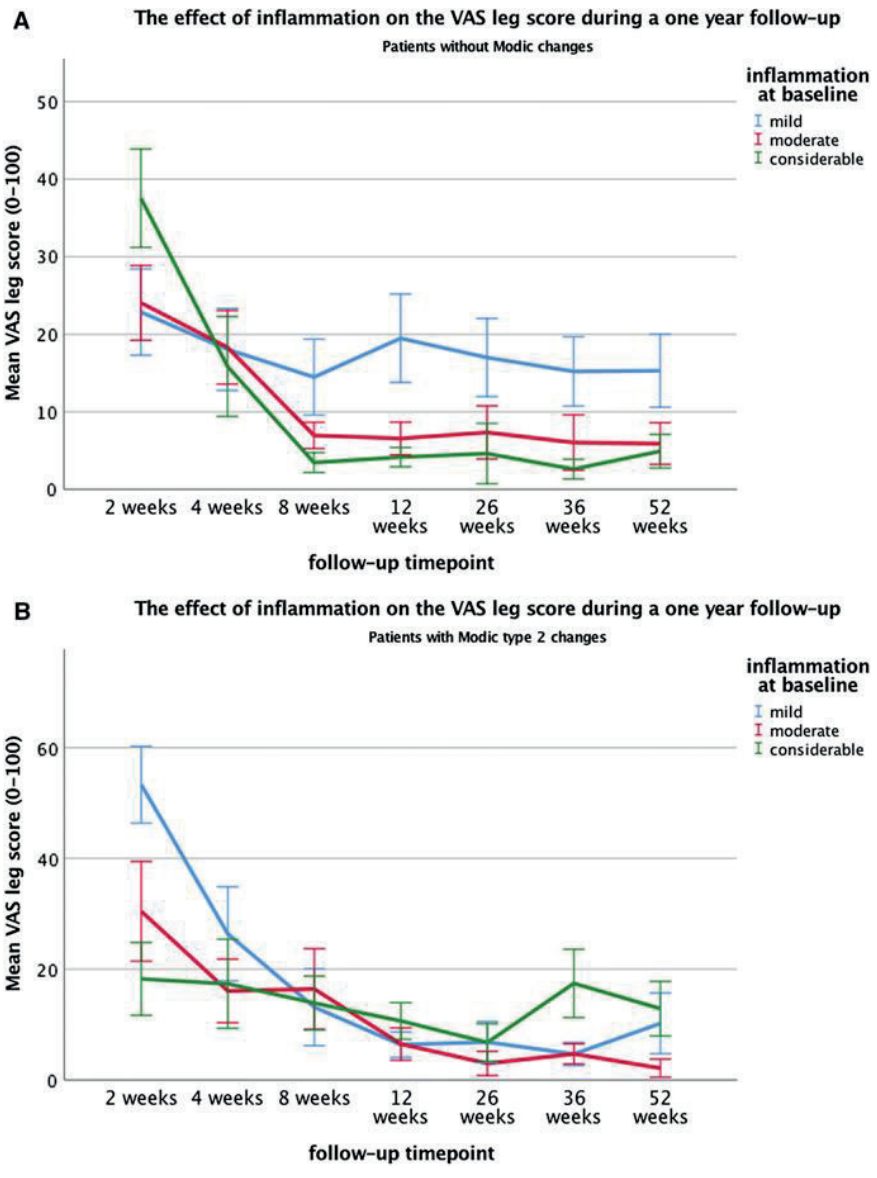
Examples of mild (0-10 macrophages per cm^2), moderate (10-100 macrophages per cm^2), and considerable (>100 macrophages per cm^2) inflammation.

Figure 2. The effect of inflammation on the RDQ score during a one-year follow-up analysis with multiple time points



2A: Error bars are SE'. No significant differences were seen. **2B**: Error bars are SE's. A significant difference was seen between mild and considerable, and between moderate and considerable inflammation.

Figure 3. The effect of inflammation on the VAS leg score during the 1-year follow-up analysis with multiple time points



3A: Error bars are SE's. A significant difference was seen between mild and moderate, and between mild and considerable inflammation. **3B**: Error bars are SE's. No significant differences were found.

Supplementary appendix

Table S1. Baseline main and interaction effects of MC2 and inflammation on clinical outcomes separate for Bulging and Extruded disc.

S1A: Baseline analysis (ANOVA)	**RDQ score F value (p-value)**	**VAS leg score F value (p-value)**	**VAS back score F value (p-value)**
Bulging			
• MC2 effect	0.33 (p=0.572)	0,79 (p=0.382)	0.13 (p=0.718)
• Inflammation effect	2.05 (p=0.162)	0,19 (p=0.666)	0.20 (p=0.656)
• Interaction effect MC2* inflammation	0.40 (p=0.532)	0.9 (p=0.350)	3.8 (p=0.061)
Extrusion			
• MC2 effect	0.32 (p=0.572)	0.60 (p=0.441)	<0.01 (p=0.980)
• Inflammation effect	0.51 (p=0.605)	0,73 (p=0.484)	0.16 (p=0.207)
• Interaction effect MC2* inflammation	0.16 (p=0.852)	0.17 (p=0.847)	2.19 (p=0.121)
S1B: Follow-up analysis (Mixed model)	**RDQ score F value (p-value)**	**VAS leg score F value (p-value)**	**VAS back score F value (p-value)**
Bulging			
• MC2 effect	0,62 (p=0.433)	0,52 (p=0.182)	0.09 (p=0.762)
• Inflammation effect	0.92 (p=0.340)	5.45 (p=0.021)	1.12 (p=0.290)
• Interaction effect MC2* inflammation	3.13 (p=0.079)	0.52 (p=0.471)	0.47 (p=0.496)
Extrusion			
• MC2 effect	4.62 (p=0.032)	0.41 (p=0.521)	0.01 (p=0.906)
• Inflammation effect	1.16 (p=0.315)	2.91 (p=0.056)	0.44 (p=0.646)
• Interaction effect MC2* inflammation	8.04 (p<0.001*)	5.27 (p=0.006*)	5.12 (p=0.007)

S1A: Two-way ANOVA was used, values are F values with p values, n total = 114**.** P-values are corrected for multiple testing according to Bonferroni in each group (9 tests, α=0.006). The follow-up measurements were conducted at 2,4, 8, 12, 16, 36 and 52 weeks post-surgery. For bulging, the amount of considerable inflammation was too low for statistical testing in MC2 (n=1), hence only mild and moderate inflammation groups were used. **S1B:** Mixed model analysis was used, values are F values with p values, N total = 93**.** P-values are corrected for multiple testing according to Bonferroni (9 tests per disc type, α=0.006), significance is indicated with '*'. For bulging the amount of considerable inflammation was too low for statistical testing in MC2 (n=1), hence only mild and moderate inflammation groups were used.

Table S2. Post-hoc test for the interaction effects on the RDQ and VAS leg score in extruded discs during the one-year follow-up.

Inflammation in extruded discs without MC:	**Mild (N = 17)**	**Moderate (N =13)**	**Considerable (N=11)**	**mild vs moderate**	**mild vs considerable**	**moderate vs considerable**
• RDQ	4.1 (2.9; 5.2)	4.0 (2.8; 5.2)	2.4 (1,1; 3.8)	p=0.923	p=0.073	p=0.091
• VAS leg	16.0 (12.2; 19.8)	8.6 (4.7; 12,4)	5.3 (1.1; 9.6)	p=0.007	p<0.001*	p=0.274
Inflammation in extruded discs with MC2:	**Mild (N = 9)**	**Moderate (N =13)**	**Considerable (N=10)**	**mild vs moderate**	**mild vs considerable**	**moderate vs considerable**
• RDQ	4.1 (2.7; 5.5)	3.2 (1.7; 4.6)	6.8 (5.2; 8.3)	p=0.372	p=0.009	p<0.001*
• VAS leg	10.8 (6.3; 15.2)	8.2 (3.7; 12.8)	14.3 (9.5; 19.1)	p=0.437	p=0.284	p=0.070

Table S2 shows which subgroups explain the main / interaction effect found in Table S2. mean RDQ and VAS leg scores are compared between different degrees of inflammation, separately for patients with and without MC2 (n = 96). Values are means (confidence interval), and p values, which are corrected for multiple testing according to Bonferroni (12 tests, α=0.004), significance is indicated with '*'.


5

Chapter 5

Gadolinium enhancement is not associated with disc inflammation in patients with sciatica

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"There are no shortcuts, everything is reps, reps, reps."
(Arnold Schwarzenegger)

Abstract

Study design
Retrospective observational histological study

Objective
To evaluate the reliability of gadolinium enhancement as a marker for inflammation by associating gadolinium enhancement findings with the degree of inflammation as measured by macrophage infiltration in disc material retrieved during disc surgery in patients with sciatica.

Summary of the background data
Disc inflammation often occurs in sciatica patients, a non-invasive tool that is used to assess disc inflammation is gadolinium enhanced MR imaging.

Methods
Disc tissue was retrieved from patients in the Sciatica trial (N = 119), A multicentre randomized controlled trial in patients with sciatica. Disc tissue was embedded in paraffin and stained with haematoxylin and CD68. Tissue samples were categorized as mild (0-10 macrophages/cm^2), moderate (10-100 macrophages/cm^2), and considerable (>100 macrophages/cm^2) inflammation. Of the 119 MRI's, 96 were additionally performed with contrast-enhanced gadolinium.

Results
74 patients showed gadolinium enhancement of the disc herniation and 26 of the nerve root. Degree of inflammation by macrophages was not associated with gadolinium enhancement of nerve roots or herniated discs. These results did not change if the patient groups with and without Modic type 2 changes were evaluated separately. Furthermore, no associations were observed between gadolinium enhancement and presence of Modic type 2 changes.

Conclusions
This study found gadolinium enhanced MRI findings to be unreliable as an indicator for inflammation of disc herniation or nerve root in patients with sciatica.

Introduction

A highly prevalent medical condition is lumbar disc herniation [1], often accompanied by sciatica, also known as radicular pain syndrome. Since 1934, it has been widely accepted that this condition is caused by compression of the nerve root of the herniated lumbar disc [2, 3]. Disruptions of the annulus fibrosus or of its attachments to the adjacent endplate may result in a herniation of nucleus pulposus tissue into the epidural space. This concept was later fine-tuned with theories involving micro traumata of the vertebral endplate and/or micro traumata leading to disruption of the annulus fibrosus. In addition to the abovementioned nerve root compression, disruption of the annulus fibrosus can also induce a foreign-body reaction aimed at the nucleus pulposus tissue in the epidural space, which is accompanied by neovascularization and macrophage infiltration [4-6]. Subsequently, infiltrated macrophages have the ability to exacerbate the sciatic symptoms by excreting pro inflammatory cytokines [7-9]. Both the nerve root compression and inflammatory response may contribute to the irritation of the nerve root and thus to pain in the innervated dermatome [10]. However, the foreign-body reaction could also have a positive effect: it may help to accelerate the healing process through resorption of the herniated material mediated by infiltrated macrophages [8, 11, 12].

In order to further explore the role of inflammation in sciatica, it would be of benefit if non-invasive tools for inflammation could be identified. Such a tool might be Magnetic Resonance Imaging (MRI) accompanied by intravenous injection of gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA). This contrast enhancement method helps visualizing vascular supply to tissue. It is widely thought that the herniated disc is only vascularized in case of an inflammatory response [13]. Consequently, some studies have suggested that this technique provides a good predictor for inflammatory reactions in the disc. However, evidence is limited [14, 15].

An additional MRI feature that is often described in sciatica patients are Modic changes. Recently, Dudli et al. added a characteristic. The authors showed that both Modic type 1 and type 2 changes are associated with an inflammatory dysmyelopoiesis with fibrogenic changes [16]. This suggests that Modic changes interact with the inflammatory process in the disc. This is supported by our previous study that showed an interaction effect of Modic type 2 changes and disc inflammation on clinical outcome in sciatica [17]. Because of this interaction, it could be that the correlation between gadolinium enhancement and inflammation is different in patients with and without Modic Changes. The aim of this study is to investigate the association between gadolinium enhanced MR imaging and the extent of disc inflammation in sciatica patients with, and without Modic changes.

Materials and Methods

Study population

Patients for this study were participants in the Sciatica Trial [18]. This was a multicentre randomized trial involving 283 patients who suffered from sciatica for 6-12 weeks and had MRI disc herniation. Patients were included if they suffered from a dermatomal pattern of pain distribution with accompanying neurological dysfunction that corresponded to the same nerve root being affected on the MRI. Exclusion criteria were cauda equina syndrome, muscle paralysis, insufficient strength to move against gravity, occurrence of another episode of symptoms similar to those of the current episode during the previous 12 months, previous spine surgery, bony stenosis, spondylolisthesis, pregnancy, or severe coexisting disease. In the trial, early surgery was compared to prolonged conservative care. 141 patients were randomized to surgery and 125 patients actually underwent surgery (16 recovered before surgery could be performed). The other 142 patients were randomized to prolonged conservative care, of which 55 patients underwent surgery within 1 year, with a mean time to surgery of 15 weeks after randomization. Thus, in the first year after randomization a total of 180 patients underwent surgery for sciatica. The protocol, which included analysis of the disc material, was approved by the medical ethics committees at all participating hospitals.

Histological analysis

Disc material of all operated patients was collected and fixed in 4% formaldehyde solution. For assessment, the tissue was subsequently embedded in blocks of paraffin. Thin slices were prepared and the tissue was evaluated histologically for inflammation by investigating the presence of macrophages. For the haematoxylin staining, a 5-µm thick slice was taken from the middle of the paraffin blocks. Each slice was stained with Harris haematoxylin according to the program from the Leica ST 5020-mulitstainer. For the immunohistochemistry, 5-µm paraffin slices were rinsed in ethanol and methanol solutions and prepared for the expression of CD68 (macrophages) (DAKO, Denmark). Immunohistochemistry was performed using a three-step indirect method. Antibodies were cooked in EDTA pH 9.0 buffer as a pre-treatment. Subsequently, a avidin-biotin complex technique was performed with the Vectastain ABC-Elite Kit (Vector Lab. USA) and the appropriate biotinylated antibodies. Visualization of the peroxidase reaction was done with DAB solution (Sigma). Moreover, the samples were counterstained with Harris haematoxylin. All of these samples were accompanied by a positive control. In control samples, primary antibodies were omitted, which resulted in the expected absence of any cellular labelling. In order to standardize the evaluation of the samples, all samples were photographed under the microscope before they were evaluated.

The evaluation was done by two independent investigators, who were blinded to clinical information and MRI. The training of these independent researchers was carried out by a senior pathologist. The number of macrophages on each sample was counted and estimated. Using this method, the tissue samples were categorized according to their inflammation grade. The categories consisted of mild (0-10 macrophages per cm^2), moderate (10-100 macrophages per cm^2), and considerable (>100 macrophages per cm^2) inflammation. Subsequently, a consensus score between the independent researchers was determined. An acceptable consensus score was predefined as 60%. It was pre-defined that all samples would be re-assessed with a senior pathologist if the consensus was less than 60%.

MRI protocol and Image evaluation of the sciatica trial

MRI scans were performed at baseline by a 1.5 Tesla scanner, and both sagittal T1- and T2-weighted images of the lumbar spine were obtained. T1 images were gathered with contrast-enhanced gadolinium dithylenetriamine penta-acetic acid [DTPA] at a standard dose of 0.1 mmol/kg body weight. Image evaluation of Modic changes was according to the criteria of Modic changes et al[19, 20]. Image evaluation was done according to a predefined protocol (Supplementary Table S1)[21]**.**MRIs were evaluated by 2 neuro radiologists and 1 neurosurgeon. All three were blinded to histological data and clinical information. The readers were not involved in the selection or treatment of the patients included. Inter-observer agreement analyses regarding the MRI findings were published earlier [22, 23]. For the statistical analyses, the majority opinion of the three independent researchers (answered by at least two of the three) was used.

The structure enhancement by gadolinium were scored in the following categories: 1. Enhancement of herniated disc: no or little enhancement, full or diffuse enhancement. 2. Enhancement of target nerve root: enhancement versus no enhancement. Regarding Modic changes, Type was scored as: 1, 2, or 3. Because in earlier findings no Modic changes were observed at level L1-L2 [24], only images from L2-L3 through L5-S1 were evaluated.

The inter-observer agreement was substantial for the Modic changes (69-97%), as described by el Barzouhi (2014) [25]. The inter-observer agreement was moderate regarding the scoring of enhancement of the herniated disc (56.2%, kappa = 0.42). Enhancement of the target nerve root inter-observer agreement was moderate (63.4%, kappa = 0.27).

Statistical analysis

Firstly, we correlated gadolinium enhancement with the presence of Modic changes. Secondly, the categorized histological findings were associated with gadolinium enhancement of the nerve root and herniated disc. In addition, we repeated the analyses separately in patients with and without Modic changes. All comparisons were done using X^2 tests for categorical data. P-values of < 0.05 were regarded as significant.

Preceding this additional subgroup analysis, the data was tested for an association between Modic type 2 changes and inflammation, no association was found ($p = 0.68$), a more detailed description was previously described [17]. All comparisons were done using X^2 tests for categorical data. P-values of < 0.05 were regarded as significant.

Results

Demographics

Of the 180 patients that underwent surgery, 119 patients disc samples were preserved and analyzed. Missing samples were due to multiple reasons: samples were either not collected during surgery, got lost after surgery, were not preserved properly, or got lost after preservation. 96 of the 119 patients received gadolinium enhanced MRI's. The baseline characteristics age, gender, BMI and duration of sciatica prior to surgery of the three inflammation groups were comparable (Table 1).

The histological data analysis

CD68 staining to identify macrophages resulted in the following distribution: 47 (39.5%) patients had 0-10 macrophages per cm^2 (indicating mild inflammation), 45(37.8%) patients had 10-100 macrophages per cm^2 (indicating moderate inflammation), and 27 (22.7%) patients had >100 macrophages per cm^2 (indicating considerable inflammation) (Table 2).The consensus score was excellent (0.96). (Table 2). Examples of the CD68 samples and their categories are shown in Figure 1.

The MRI data analysis

Of the 119 patients, a total of 1 patient showed Modic type 1 changes, 33 patients Modic type 2 changes, no patients Modic type 3 changes, and 85 patients had no Modic changes. Because only one patient showed Modic type 1 changes, this sample was excluded from the statistical analyses and thus only Modic type 2 changes were taken into account. Out of the 96 patients with gadolinium enhanced MRI's, 26 showed gadolinium enhancement of the root, and 74 showed gadolinium enhancement of the herniated disc.

Association between Modic changes and gadolinium

No significant associations were found between the presence of Modic type 2 changes and enhancement of the nerve root ($p = 0.51$) or herniated disc ($p = 0.61$) (Table 3).

Association between inflammation and gadolinium

The degree of inflammation by macrophages was not associated with enhancement of nerve root in the entire population ($p = 0.66$), and neither in the subgroups (no Modic changes $p = 0.90$ and for Modic Type 2 changes $p = 0.66$) (Table 4). Enhancement of the herniated disc was also not associated with the degree of inflammation in the entire population ($p = 0.30$) or in the subgroups (no Modic versus Modic Type 2 changes) (Table 5).

Discussion

The most important finding of this study is that gadolinium enhancement assessed on MRI is not associated with disc inflammation by macrophages. This indicates that gadolinium enhancement is probably unreliable as a marker tool for inflammation in sciatica.

In contrast with previous literature, the expected association between histologically defined inflammation by macrophages and gadolinium enhancement on MRI was not observed in this study. This questions the reliability of gadolinium as a proper indicator for disc inflammation, as it was suggested earlier [14, 15]. In addition, we did not observe an association between gadolinium enhancement and the presence of Modic type 2 changes, which is also in contradiction with previous research in which an increase of enhancement was found in patients with Modic changes [26]. However, in that study the investigators did not make any difference between type 1 and 2 Modic Changes [26]. The correlation might not have been caused by Modic type 2 but by Modic type 1, or that gadolinium enhancement is not sensitive enough to point out the difference in vascularization in patients with and without Modic changes. As we also did not observe an association between gadolinium enhancement and histologically defined inflammation by macrophages, we expect the latter to be more likely [26].

Our findings may be explained by the great inter-observer variability among the evaluators with respect to gadolinium enhancement. This indicates that it is often unclear in which enhancement category a sample fits best. the fact that most other MRI parameters that were observed during this study demonstrated a good to excellent inter-observer agreement[22, 23, 27], makes it more likely that the poor inter-observer agreement was due to visibility of gadolinium instead of the incompetence of the evaluators. However, before we conclude that gadolinium is unreliable as an imaging tool in disc herniation, we must take the possibility into account that the lack of associations was due to methodological or equipment reasons. This stresses the importance of replication studies.

This study has several strong points. For the histological analysis we used multiple evaluators and demonstrated substantial consensus score. Moreover, this study was the first study to compare histology to gadolinium enhancement between patients with and without Modic type 2 changes. This study also has some limitations. the inter agreement analysis of gadolinium revealed only moderate kappa values, which points out that human error remains an obstacle in the present research environment that has yet to be overcome. The samples used for the pathological analysis were old, which might have reduced their quality. Nevertheless, the CD68 staining did show properly identifiable macrophages. Hence we assume that the age of the sample did not alter our result. Also, since this study measured the degree of inflammation in number of macrophages, it cannot be out ruled that T or B lymphocytes would associate differently with gadolinium enhancement. However, previous findings have shown that macrophages are the main type of inflammatory cells found in discus samples [28] [14], and macrophages in disc material are associated with neovascularization [14], we do not expect T and B lymphocytes to associate differently with gadolinium enhancement.

This study points out the limited and now contra dictionary evidence available on the reliability of gadolinium enhancement as an indicator for inflammation in patients with sciatica due to a lumbar disc herniation [14, 15, 20]. In order to put our findings in perspective, future studies should focus on evaluating the usage of gadolinium in disc herniations.

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Conflict of interest
None of the authors has any conflict of interest. No funding was received for the conductance of this study.

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Appendix

Table 1. Baseline characteristics of the three histologically defined inflammation groups

	Mild (N=48)	moderate (N=45)	considerable (N=27)
Age	40.4±9.6	42.1±10.3	43.7±6.6
Male gender	36 (75.0%)	28 (62.2%)	22 (81%)
Body-mass index	26.0±4.1	25.8±3.5	25.9±3.2
Duration of sciatica in weeks	9.6±1.9	9.7±2.3	8.7±2.1
Received gadolinium	37 (77.1%)	40 (88.9%)	19 (70.4%)

Values for gender are n and (%) or means ± SD.
No significant baseline differences were observed between the three inflammation groups.

Table 2. Consensus score of the pathological findings

	A vs. B % agreement
Inflammation at baseline (3 categories)	95.8
Mild	96.8
Moderate	94.6
considerable	96.2

A and B both represent independent observers. The agreement was assessed after the consensus reading.

Table 3. Association between Modic changes and gadolinium enhancement of the herniated disc

X^2 test	No MC (N = 67)	MC2 (N = 26)	P value
Nerve root enhancement			0.51
• No enhancement	47 (70.1%)	20 (76.9%)	
• Enhancement	20 (29.9%)	6 (23.1%)	
X^2 test	**No MC (N = 67)**	**MC2 (N = 26)**	**P value**
Disc herniation enhancement			0.61
• No or little enhancement	42 (63.6%)	18 (69.2%)	
• Full or diffuse enhancement	24 (36.4%)	8 (30.8%)	

95 of 96 Gd-MRI's were scored for MC 2 changes. X^2 tests for no MC vs MC2 compared to enhancement of the nerve root and herniated disc.

Table 4. Association between gadolinium nerve root enhancement and inflammation for all patients and separately for patients with and without Modic changes

X^2 test (All patients)	Mild (N = 37)	Moderate (N = 40)	Considerable (N = 19)	P value
Nerve root enhancement				0.66
• No enhancement	25 (67.6%)	30 (75%)	14 (77.8%)	
• Enhancement	12 (32.4%)	10 (25%)	4 (22.2%)	
X^2 test (without MC*)	**Mild (N = 28)**	**Moderate (N = 27)**	**Considerable (N = 12)**	**P value**
Nerve root enhancement				0.90
• No enhancement	19 (67.8%)	19 (70.4%)	9 (75%)	
• Enhancement	9 (32.2%)	8 (29.6%)	3 (25%)	
X^2 test (with MC2 changes)**	**Mild (N = 9)**	**Moderate (N = 12)**	**Considerable (N = 6)**	**P value**
Nerve root enhancement				0.66
• No enhancement	6 (66.7%)	10 (83.3%)	4 (80%)	
• Enhancement	3 (33.3%)	2 (16.7%)	1 (20%)	

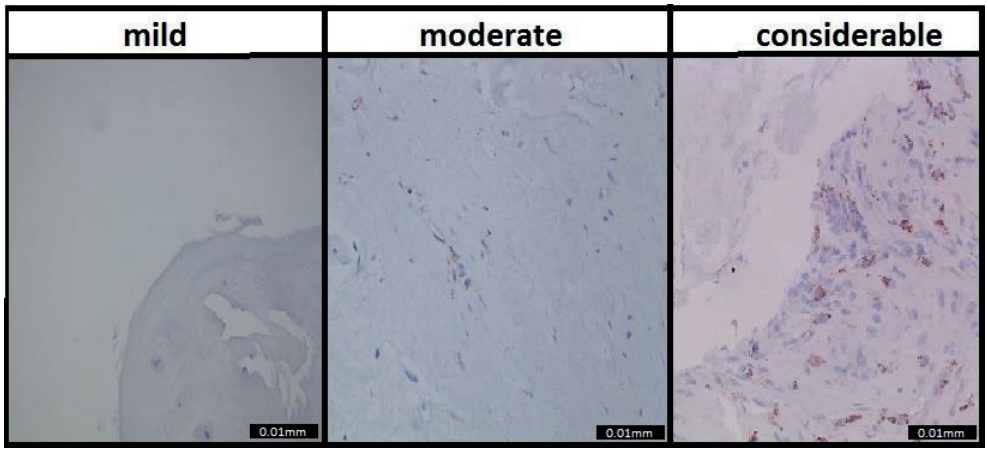
X^2 tests for mild vs moderate vs considerable inflammation compared to enhancement of the nerve root. First in all patients, then in patients without MC and last in patients with MC2.
* One value missing for patients without MC, in the moderate inflammation group.
** One value missing for patients with MC2, in considerable inflammation group.

Table 5. Association between gadolinium enhancement of the herniated disc and inflammation for all patients, and separate for patients with and without Modic changes

X^2 test for all patients	Mild (N = 37)	Moderate (N = 40)	Considerable (N = 19)	P value
Disc herniation enhancement				0.30
• No or little enhancement	20 (55.6%)	29 (72.5%)	12 (66.7%)	
• Full or diffuse enhancement	16 (44.4%)	11 (27.5%)	6 (33.7)	
X^2 test (without MC*)	**Mild (N = 28)**	**Moderate (N = 27)**	**Considerable (N = 12)**	**P value**
Disc herniation enhancement				0.60
• No or little enhancement	16 (57,1%)	19 (70.4%)	7 (63.6%)	
• Full or diffuse enhancement	12 (42.9%)	8 (29.6%)	4 (36.4%)	
X^2 test (with MC2 changes)**	**Mild (N = 9)**	**Moderate (N = 12)**	**Considerable (N = 6)**	**P value**
Disc herniation enhancement				0.45
• No or little enhancement	4 (50%)	10 (83.3%)	4 (66.7%)	
• Full or diffuse enhancement	4 (50%)	2 (17.7%)	2 (33.3%)	

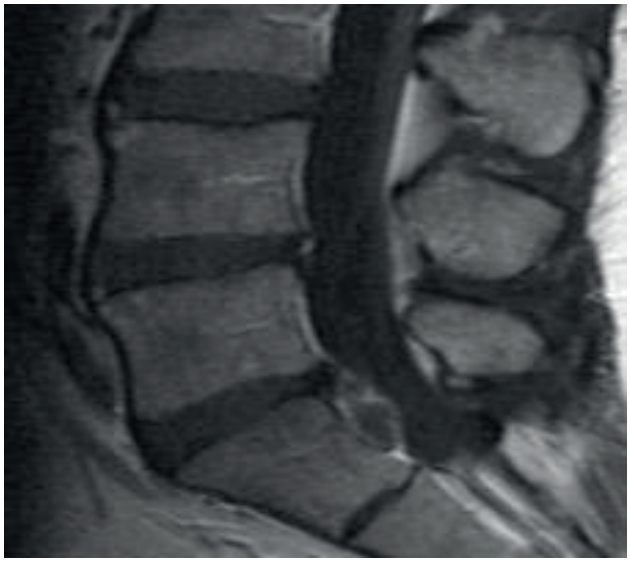
X^2 tests for mild vs moderate vs considerable inflammation compared to enhancement of the nerve root. First in all patients, then in patients without MC and last in patients with MC2**.**
* Disc bulging was scored once in the considerable inflammation group.
** Disc bulging was scored once in the mild inflammation group.

Figure 1. Macrophage infiltration categories



Examples of mild (0-10 macrophages per cm^2), moderate (10-100 macrophages per cm^2), and considerable (>100 macrophages per cm^2) inflammation.

Figure 2. Example of gadolinium enhancement



A patient who showed circumferential gadolinium enhancement of a disc herniation at level L5-S1.

Supplementary appendix

Table S1. MRI study variables (gadolinium)

MRI variable	Type	Categories
Disc level that most likely caused the lumbosacral radicular syndrome of the patient	Disc level	1. L2L3 2. L3L4 3. L4L5 4. L5S1 5. Not applicable, all disc levels have a normal disc contour: no disc extension beyond the normal margins of the intervertebral disc space at any disc level
	Disc contour at this disc level	1. Normal: no disc extension beyond the normal margins of the intervertebral disc space 2. Bulging: presence of disc tissue circumferentially (50-100%) beyond the edges of the ring apophyses 3. Consideration of a disc herniation: localized displacement of disc material beyond the normal margins of the intervertebral disc space
	Certainty about the presence of a disc herniation	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but probability > 50% 3. Possible about the presence: reason to consider but probability < 50% 4. Definite about the absence: no doubt about the absence of a disc herniation.
If a herniation at the disc level is considered	**Gadolinium enhancement of the intervertebral disc herniation**	**1. No enhancement** **2. Any edge enhancement** **3. Complete circumferential enhancement** **4. Diffuse staining**
Nerve root compression	Probability of nerve root compression	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but likelihood > 50% 3. Possible about the presence: reason to consider but likelihood < 50% 4. Definitely no nerve root compression
	If nerve root compression is present, which nerve root is affected	1. L3 2. L4 3. L5 4. S1 5. Not applicable, definitely no nerve root compression
	Side nerve root compression	1. Right 2. Left
	Gadolinium enhancement of the affected nerve root	1. No enhancement 2. Yes, mild enhancement 3. Yes, considerable enhancement
	Nerve root thickness distal to the site of compression	1. Normal 2. Thickened 3. Narrowed

Table S2. MRI study variables (Modic changes)

Disc level	Variable	Category
Disc level with the most severe nerve root compression	Disc level	1. Not applicable: no nerve root compression 2. L2L3 3. L3L4 4. L4L5 5. L5S1
	Disc contour at this level	1. Bulging: presence of disc tissue circumferentially (50-100%) beyond the edges of the ring apophyses 2. Herniation: localized displacement of disc material beyond the normal margins of the intervertebral disc space
	Certainty about the presence of disc herniation	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but probability > 50% 3. Possible about the presence: reason to consider but probability < 50% 4. Definite about the absence: no doubt about the absence
	Loss of disc height at this level	1. Yes 2. No
	Signal intensity of nucleus pulposus on T2 images at this level	1. Hypointensity 2. Normal 3. Hyperintensity
	Certainty about the presence of nerve root compression	1. Definite about the presence: no doubt about the presence 2. Probable about the presence: some doubt but probability > 50% 3. Possible about the presence: reason to consider but probability < 50% 4. Definite about the absence: no doubt about the absence
	Spinal canal stenosis	1. Yes 2. No
	Disappearance of epidural fat	1. Completely disappeared 2. Partly disappeared 3. No disappearance
	Presence of vertebral end plate changes and its extent	1. No VESC (Vertebral Endplate Signal Changes) 2. VESC type 1: hypointense on T1-weighted sequences and hyperintense on T2-weighted sequences 3. VESC type 2: increased signal on T1 weighted sequences and isointense or slightly hyperintense signal on T2 weighted sequences 4. VESC type 3: hypointense both on T1- and T2-weighted sequences 5. VESC type 1 and 2

Chapter 6

Influence of different endplate pathologies on the inflammation profile of herniated discs: a proteomic approach

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Submitted

"He who has a why to live for can bear almost any how"
(Friedrich Nietzsche)

Abstract

Purpose

The aim of this observational radiographic and proteomic study is to explore the influence of both MC and EPA on the inflammation profile of herniated discs using a proteomic and bioinformatics approach.

Methods

15 nuclei pulposi (NP) harvested from surgery underwent LC-MS/MC analysis, the proteome was subsequently scanned for inflammatory pathways using a bioinformatics approach. All proteins that were identified in inflammatory pathways and gene ontology, and present in >7 samples were integrated in a multiple regression analysis with MC and EPA as predictors. Significant proteins were imputed in an interaction and pathway analysis.

Results

Compared to AFT, 6 proteins were significantly altered in EPA: Catalase, Fibrogen beta chain, Protein disulfide-isomerase, Pigment epithelium-derived factor, Osteoprotegerin and lower expression of Antithrombin-III, all of which corresponded to an upregulation of pathways involved in coagulation and detoxification of reactive oxygen species (ROS). Moreover, presence of MC resulted in a significant alteration of 9 proteins compared to patients without MC. Patients with MC showed a significantly higher expression of Clusterin and Lumigan, and lower expression of Catalase, Complement Factor B, Fibrogen beta chain, Protein disulfide-isomerase, Periostin, Alpha-1-antitrypsin and Pigment epithelium-derived factor. Together these alternated protein expressions resulted in a downregulation of pathways involved in detoxification of ROS, complement system and immune system.

Conclusion

Both EPA and MC status significantly influence disc inflammation. The beneficial inflammatory signature of EPA illustrates that endplate pathology does not necessarily have to worsen the outcome, but that the pathological inflammatory state is dependent on the presence of MC.

Introduction

Herniation of the lumbar intervertebral disc is a highly prevalent disease, during which the herniated disc compresses the adjacent nerve root. Patients with herniated discs experience debilitating back pain and often excruciating leg pain that radiates down the dermatome. The severity of these symptoms can vary widely between patients irrespective of the degree of compression. In search for an explanation for this wide variety in symptoms, research has focused on inflammation of the intervertebral disc.

Disc inflammation seems to function as a double-edged sword [1]. In which it shows beneficial effects on one end: For example, through a resorption process of the herniated material initiated by macrophages, which is associated with a faster regression of the disc material [2]. On the other end, inflammation may lead to exacerbation of pain symptoms, through sensitization of the nerve root by pro inflammatory cytokines excreted in the disc [3]. Because of the clinical significance of disc inflammation, it is essential to develop a non-invasive tool that identifies different stages of inflammation.

Recently, there has been an increase in attention for pathology of the endplate, how it may influence the course of the herniation and the rate of recovery. Most research on this topic has focused on vertebral endplate signal changes on MRI, more commonly referred to as Modic changes (MC) [4, 5]. MC represent inflammatory or fibrotic changes in the endplate [6], and have been associated with a slower rate of recovery [7, 8]. This could be explained by intrusion of cartilage pieces of the disrupted endplate in the herniated nucleus pulposus, which subsequently prevent neovascularisation and macrophage infiltration in the disc [9, 10]. Moreover, others have associated MC with detrimental effect of infiltrating macrophages on clinical outcomes [1]. Taken together, the presence of MC seems to be an indication that the type of inflammation has gone from the beneficial type, towards a type of inflammation that may exacerbate pain symptoms and reduce the rate of recovery. However, the current evidence on this is still inconclusive.

A different, relatively underexposed, pathology of the endplate is endplate avulsion. A disc can herniate in two ways: Either through an annulus fibrosus tear (AFT) or through an endplate avulsion (EPA) [11]. During the latter, the annulus fibrosus is torn from the endplate due to a defect of the endplate. This pathology also resulted in pieces of cartilage in the herniated disc but only has a moderate association with MC [11]. At present, it remains unknown whether EPA has similar effects on inflammation of the disc or whether these concepts should be completely separated.

Hence, the aim of this study was to explore the effects of both MC and EPA on the inflammatory signature of the herniated disc using a proteomic and bioinformatics analysis of nucleus pulposus samples.

Materials & Methods

Patient population

IRB approval and informed consent was obtained. 15 nucleus pulposus samples were harvested from patients undergoing discectomy for radicular pain symptoms due to an extruded herniated disc.

Sample Collection

MRI scans were performed pre-surgery by a 1.5 Tesla scanner, and both sagittal T1- and T2-weighted images of the lumbar spine were obtained. Image evaluation of EPA was dichotomized into an intact endplate (AFT group), and an avulsed endplate (EPA group). Evaluation of MC status was according to the criteria of Modic et al [4, 5]. Samples were harvested during surgery, after the removal of the herniated disc material, nucleus pulposus material was separated from annulus fibrosus material and directly transferred to sterile cryopreservation vials, and snap frozen in liquid nitrogen before transport to the research laboratory.

Sample Processing

Around 100 mg Nucleus pulposus tissue from the 15 discs was subjected for extraction of total proteins and subjected to ESI-LC-MS/MS with conditions as described in our earlier report: Rajasekaran S et al., 2017 [12].

Bioinformatics analysis

A detailed description of the bioinformatic analysis and normalisation by spectral count was published earlier: Rajasekaran et al., 2020 [13].

Quantitative analysis

Out of the proteomic database, all proteins with > 2 unique peptide or 1 unique peptide with a PSM ≥ 10 were included in the analysis [14]. These selected proteins were subsequently integrated in a Gene Ontology and Pathway enrichment analysis using both STRING and DAVID databases, which allowed us to identify all pathways and protein functions that are involved in inflammatory processes. Moreover, since Mass Spectrometry will regularly fail to detect proteins that are expressed in low quantities, our results will contain a large amount of missing data for the less abundant proteins. Therefore, only proteins that were expressed in at least 8 samples were integrated in the statistical analysis.

Statistical analysis

Data analysis was performed using SPSS software version 25. Effects of EPA and MC status on protein expression were analysed using a multiple regression, for this analysis, protein expression (normalized PSM) was Log10 transformed, Assumptions of normalized residuals, influential cases (cook's distance >1), and homogeneity of variance had to be met. Two-tailed alfa level was set at 0.05. samples with missing values were excluded from the analysis.

Interaction analysis

All significant proteins were integrated in an Interaction analysis using string database. Subsequently, up or down regulations of relevant pathways corresponding to the identified interactions are evaluated.

Results

Patient characteristics

Out of the 15 included patients, 6 patients were characterized as AFT on MRI (Mean age 45.2±19.2 SD, 33% male) and 9 patients as EPA (Mean age 32±5.8 SD, 67% male). Mann Whitney U test showed that neither the difference in age (p=0.224) nor sex (p=0.205) was significant. Moreover, 8 patients did not show any MC on MRI (Mean age 41.5±17.9 SD, 50% male), and 7 patients did show MC, (Mean age 32.4±5.3 SD, 43% male). Again, the differences in age and sex were not significant. (Age: p=0.908, Sex: p=0.447). In addition, the distribution of EPA in patients with MC was similar as in patients without MC (Fisher exact: p=0.608). Lastly, neither EPA (p=0.747), nor MC (p=0.800) was associated with the extent of disc degeneration according to classification by Pfirmann et al [15]. All disc herniations were characterized as the extruded type according to Fardon et al (2014) [16]. An overview of all patient characteristics can be found in table 1.

Pathway analysis

The Gene ontology and Pathway analysis identified 31 pathways that were involved in inflammation related processes (Table S1). In these 31 pathways combined, 147 inflammation related proteins were identified. Out of which 41 were eligible for statistical analysis (Supplementary table S2).

Comparing protein expression

Out of the 41 proteins, 5 proteins were significantly affected by EPA status. EPA patients had significantly higher levels of Catalase (CAT) (p=0.005) and FGB (p=0.007), Protein disulfide isomerase (P4HB) (p=0.031), Pigment Epithelium derived factor (SERPINF1) (p=0.023) and Osteoprotegerin (TNFRSF11B) (p=0.014), and significantly lower expression of Antithrombin-III (SERPINC1) (p=0.002) (Figure 1A, Table 2).

Furthermore, compared to those without MC; MC patients showed significantly higher expression of Clusterin (CLU) (p=0.019) and Lumican (LUM) (p=0.029), and significantly lower expression of Complement factor B (CFB) (p=0.022), (P4HB) (p=0.029), Periostin (POSTN) (p=0.012) and Alpha-1-antitrypsin (SERPINA1) (p=0.047) (Figure 1B). At last, SERPINF1 (p=0.029), CAT (p=0.035) and FGB (p<0.001), which were all upregulated in EPA patients, were downregulated in MC patients (Figure 1B, Table 2).

Interaction analysis

Out of the 6 proteins that were significantly up/downregulated in EPA: CAT, P4HB, FGB and SERPINC1 revealed an interaction. The reactome pathway analysis revealed that this corresponded to an upregulation in: fibrin clot formation and detoxification of reactive oxygen species (ROS). SERPINF1 and TNFR11B did not show direct interactions with any of the other 5 proteins (Figure 2A).

Out of the 7 proteins significantly up- or downregulated in patients with MC, 2 interaction cascades were found. One cascade including CAT, P4HB, SERPINA1, FGB, CFB, and CLU, which were involved in several pathways defined by Reactome.org. Based on whether these proteins were up or downregulated in patients with MC, it could be concluded that the pathways involving Complement/coagulation cascade, detoxification of ROS, and the immune system functions were all downregulated in MC patients. The other interaction cascade, which included POSTN, LUM and SERPINF1, interacted due to often reported co-expression but were not involved in the same pathway (Figure 2B). An overview of the relevant pathways and their involved proteins can be found in Table 3.

Discussion

This study explored the influence of EPA type herniation and MC on the inflammatory signature of the disc. The most important findings of this study are the different effects that the two endplate pathologies have on the inflammation profile. EPA patients showed an upregulation coagulation and detoxification of ROS compared to AFT. By contrast, the detoxification of ROS, complement system and immune system were all downregulated in MC compared to patients without MC.

Coagulation

The increase of coagulation in EPA as compared to AFT was illustrated by an increase in FGB, which is one of the fibril components necessary for clot formation [17], and a decrease in SERPINC1, a protein that inhibits thrombin activity [18]. The increase in this pathway compared to AFT could be very well explained by the endplate being heavily vascularised, which requires increased coagulation to heal the wound after avulsion. In contrast, in the AFT type, no or little blood vessels are ruptured, and thus upregulation of proteins involved in coagulation is less required. Interestingly, the protein alterations in MC patients suggested a downregulation of coagulation, which was illustrated by a decrease in FGB, and SERPINA1, which has some inhibiting effects on thrombin activity [19]. However, this was accompanied by an increase in CLU, a protein excreted by platelets, of which the exact role remains to be elucidated. Therefore, the current evidence seems insufficient to conclude whether coagulation is downregulated in MC.

Detoxification of reactive oxygen species

Moreover, an EPA type herniation was correlated with an upregulation of detoxification of ROS. This upregulation was illustrated by an increase in P4HB, which functions as a chaperone at high concentrations [20], and an increase in CAT, a protein often excreted by macrophages with anti-oxidative and anti-inflammatory effects while preserving phagocytic and digestive capacities [21, 22]. In contrast, when comparing patients with MC to those without MC: CAT and P4HB were down regulated, and consequently detoxification of ROS was also downregulated.

From a clinical perspective, the upregulation of ROS detoxification illustrates an increase of 'beneficial inflammation' in avulsed endplate herniations as compared to the annular tear type. This increase of beneficial inflammation could be explained by the increased exposure to neovascularisation from the highly vascularised endplate [23]. Unfortunately, not all herniated material can be absorbed in equal efficiency. This depends on the amount of cartilage pieces, and the quantity and functionality of the immune cells [9, 22]. Such an inadequately absorbed herniation may stimulate nucleus pulpous cells to induce a chronic inflammation process [24, 25], characterized by an increase in pro-inflammatory cytokines, reactive oxygen and fibrotic changes [6, 26]. This chronic inflammation process can be identified on the MRI as MC [6], and can explain the reduced recovery rate.

Immune system

In line with the immune-modulating effects of CAT, SERPINA1 is also known for its immune-modulating capacity. The expression of this protein is increased by immune cells during an inflammatory response to balance the pro-inflammatory cytokines and oxidative stress [27]. In addition, it has been shown to switch the type of microglia activity away from oxidative stress and pro inflammatory cytokines towards tissue remodelling and phagocytosis [28, 29]. The lower expression of SEPRINA1 in MC patients compared to those without MC thus suggest an alteration in the type of infiltrating immune cells.

Also in line with the altered immune cell infiltration in MC patients, is the downregulation of the complement system in MC, illustrated by a significant decrease in CFB, and an almost significant decrease in C3. This was accompanied by an increase in CLU, which is an inhibitor of the complex system cascade [30]. Moreover, together with the decrease in the detoxification of ROS, a downregulation of immune response may together indicate a malfunctioning immune response. This may create opportunities for subclinical infections with anaerobic bacteria, which is in line with the emerging evidence that MC is associated with bacterial infections [31, 32].

Tissue resorption

In addition to the inflammatory pathways, the alterations in protein expression also illustrated differences in tissue resorption. In MC patients, SERPINF1 was downregulated, illustrating a deficiency in cartilage clearance, which is, again, in line with the reduced recovery rate associated with MC [8]. This cartilage clearance was also confirmed by the decrease in MC of POSTN, a protein participating in post-injury tissue regeneration processes, during which, it stimulated degradation of ECM through upregulation of matrix metalloproteases [33]. LUM belongs to the family of small-leucine rich proteins, which could get accumulated as a part of healing response as its increased expression has been documented in fibrotic lesions previously secondary to stimulation from inflammatory molecules such as TNF-A. Lumican has also been shown to modulate host response and play an important role activation of an innate immune mechanisms in response to bacterial lipopolysaccharides (LPS) and other pathogen associated molecular patterns [34]. Further LUM has been documented to have an important role in inflammatory bowel diseases such as colitis and is believed to promote intestinal homeostasis by aiding innate immune and inflammatory responses [35]. The accumulation of LUM in MC in this study adds evidence to a pro-inflammatory status in these discs which get activated probably due to infective aetiology.

As this was the first study to compare protein expression between AFT and EPA, no comparisons with previous literature could be made. Regarding MC, even though our study found a great variety of proteins involved in inflammation, none of the proteins reported by Dudli et al. (2017) were found in our analysis [6]. This can be explained by proteomics being less sensitive than a gene expression method, which prevents it from detecting proteins that are expressed in low quantities reported in previous studies [6, 7, 36, 37]. Nevertheless, similar to our results, Dudli et al (2017) also showed that only a limited amount of proteins were altered, thereby indicating that the differences are rather subtle [6]. Another limitation of this study is the limited sample size and absence of correction for multiple testing. These results should therefore be interpreted as high-grade evidence, but instead as a starting point for more extensive research on the newly identified proteins and pathways outlined in this paper.

In summary, the proteomic inflammatory signature of AFT and EPA patients differed significantly, with EPA illustrating an increase in a beneficial inflammatory response. With regard to MC, those with MC showed a shift away from beneficial and likely towards detrimental inflammatory response. Taken together, the evidence presented in this paper portrays that endplate pathology does not necessarily lead to reduced recovery, but that the presence of MC illustrates a shift in the inflammatory proteome that makes spontaneous resorption less likely. Future studies should focus on validating these findings in a large study cohort, and preferably integrate a cytokine essay and immune cell staining analysis.

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Appendix

Table 1. Patients characteristics

Case ID	Age	Sex	Pfirmann	MC	AFT/EPA
1	29	F	3	no	AFT
3	70	F	5	no	AFT
5	45	M	3	no	AFT
6	67	F	3	no	AFT
7	27	M	3	no	EPA
8	26	M	4	no	EPA
9	40	M	3	no	EPA
10	28	M	4	no	EPA
11	29	F	4	type 1	EPA
12	34	M	4	type 2	AFT
13	26	F	4	type 2	AFT
14	32	M	4	type 2	EPA
15	43	F	4	type 2	EPA
16	31	M	3	type 2	EPA
17	32	F	2	type 2	EPA

Patient characteristics at baseline, age, sex, Disc degeneration (Pfirrmann grade), AFT/EPA- and MC status are displayed for all patients.

Table 2. Effect of AFT/EPA and MC status on protein expression

Protein Gene symbol	N (AFT/EPA)	Beta (P-value) AFT vs EPA	N (no MC/MC)	Beta (P-value) no MC vs MC
A1BG	5/5	-0.021 ±0.171 (0.907)	6/4	-0.216 ±0.175(0.258)
A2M	6/9	0.119 ±0.296 (0.696)	8/7	-0.366 ±0.291 (0.232)
ACTB	2/7	-0.077 ±0.221 (0.739)	5/4	-216 ±0.185 (0.285)
AGT	3/8	0.094 ±0.197 (0.647)	6/5	-0.214 ±0.176(0.258)
ANXA2	6/8	0.089 ±0.157 (0.584)	8/6	-0.306 ±0.157 (0.077)
APOA1	5/7	-0.184 ±0.209 (0.401)	6/6	0.373 ±0.206 (0.104)
C3	6/9	-0.077 ±0.131 (0.565)	8/7	-0.269 ±0.129 (0.058)
C5	4/5	-0.427 ±0.193 (0.077)	6/3	0.164 ±0.215(0.480)
CA1	6/6	-0.081 ±0.146 (0.591)	6/6	0.269 ±0.144 (0.094)
CA2	6/2	0.236 ±0.292 (0.456)	2/6	0.064 ±0.337 (0.856)
CAT	5/7	0.393 ±0.105 (0.005)**	6/6	-0.257 ±0.103 (0.035)*
CFB	6/7	0.235 ±0.156 (0.163)	8/5	-0.434 ±0.160 (0.022)*
CLU	6/9	0.031 ±0.067 (0.649)	8/7	0.177 ±0.066 (0.019)*
COL2A1	6/8	0.124 ±0.296 (0.684)	7/7	0.038 ±0.289(0.897)
FGB	5/6	0.385 ±0.097 (0.007)**	5/6	-0.72 ±0.097 (<0.001)**
FGG	6/8	0.163 ±0.211 (0.455)	8/6	-.347 ±0.211 (0.128)
FN1	6/9	0.144 ±0.093 (0.146)	8/7	-0.084 ±0.091 (0.373)
GAPDH	6/9	0.048 ±0.129 (0.717)	8/7	-0.100 ±0.126 (0.446)
GSN	6/9	0.065 ±0.194 (0.742)	8/7	-0.292 ±0.190 (0.151)
HBB	6/9	-0.016 ±0.132 (0.907)	8/7	0.098 ±0.130 (0.467)
HP	6/9	-0.058 ±0.169 (0.736)	8/7	-0.82 ±0.629 (0.629)
HPX	6/8	-0.038 ± 0.150 (0.807)	8/6	-0.120 ±0.150 (0.441)
HSPG2	5/3	0.051 ±0.322 (0.880)	4/4	0.087 ±0.312 (0.792)
HTRA1	6/9	-0.186 ±0.151 (0.243)	8/7	0.178 ±0.148 (0.253)
KRT1	6/9	0.167 ±0.105 (0.138)	8/7	-0.077 ±0.103 (0.469)
KRT16	3/7	0.19 ±0.151 (0.249)	4/6	-0.101 ±0.141 (0.498)
KRT6A	2/6	0.162 ±0.168 (0.381)	4/4	0.025 ±.146 (0.868)
LUM	6/9	0.061 ±0.075 (0.431)	8/7	0.183 ±0.074 (0.029)*
LYZ	6/5	-0.207 ±0.156 (0.220)	5/6	0.031 ±0.156 (0.848)
P4HB	5/5	0.507 ±0.189 (0.031)*	6/4	-0.439 ±0.193 (0.038)*
PKM	4/4	0.098 ±0.268 (0.730)	3/5	-0.571 ±0.277 (0.094)
POSTN	6/6	-0.291 ±0.205 (0.120)	7/5	-0.650 ±0.208 (0.012)*
PRG4	4/9	-0.286 ±212(0.207)	7/6	0.138 ±0.197 (0.500)
SERPINA1	6/9	-0.014 ±0.088 (0.875)	8/7	-0.192 ±0.087 (0.047)*
SERPINC1	5/6	-0.629 ±0.135 (0.002)**	6/5	0.105 ±0.135 (0.460)
SERPINF1	6/8	0.527 ±0.200 (0.023)*	7/7	-0.499 ±0.198 (0.029)*
SERPING1	6/8	0.035 ±0.159(0.831)	8/6	-0.274 ±0.159 (0.112)
THBS1	5/7	-0.081 ±0.225 (0.726)	7/5	-0.118 ±0.225(0.614)

Protein Gene symbol	N (AFT/EPA)	Beta (P-value) AFT vs EPA	N (no MC/MC)	Beta (P-value) no MC vs MC
TNFRSF11B	5/4	0.289 ±0.084 (0.014)*	5/4	-0.044 ±0.084 (0.620)
VIM	5/5	-0.117 ±0.256 (0.660)	6/4	-0.446 ±0.261 (0.117)
VTN	3/9	-0.215 ±0.260 (0.430)	6/6	-0.056 ±0.225 (0.810)

Table 2 displays the results of the multiple regression analysis in which EPA and MC status were used as predictor for the listed protein expression. Beta ±SE and p-values are given, * indicates P<0.05, ** indicates P<0.01.

Table 3. Up/down regulation of pathways in EPA vs AFT and MC vs no MC

A			
EPA pathways interaction analysis	**P-value**	**Matching proteins**	**Change in EPA**
Common Pathway of Fibrin Clot Formation	0.0012	FGB ,SERPINC1	upregulated
Detoxification of Reactive Oxygen Species	0.0015	CAT,P4HB	upregulated
Immune System	0.0114	CAT,FGB,P4HB,TNFRSF11B	inconclusive
B			
MC pathways interaction analysis	**P-value**	**Matching proteins**	**Change in MC**
Platelet degranulation	0.0018	CLU, FGB, SERPINA1	downregulated
Detoxification of Reactive Oxygen Species	0.0020	CAT, P4HB	downregulated
Regulation of Complement cascade	0.0025	CFB, CLU	downregulated
Immune System	0.0018	CAT, CFB, CLU, FGB, P4HB, SERPINA1	downregulated

Table 3 shows the reactome pathways in which the significant up or downregulated proteins are involved. **3A** shows the pathway results of the 6 proteins that were altered in EPA, **3B** shows the pathway results of the 9 proteins that were altered in MC. The first column describes the name of the pathway, the second the p-value of the enrichment of the pathway provided by reactome, the third column lists the proteins that were picked up in the respective pathway, and the last column shows whether the pathway is up or downregulated in EPA(3A) / MC(3B). The up or down regulation of the pathway was based on the up/downregulation of the involved proteins combined with their specific role in the pathway (stimulating or inhibiting the pathway. Change in a pathway is scored inconclusive when both stimulatory- and inhibitory proteins are upregulated, and thus no clear up or downregulation could be identified.

Figure 1. Significantly altered protein expression

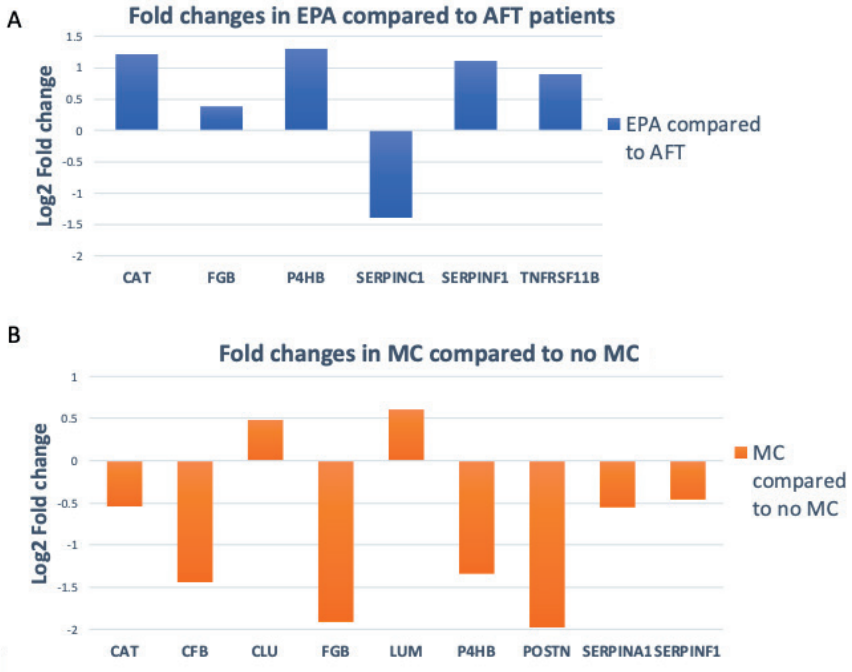

Figure 1 illustrates the significantly different expressed proteins between AFT vs EPA and no MC vs MC, as assessed by a multiple regression with EPA and MC status as predictor. Figure **1A** shows the 7 proteins that differed significantly between AFT and EPA. AFT is used as baseline to illustrate the Log2 fold changes in EPA as compared to AFT, all proteins symbols are shown on the X axis, Log2 fold changes in protein expression (nPSM) are shown on the Y axis. Figure **1B** displays the 9 proteins significantly altered in MC compared to no MC. No MC is used as baseline to show the Log2 fold changes in MC compared to no MC. all proteins symbols are shown on the X axis, Log2 fold changes in protein expression (nPSM) are shown on the Y axis.

Figure 2. Protein-protein interaction analysis by STRING database

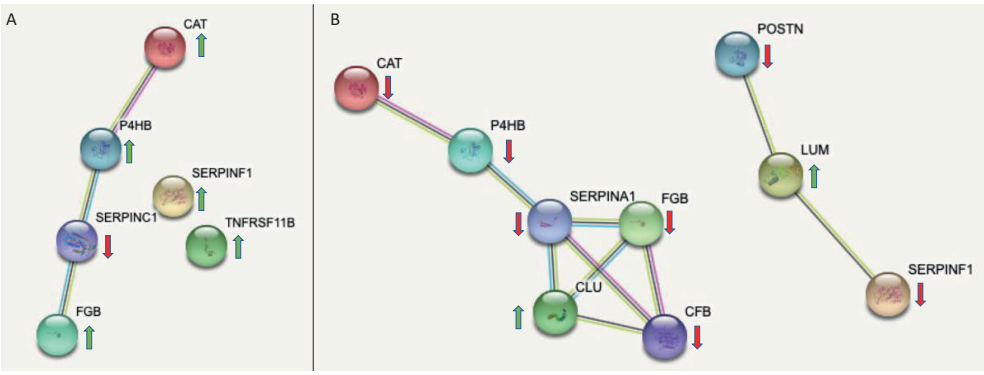

Figure 2 illustrates the protein-protein interactions revealed by STRING database between AFT vs EPA and no MC vs MC, as assessed by a multiple regression with EPA and MC status as predictor Figure **2A** shows the interactions between the proteins that were significantly up or down regulated in EPA compared to AFT. Lines between the proteins illustrate a direct functional interaction between the two proteins, with either a stimulating of inhibiting effect, which connects 4 out the 6 proteins in an interaction cascade. Arrows indicate up or downregulation in EPA compared to AFT. **2B** shows the interactions between the proteins that were significantly up or down regulated in patients with MC compared to those without. Lines between the proteins illustrate a direct functional interaction between the two proteins, with either a stimulating of inhibiting effect, which results in 2 interaction cascades, one with 6 and one with 3 out the 9 proteins. Arrows indicate up or downregulation in MC compared to no MC.

Supplementary Appendix

Table S1. Identification of Inflammation related pathways in the total NP proteome

Pathway	P-value	Proteins identified
leukocyte mediated immunity	2,76E-30	57
immune effector process	1,46E-26	39
immune response	5,48E-26	79
neutrophil degranulation	1,86E-22	52
myeloid leukocyte activation	4,99E-21	71
myeloid leukocyte activation	4,99E-21	56
leukocyte activation involved in immune response	1,97E-20	8
regulation of complement activation	2,85E-20	23
complement activation	1,73E-19	22
regulation of humoral immune response	3,96E-19	24
regulation of acute inflammatory response	6,42E-18	25
complement activation, classical pathway	7,18E-17	18
regulation of inflammatory response	1,13E-13	35
regulation of immune response	4,38E-13	55
activation of immune response	1,12E-12	36
positive regulation of immune system process	2,32E-11	52
regulation of immune effector process	3,04E-10	31
complement activation, alternative pathway	2,97E-09	9
innate immune response	9,44E-09	31
acute inflammatory response	1,23E-07	13
inflammatory response	1,31E-07	101
antimicrobial humoral response	1,36E-07	17
adaptive immune response	7,99E-07	22
interleukin-12-mediated signaling pathway	8,37E-05	40
toll-like receptor signaling pathway	0,00013	10
positive regulation of apoptotic cell clearance	0,00035	4
regulation of transforming growth factor beta receptor signaling pathway	0,00043	10
antimicrobial humoral immune response mediated by antimicrobial peptide	0,00055	10
negative regulation of immune system process	0,0088	18
positive regulation of cytokine production	0,0089	17
positive regulation of NF-kappaB transcription factor activity	0,0114	9

Table S1 lists all the pathways and gene ontology processes from DAVID and STRING database related to inflammation, which were found in the total NP proteome. Proteins involved in these pathways were subsequently included in the study analysis. The name of the pathway is listed in the first column, the second column provides the p-value of the pathway/process enrichment, the 3th column shows the nr. of proteins of that pathway that were present in the study sample.

Table S2. List of protein names and gene their corresponding gene symbols

Gene Symbol	Protein Name
A1BG	Alpha-1B-glycoprotein
A2M	Alpha-2-macroglobulin
ACTB	Actin, cytoplasmic 1
AGT	Angiotensinogen
ANXA2	Annexin A2
APOA1	Apolipoprotein A-I
C3	Complement C3
C5	Complement C5
CA1	Carbonic anhydrase 1
CA2	Carbonic anhydrase 2
CAT	Catalase
CFB	Complement factor B
CLU	Clusterin
COL2A1	Collagen alpha-1(II) chain
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
FN1	Fibronectin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSN	Gelsolin
HBB	Hemoglobin subunit beta
HP	Haptoglobin
HPX	Hemopexin
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein
HTRA1	Serine protease HTRA1
KRT1	Keratin, type II cytoskeletal 1
KRT16	Keratin, type I cytoskeletal 16
KRT6A	Keratin, type II cytoskeletal 6A
LUM	Lumican
LYZ	Lysozyme C
P4HB	Protein disulfide-isomerase
PKM	Pyruvate kinase
POSTN	Periostin
PRG4	Proteoglycan 4
SERPINA1	Alpha-1-antitrypsin
SERPINC1	Antithrombin-III
SERPINF1	Pigment epithelium-derived factor
SERPING1	Plasma protease C1 inhibitor
THBS1	Thrombospondin-1
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B; Osteoprotegerin
VIM	Vimentin
VTN	Vitronectin

Table S2 lists the proteins from the inflammation related pathways that were eligible for statistical analysis. Column 1 displays the gene symbols of the proteins, the protein names are listed in column 2.

Chapter 7

Exploring macrophage differentiation in herniated disc tissue in patients with radiculopathy; is this associated with Modic changes?

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Submitted

"Failure is an option here. If things are not failing, you are not innovating enough." (Elon Musk)

Abstract

Background & Objective

Cervical- and lumbosacral radiculopathy symptoms due to disc herniation are likely to be influenced by degenerative endplate changes and macrophage infiltration of the herniated disc. The present study was conducted to assess the efficacy of immunohistological methods to discern pro-inflammatory M1- and anti-inflammatory M2 macrophage differentiation patterns in herniated intervertebral disc tissue and to evaluate their associations with Modic changes (MC) of the vertebral endplates.

Methods

Herniated disc samples were collected from 38 patients undergoing surgery for cervical- or lumbosacral radiculopathy. Samples were processed for immunohistochemistry and stained for the presence macrophages: CD68 (macrophage marker), CD163 (M2), CD40 (M1), Arg1 (M2) and iNos (M1). In order to assess whether other immune cells also play a pivotal role, T-cells (CD3) and neutrophil (CD15) expressions were studied additionally.

Results

CD68 positive cells were present with a density of 10-100/cm2 in 10 samples and of >100/cm2 in 18 samples. In 12 of these samples 10-50% of the CD68+ cells were M2-type (CD163+) and in 6 of these samples this was even more than 50%. Whereas in 7 of the CD68+ samples, 10-50% of the CD68+ cells were M1-type (iNOS+), and in none of the samples more than 50%. Arg1 and CD40 were expressed in minimal quantities. Expression levels for CD68 were slightly higher in lumbar than cervical patients (OR=2.7, p=0.18). Presence of Modic changes was associated with higher levels of CD68+ cells (OR=6.0, p=0.023) and with lower relative expression of CD163 (OR=0.123, p=0.02). T-cells (CD3) and neutrophils (CD15) were present in a limited number of samples.

Discussion

The relative high expression of CD163 (M2 marker) indicates predominance of an anti-inflammatory over a pro-inflammatory macrophage presence in symptomatic disc herniations, both at lumbar and cervical level. The association of M2 marker positive cells with absence of Modic changes implies possibilities for prediction of rate of recovery in radiculopathy patients. However, the opposite, an association between presence of Modic changes and pro-inflammatory macrophages could not be established.

Introduction

Herniation of the intervertebral disc is common phenomenon that causes a major burden for society worldwide. With an incidence of 1-3% it occurs most often in lumbar discs [1], followed by cervical discs with an incidence of 0,018% [2]. Lumbar patients suffer from disabling leg pain that radiates down the dermatome, whereas for cervical patients this radiating pain is localized in the arm. These symptoms seem to be caused by a multiple factors and vary tremendously in intensity and duration between patients. Recent evidence has indicated that an important factor that may influence radicular symptoms is infiltration of the herniated disc with inflammatory cells [3, 4], which are mostly macrophages [5, 6].

Macrophages have shown to considerably aid disc resorption through phagocytosis of herniated tissue [7], thereby increasing the rate of recovery. In contrast, they may also excrete pro-inflammatory cytokines [8], which may sensitize the nerve root and have been associated with a decreased rate of recovery [9]. The discrepancy in these effects can potentially be explained by the various differentiation profiles of macrophages. Each set of environmental cues will lead to distinct macrophage phenotypes, which show unique behaviors and expression profiles [10]. Even though each differentiation profile produces a unique phenotype, they can be polarized to pro-inflammatory(M1) or anti-inflammatory (M2) macrophages[11]. M2 macrophages express markers such as CD163, and may be responsible for the abovementioned beneficial effects on recovery through expression of anti-inflammatory factors like IL-4 or arginase-1 [11-13] and phagocytosis factors such as IL-10 [13]. By contrast, M1 macrophages express markers such as CD40 and may exacerbate pain symptoms through expression of pro-inflammatory cytokines such as, IL-1β, IL-6 and TNF- α [9, 11, 13].

Since not all herniations are of the same etiology, it is possible that macrophage differentiation may vary depending on the characteristics of the disc lesion and condition of the vertebral end plates. Vertebral endplate changes are visible on MRI (Modic changes; MC) and are considered to be a sign of inflammatory fibrosis and/or edema [14], possibly induced by decreased vascularization. In a previous study, we demonstrated that in patients without MC, macrophage infiltration was associated with faster recovery after surgery, whereas in patients with MC, macrophage infiltration was associated with a decreased recovery rate after surgery [4]. Based on these findings, a higher percentage of M2 macrophages is expected in patients without MC, whereas in those with MC, M1 macrophages are likely to be present more abundantly. In this study we aim to verify this by characterizing the macrophages present in symptomatic herniated discs of patients that were subjected to surgery for radiculopathy. Additionally, In order to explore whether other immune cells may also play a pivotal role in radiculopathy, T-cells and neutrophils will also be analyzed.

Another factor that could be of influence is the location of the herniation: cervical herniated discs have been proposed to contain a lower degree of neovascularization after herniation compared to lumbar ones [15], which likely results in a lower degree of macrophage infiltration in cervical as compared to lumbar patients. Moreover, In cervical spine herniated disc pathology, MC are observed less frequently [16]. If true, this would imply predominance of M2 macrophages in cervical herniated discs.

A better understanding of macrophage differentiation in the herniated disc will help to understand its role in recovery, and may open new doors for treatment possibilities. Inducing a shift towards M2 macrophage presence in radiculopathy patients could fasten the resorption process of herniated discs and alleviate radiculopathy symptoms. Additionally, the presence of MC may be indicative of the dominant type of macrophage present and predict the natural course of clinical symptoms. Therefore, the aim of this study is to immunohistochemically explore macrophage differentiation in both cervical and lumbar disc herniations and investigate the association with Modic changes.

Methods

Study population

Discectomy patients were included if they suffered radicular symptoms due to an intervertebral disc herniation, verified by MRI, for 8 or more weeks. In cervical radiculopathy patients, an anterior approach was performed for discectomy and the bulk of the disc was removed. In lumbar radiculopathy patients, a posterior approach was performed using a standard unilateral transflaval approach. The herniated part and some of the intervertebral part of the disc was collected. Study was approved by the medical ethics committee.

Sample processing

All harvested discs were fixed in a 4% formaldehyde solution for 3-7 days. Tissue was subsequently embedded in paraffin blocks and a 5-µm thick slices were taken from the middle of the block for hematoxylin staining, which was performed according to the Leica ST 5020-mulitstainer standard protocol. Samples were evaluated under the microscope for clear signs of inflammatory cells. If tissue from one sample exceeded the capacity of 1 paraffin block, multiple blocks were formed and a slide of each block was evaluated.

Immunohistochemistry

From each disc containing inflammatory cells, the slide with the most inflammatory cells was submitted to further analysis using immunohistochemistry: Macrophages were characterized using CD68, M1 macrophages were identified using CD40 and iNOS, M2 macrophages were identified using CD163 and Arginase 1(ARG1), T-cells were identified using CD3 and neutrophils using CD15. 5-µm paraffin slices were rinsed in ethanol and methanol solutions and prepared for the expression of CD68 (DAKO, Denmark), CD40 (Sanbio, Netherlands), iNOS (Spring bioscience, USA) CD163 (Abcam, Netherlands), ARG1 (Spring bioscience, USA), CD3 (DAKO, Denmark). Immunohistochemistry was performed using a three-step indirect method. Antibodies CD68, Arg1, iNOS and CD3 were cooked in Citrate pH 6.0 buffer, CD163 and CD15 in EDTA pH 8,5 and CD40 in pronase as a pre-treatment. Subsequently, an avidin-biotin complex technique was performed with the Vectastain ABC-Elite Kit (Vector Lab. USA) and the appropriate biotinylated antibodies. Visualization of the peroxidase reaction was done with DAB solution (Sigma). Samples were counterstained with Harris hematoxylin. All samples were accompanied by a positive control, which was atherosclerosis tissue for all macrophage markers, and tonsil tissue for T-cells and B-cells. In order to standardize the evaluation of the samples, all samples were photographed under a light microscope before they were evaluated, using Philips ultra fast scanner. Since previous studies have reported the expression of CD68, CD40 and iNOS by nucleus pulposus cells and chondrocytes [17-19], cells were analyzed based on morphological features and only macrophages were photographed and evaluated. The same approach was used for CD163 and Arg1. For CD3 and CD15 morphological features of T-cells and neutrophils respectively were taken into account.

MRI

For the evaluation of Modic Changes, a 3T MRI was used. Both sagittal T1- and T2-weighted images of the lumbar or cervical spine were obtained. Image evaluation of MC was according to the criteria of Modic et al [20, 21]. Image evaluation was done by two independent researchers in a blinded manner (ND & CVL). Inter agreement analysis was performed and kappa values were calculated. Upon disagreement, a third observer was consulted (GL).

Data analysis

Cell counts were performed using ImageJ and evaluation was executed by two independent researchers (ND & GL). Inter-observer correlation coefficients were calculated for each staining separately. If an intercorrelation of >0.8 was found, a third observer was consulted (SVD). For each antibody 50 pictures were evaluated by hand in the above-mentioned manner. Subsequently an automated cell count algorithm in ImageJ was matched on the average count of the two observers with a correlation coefficient of >0.8. This algorithm was used to evaluate the remaining bulk of pictures. Positive Macrophage / lymphocyte counts were divided by the surface of the herniated disc in cm^2. First the total amount of macrophages was counted using CD68, subsequently, M2 dominance was determined as the fraction of positive CD163 or ARG1 macrophages out of the CD68 positive macrophages. M1 dominance was determined as fraction of positive CD40 or iNOS macrophages out of the CD68 positive macrophages.

For statistical analysis CD68, CD3 and CD15 positive cells were each categorized as: No infiltration: (<10 cells/$cm^{2)}$), moderate infiltration (10-100 cells/cm^2) and considerable infiltration (>100 cells/cm^2). For all analyses that focus on the quantification of differences in M1 and M2 macrophage marker expression (CD163 Arg1, CD40, iNOS), only samples of patients with at least moderate inflammation were used (>10 CD68+ cells/cm^2) and the positive marker expression was presented as a percentage of the CD68+ cells present in that sample. These were subsequently categorized as: low expression (<10% of CD68+), medium expression (10-50% of CD68+) and high expression (>50% of CD68+). The effect of Modic changes and location of herniation (cervical/lumbar) on the absolute and relative expression categories were tested in ordinal logistic regressions, for which assumptions were met. In this model, type of herniation (bulging/extrusion/sequester) was included as a covariate, since this is known to influence macrophage infiltration. At last, the correlations between duration of symptoms and expression of inflammatory markers were evaluated using Spearman's correlation. For all analysis, alpha was set at 5%.

Results

Study population

Herniated disc samples were retrieved from 38 patients that consecutively underwent discectomy for radiculopathy in 2018 and fulfilled the inclusion criteria. Twenty two patients underwent lumbar discectomy for sciatica, and 16 patients underwent anterior discectomy for a cervical disc herniation with radiculopathy. No statistically significant difference was found in age, gender and symptom duration between lumbar and cervical patients, nor between patients with and without MC (Table 1).

Histopathology

The tissue that was removed, embedded and stained mostly consisted of nucleus pulposus (NP) material with varying degrees of Annulus fibrosus (AF) and cartilage endplate (EP) present. 29/38 samples (76%) showed inflammatory cells; which were localized in the edges of NP tissue (Figure 1a). Only CD163 showed to be specific for macrophages. CD68, CD40, iNOS and Arg1 also stained nucleus pulposus cells and chondrocytes (Figure 1b). Most of the immune cells stained positive for macrophage marker CD68. A limited number of immune cells stained positive for the T-cell marker CD3 and neutrophil marker CD15. CD3 and CD15 exclusively stained the T-cells and neutrophils respectively. CD68, CD163, CD40 and CD3 stained cells with high intensity, whereas iNOS and Arg1 staining was of lower intensity and both intra and extracellular (Figure 1c-h).

Inter observer agreement and algorithm efficacy

The number of pictures taken from each sample varied from 0 (if no inflammatory cells were present) till >200. For all macrophage markers, an inter observer correlation coefficient of >0.8 was found between the two observers for 50 randomly chosen pictures from different samples. For CD68, CD163 and CD40, a correlation coefficient of >0.8 was also achieved between the average count of the observers and ImageJ automated cell count. Hence the automated cell count was used for the remaining pictures. For iNOS and Arg1 however, the

required correlation coefficient could not be achieved due to the low staining intensity and extracellular staining. The extracellular staining was namely often (falsely) counted as 'positive' by the algorithm; hence all pictures were evaluated manually. Regarding CD3 and CD15, the total number of pictures with positive cells was <50, hence no automated cell count could be validated and cell counts were performed manually (Table S1). For all manual counts, average counts of the observers were used for data analysis.

Quantification of inflammation markers

Expression levels of CD68 varied widely between samples (median=48,3/cm^2).

Of the 38 samples, 10 showed mild (<10/cm^2), 10 moderate (10-100/cm^2), and 18 considerable infiltration (>100/cm^2). The distribution of M1/M2 markers (as% of CD68+) is as follows: highest levels were seen in CD163 (M2 marker): 11 showed low (<10%), 12 showed medium (10-50%) and 6 showed high expression (>50%). Whereas for Arg1 (M2 marker) 27 samples showed mild, 1 medium and 1 high expression. CD40 (M1 marker) also showed limited expression with 24 samples in the mild, 4 in the medium and 1 in the high expression group. Comparable results were seen for iNOS (M1 marker): 22 samples were scored as mild, 7 as medium and 0 as high expression (Table 2).

CD3 and CD15 were mostly expressed in limited quantities: CD3 median<1/cm^2 (32 mild, 6 moderate), CD15 median=1/cm^2 (25 mild, 10 moderate and 3 considerable). Further, high expression of CD68 correlated with higher levels of CD3 (p<0,001) and CD15 (p<0,001) (Table 2).

Inter observer agreement MRI

For the presence of MC, inter observer agreement was moderate with an agreement percentage of 78% (Cohens Kappa = 0,58). When results were separated for location of herniation, a strong inter observer agreement was seen in cervical patients with an agreement percentage of 94% (Cohens Kappa = 0,82), whereas in lumbar patients this was 71% (Cohens Kappa = 0,42).

Association of macrophage type and Modic changes

Cervical patients showed a median expression/cm^2 of 16 in patients without MC (MC-) and 40 in patients with MC (MC+). This was lower than in lumbar patients, where MC- patients showed a median cell count/cm^2 of 223 and MC+ a median of 231. Regarding the expression of M1 and M2 markers relative to CD68, cervical MC- patients showed the highest levels of CD163 (median of 59%), in cervical MC+ patients this percentage was 21%. In lumbar patients, MC- patients had 26% CD163, whereas in MC+ patients this was 8%. Arg1 and CD40 expression was very low in all subgroups. iNOS expression was also low: 5,6% for MC- patients and 3,7% for MC-. An overview of the medians per subgroup can be found in table 3 and an overview of all expression levels per sample in table S2.

Ordinal logistic regression illustrated that the presence of MC was associated with higher levels of CD68 (OR=6.0, p=0.023) and with lower relative levels of M2 marker CD163 (OR=0.123, p=0.02) compared to patients without MC. No significant differences between cervical and lumbar samples were seen with regard to expression levels of CD68, or the relative expression levels of CD163, Arg1, CD40 or iNOS (Table 4). No other significant results were seen.

T-cell count (CD3) was <1 in all subgroups. The median cell count/cm^2 of CD15+ neutrophils was 3.45 in MC- lumbar patients and 2.95 in MC+ lumbar patients, whereas it was <1 in MC- cervical patients and 2.15 in MC+ cervical patients. Moreover, the ordinal regression analysis showed no differences for CD3. However, it revealed that lumbar samples had higher levels of CD15 positive neutrophil infiltration as compared to cervical samples (OR=12.6, p=0.038).

Influence of time on macrophage differentiation

The median duration of symptoms was 6.5 months (IQR: 5 – 13). Neither the absolute expression of CD68, CD3 or CD15 nor the relative expression (% of CD68) of inflammatory markers was correlated to the duration of symptoms (Table 5).

Discussion

The present study indicates that M2 macrophages (CD163+) are the dominant type of inflammatory cells in herniated intervertebral disc tissue harvested from cervical and lumbar radiculopathy patients, and are more dominant in MC- as compared to MC+ patients. By contrast, the M1 phenotype (iNos+ and CD40+) only forms a small portion in all inflammatory cells identified, and we were unable to associate this with MC+ patients. Based on our findings, we conclude that CD163 is a suitable marker for M2 macrophages in both lumbar and cervical radiculopathy patients, whereas Arg1 was deemed unsuitable as M2 marker due to its limited expression and extracellular staining. Further, both iNOS and CD40 can be used as M1 markers, but with some limitations: iNOS showed the highest expression levels, but also illustrated extracellular reactivity and limited staining intensity, making it unsuitable for algorithm counting. CD40 was expressed in lower quantities, but staining was intracellular and the intensity high, thereby making it suitable for algorithm counting. Further, Both CD3 and CD15 have shown to be proper markers for T-cells and neutrophils respectively. Nonetheless, as they are only expressed in limited numbers, their relevance in the inflammation response of the herniated disc is questionable.

Our results imply a higher degree of macrophage infiltration in lumbar herniated discs, compared to cervical discs. This difference among the locations are in line with results from Chitkara et al (1991), who reported a lower degree of neovascularization in cervical discs [15], and could be the result of morphological and pathological differences affecting vascular structure and growth, which have yet to be elucidated.

Additionally, the present study showed that inflamed cervical herniated discs contain a larger proportion M2 (CD163+) macrophages and a smaller proportion of M1 (iNOS+) macrophages when compared to lumbar ones, which was in line with our hypothesis. Since M2 macrophages tend to advance recovery by disc resorption through phagocytosis of the herniated tissue [12, 13] and M1 macrophages delay recovery by exacerbating pain symptoms [9], cervical patients might benefit from inflammation more than lumbar patients. This could be the result of structural differences between cervical and lumbar HNPs. That act on phenotypic differentiation of macrophages, but the exact mechanism yet has to be unraveled.

Moreover, present findings demonstrate a higher proportion of M2 (CD163+) macrophages in MC- patients. This is in accordance with previous findings [4], proposing that patients without MC benefit from macrophage infiltration as this was associated with faster recovery after surgery, whereas in MC+ patients, macrophage infiltration was associated with decreased recovery after surgery. Hence we expected MC+ to be associated with an increase in M1 macrophages. The fact that we were not able to find this association could be due to our limited sample size, as both iNOS and CD40 were rather insensitive marker and only few discs were found positive. Alternatively, it could be that the wrong M1 markers were used, and that other M1 markers such as CD64, CD80,CD86 or CD192 would provide better results [11, 13, 22].

Because the proportion of M2 macrophages is higher in patients without MC, It seems that MC status affects the macrophage differentiation process, possibly through the creation of a degenerative and pro inflammatory environment that could induce differentiation towards M1 [10, 14]. Vice versa, decreased M2 differentiation could also resemble a malfunction of the immune system that upon infiltration of the herniated disc material also creates endplate damage and thereby induces MC. Another scenario is that both observations are the result of a poor vascular status, which may have induced the degenerative changes of the endplate and alter immune function [23]. Another explanation, which is in line with a poor vascular status that leads to a degenerated endplate, would be the presence of anaerobic bacteria in the endplate, mostly Propionibacterium Acnes [24]. The presence of such bacteria has been associated with MC+ [24], and may induce a pro-inflammatory environment that could prevent macrophage differentiation towards M2. Unfortunately, these theories are still in concept, and in order to improve our understanding, more experimental research and knowledge of the vascular status of radiculopathy patients are required.

Moreover, the total number of macrophages was significantly higher in MC+ patients, whereas in our previous study we were not able to find this difference [4], and others have even reported lower numbers of macrophages in MC+ patients [1]. Due to this incongruency, we must conclude that more large studies are required in order to draw a conclusion on this association. In the present study, symptom duration did not affect results. In a normal wound healing response, the initial response is dominated by M1 macrophages on day 1[25], and switches to M2 in the following days [26, 27]. As patients in this study had symptoms >2 months, it is assumed that the initial switch from M1 to M2 is finalized, and that in this stage of chronic symptoms, a longer duration does not further influence macrophage differentiation. Nevertheless, even though our findings are in line with the literature, it should be noted that this analysis was conducted with a small number of samples and that data was obtained retrospectively during the intake visit, making it prone for recall bias.

A limitation of this study lies within the methodology of tissue processing and immunohistochemistry. For example, for each antibody a new slide of the same paraffin block was used, and sometimes the total number of macrophages was larger in the M1/2 marker slide compared to the CD68 slide. Because the CD68 slide was used as a reference number to calculate the percentage of M1/M2, the positive fraction of M1/M2 could exceed 100% of CD68+. Moreover, from some patients, more tissue could be collected than from others, resulting in multiple paraffin tissue blocks, of which only the one with the most inflammation was submitted for evaluation. This may have resulted in an overestimation of the number of inflammatory cells in patients with large amounts of tissue. Nonetheless, during surgery, not only the herniated tissue, but also some intervertebral disc tissue without any inflammatory cells is removed. Thus the block with the most inflammatory cells logically resembles the sample with the most herniated tissue, thereby making it the most representable sample. Another limitation of this study is caused by the absence of correction by multiple testing, which we deemed unsuitable for the exploratory nature of this study.

Conclusion

M2 (CD163+) macrophages are abundantly present in intervertebral disc tissue that is herniating, compressing the nerve and associated with radiculopathy both at lumbar and cervical level. Moreover, M2 (CD163+) macrophages are more abundant in MC- patients which supports previous data and suggests patients without MC with macrophage infiltration have a quicker recovery rate after surgery. In order to further explore the role of inflammation and MC in recovery of surgical patients with lumbar and cervical radiculopathy, a large prospective trial with elaborate clinical follow-up is required.

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Appendix

Figure 1. Examples of immunohistochemistry staining results

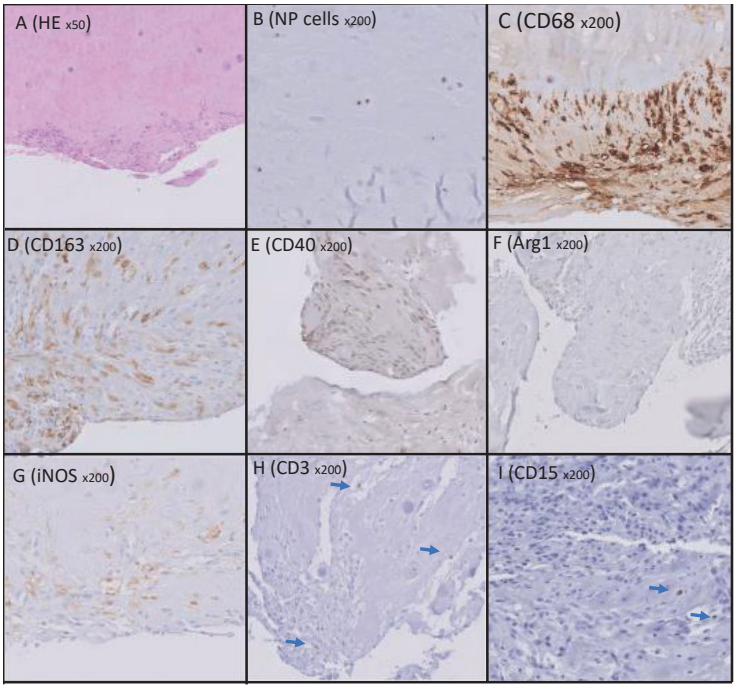

Figure 1 displays examples of the different antibody staining results. **1A** shows a HE coupe with infiltrating inflammatory cells in NP tissue. **1B** displays positively stained NP cells with CD68. **1C** shows infiltrating macrophages, intracellularly stained with CD68. **1D** shows infiltrating macrophages of which a large percentage is intracellularly stained with CD163. **1E** reveals infiltrating macrophages, some of which are stained intracellular with CD40. **1F** displays infiltrating macrophages of which a few are Arg1 positive, ECM surrounding Arg1 positive cells is also stained positively. **1G** illustrates infiltrating macrophages of which some are iNOS positive, ECM surrounding iNOS positive cells is also stained positively. **1H** reveals intracellular staining of infiltrating t-cells. **1I** shows intracellular staining of infiltrating neutrophils.

Table 1. Baseline characteristics

1A	Cervical	Lumbar	p-value
N	16	22	
Age	55	52	0.54
% male	31%	45%	0.51
Duration of symptoms (months)	7.7	13.2	0.63
% Modic Changes	27%	43%	0.48
1B	**MC**	**No MC**	**p-value**
N	13	21	
Age	49	55	0.45
% male	46%	41%	1.00
Duration of symptoms (months)	10.2	11.7	0,77
% Cervical discs	31%	48%	0.48

1A displays the baseline characteristics for Cervical and Lumbar patients separately. **1B** shows baseline characteristics for patients with and without Modic changes separately.

Table 2. Distribution of samples over the immune cell infiltration categories

A			
Marker	**<10/cm²**	**10-100/cm²**	**>100/cm²**
CD68	10	10	18
CD3	32	6	0
CD15	25	10	3
2B			
Marker as % of CD68	**<10%**	**10-50%**	**>50%**
CD163%	11	12	6
Arg1%	27	1	1
CD40%	24	4	1
iNOS%	22	7	0

Table **2A** displays for each immune cell marker the number of samples in each infiltration category. Table **2B** shows the relative expression of M2 markers (CD163 and Arg1) and M1 markers (CD40 and iNOS) relative to CD68 expression in all samples that showed at least moderate CD68 infiltration (>10 cells/cm²).

Table 3. Overview of inflammatory marker expression in subgroups for location of herniation and MC status

Subgroups	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
Cerv_MC+	40	0	2	21%	0%	0%	0%
Cerv_MC-	16	0	0	59%	0%	2%	0%
Lumb_MC+	231	0	3	8%	2%	0%	4%
Lumb_MC-	223	0	3	26%	0%	0%	6%

The values are medians for CD68, CD3 and CD15, and percentages of CD163, Arg1, CD40 and iNOS are relative expressions compared to CD68+ cells.

Table 4. Results of ordinal logistic regression

	MC		Herniation location	
Marker	**p-value**	**Odds-ratio (95% CI)**	**p-value**	**Odds-ratio (95% CI)**
CD68	0,023*	6,0 (1,276 - 28.49)	0,18	2,7 (0,623 - 12,49)
CD3	0,172	5,0 (0.495 - 50.6)	0,999	1,3E9(0 - > 1E20)
CD15	0,124	4,08 (0,681 - 24,47)	0,038*	12,6 (1,145 - 139.08)
CD163%	0,020*	0,123 (0,021 - 0,721)	0,275	0,383 (0,068 - 2,145)
Arg1%	0,999	1,3E8 (0 - >1E20)	0,999	1,7E8 (0- > 1E20)
CD40%	0,748	0,702 (0,081 - 6,083)	0,509	2,3 (0,190 - 28,49)
iNOS%	0,998	2,1E-9 (0- > 1E20)	0,998	1,1E17 (0- > 1E30)

Table 4 lists the result of an ordinal logistic regression in which location of herniation and MC were used as independent predictors, and the type of hernia(bulging/extrusion/sequester) was taken into account as a covariate. For both MC and location of herniation, estimator p-values and OR are provided. For the OR, 'no MC' and 'cervical samples' were used as reference category). % means relative to CD68 expression.

Table 5. Correlations between duration of symptoms and inflammatory markers

Absolute count	Spearman's rho	p-value
CD68	-0,016	0,924
CD163	-0,069	0,683
Arg1	0,119	0,497
CD40	0,143	0,397
iNOS	-0,124	0,465
CD3	-0,028	0,866
CD15	-0,158	0,343
Relative count		
CD163%	-0,198	0,323
Arg1%	0,042	0,834
CD40%	0,138	0,492
iNOS%	-0,178	0,364

Table 5 displays the correlation coefficients and p-values for the Spearman correlation tests between the duration of symptoms and macrophage, T-cell and neutrophil markers. Row 2-8 display the absolute cell counts whereas row 10-13 display expression as a percentage of CD68 positive cells.

Supplementary appendix

Table S1. Inter observer correlation and algorithm validation for cell counts

Marker	ND count	GL count	R (ND-GL count)	algorithm count	R (algorithm - average ND&GL)
CD68	3287	3563	0,95	3499	0,95
CD163	1716	1722	0,85	1.399	0,86
CD40	606	694	0,95	885	0,83
iNOS	615	678	0,81	634	0,61
Arg1	562	706	0,90	701	0,63
CD3	237	121	0,84	/	/
CD15	707	667	0,87	743	0,92

Table 1 displays the inter-observer correlation between the two independent researchers and the automated cell count from image J. The first column lists the antibody used, the second and third list the total positive cell count from 50 pictures by the two observers. The fourth column lists the correlation coefficient between the counts of the two observers (ND and GL). The fifth column shows the total cell count by the automated algorithm. In the last column, the correlation coefficient is listed for the correlation between the automated cell count and the averaged count of both observers. '/' illustrates that less than 50 pictures were available and no algorithm validation could be performed.

Table S2. Raw data file

CaseID	location	MC	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
1	cervical	No	172	1	1	44%	1%	2%	0%
2	cervical	no	0	0	0				
3	cervical	no	145	0	5	71%	1%	8%	3%
4	cervical	No	0	0	0				
5	cervical	No	0	0	0				
6	cervical	No	324	0	43	101%	0%	13%	27%
7	cervical	no	0	0	0				
8	cervical	No	8	0	0	80%	0%	0%	0%
9	cervical	No	15	0	33	46%	0%	22%	0%
10	cervical	No	168	0	0	11%	0%	3%	2%
11	cervical	No	17	1	29	100%	0%	0%	0%
12	cervical	No	36	0	0	0%	0%	0%	0%
13	cervical	Yes	47	0	0	18%	0%	0%	0%
14	cervical	Yes	404	0	427	46%	0%	6%	0%
15	cervical	Yes	34	0	0	9%	0%	0%	0%
16	cervical	yes	25	0	4	25%	0%	0%	0%
17	lumbar	Missing	9183	0	3	1%	0%	0%	2%
18	lumbar	No	0	0	0				

CaseID	location	MC	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
19	lumbar	No	396	14	15	46%	0%	0%	4%
20	lumbar	no	4708	0	190	5%	4%	0%	1%
21	lumbar	no	35	0	4	464%	0%	0%	20%
22	lumbar	No	1166	0	46	6%	0%	0%	7%
23	lumbar	No	19341	17	61	42%	0%	0%	3%
24	lumbar	no	0	0	0				
25	lumbar	No	1747	60	77	49%	0%	11%	22%
26	lumbar	no	0	0	0				
27	lumbar	No	0	0	0				
28	lumbar	No	50	1	1	10%	0%	0%	44%
29	lumbar	Yes	536	0	0	8%	0%	0%	10%
30	lumbar	Yes	0	0	0				
31	lumbar	Yes	148	0	5	2%	21%	10%	0%
32	lumbar	Yes	14	0	0	0%	100%	0%	37%
33	lumbar	Yes	4868	17	41	25%	4%	2%	4%
34	lumbar	Yes	983	1	1	38%	3%	0%	15%
35	lumbar	Yes	45	19	12	62%	0%	8%	12%
36	lumbar	Yes	225	0	0	9%	0%	0%	0%
37	lumbar	yes	14868	33	1097	4%	1%	0%	2%
38	lumbar	Yes	237	3	19	1%	2%	145%	1%

This table displays the location of the disc sample, MC status and absolute or relative (% of CD6+ cells) expression levels of all immune cell markers for all samples separately. If a percentage exceed 100%, slide with the M1/M2 marker had a higher total number of macrophages than the slide that was stained with CD68.

Chapter 8

Conclusion and discussion

"Blessed are the hearts that can bend; they shall never be broken." (Albert Camus)

This study has investigated, how macrophage infiltration of the herniated disc influences the clinical and radiological outcomes in sciatica. More specifically, the association between alterations in macrophage differentiation and inflammatory protein expression and radiological outcomes was investigated. For this purpose, herniated lumbar disc samples harvested during discectomy surgery were examined. This allowed us to study the grade of inflammation using immunohistochemistry and proteomics and relate these findings to clinical and radiological outcomes, both at baseline and at follow-up. Through evaluation by immunohistochemistry we learned that the extent of macrophage infiltration is related to the type of disc herniation, with extruded disc material containing more cells than bulging disc material. Furthermore, we found that a higher number of macrophages present in the lesioned disc material associates with less disc herniation on MRI one year post surgery, which we interpreted as the macrophages contributing to the resorption process. In terms of clinical outcomes, a remarkable discrepancy in the effect of patients with Modic Changes (MC) and those without MC was demonstrated. In patients with MC, more infiltrated macrophages resulted in a less satisfactory recovery. On the other hand, in patients without MC, more infiltrated macrophages resulted in a more satisfactory recovery.

Furthermore, in an additional immunohistochemistry study, we found that this discrepancy in effect of macrophage infiltration between patients with and without MC seems to be attributed to a difference in macrophage differentiation: patients with MC have a considerably lower fraction of CD163+ positive macrophages, which is a marker for tissue remodeling. In addition, in the proteomic study, examination of protein expression in nucleus pulposus samples revealed that patients with MC showed a decrease in detoxification of reactive oxygen species (ROS) compared to patients without MC. ROS is a marker for inflammation and tissue damage, and a decrease in the detoxification of ROS illustrates that there exists dysfunction of the immune response. This is in line with the immunohistochemistry results that showed a lower percentage of CD163+M2 macrophages in MC+ patients, and with our earlier findings that show a slower recovery of MC patients with inflammatory cells. At last, we found that a large % of the identified macrophages did not stain positively for any of the M1(iNOS/CD40) or M2 (CD163/Arg1) markers. Whether this is due to an absence of differentiation or because the limited number of included markers could not cover all macrophage differentiations, remains a question for further study.

Since only surgical samples were examined to answer the research question, the abovementioned conclusions may be limited in their generalizability. Surgery is only indicated for severe sciatica cases, hence the distribution of inflammation and the importance of its effect on clinical outcomes described in this study may be different from that in a population with mild complaints. These limitations emphasize the importance of careful evaluation of the results. Hence, in the paragraphs below, we will not only discuss the study findings but also advantages and disadvantages of the methodologies used in this study. In addition, the implications and future perspectives of the results of this study will also be discussed.

Macrophage differentiation

M1 and M2 macrophages, an effective dichotomization?

Macrophage differentiation or polarization has recently emerged as a topic of major interest in multiple fields [1]. This complex phenomenon is influenced by numerous factors, and results in a wide variety of macrophage phenotypes with according behaviors and expression profiles including: antimicrobial activity, immune cell activation, tumor resistance, allergy, immune modulation and tissue remodeling [2]. It seems an oversimplification to polarize these phenotypes as M1 (pro-inflammatory/anti-microbial) and M2 (anti-inflammatory / tissue remodeling), yet it is still broadly used in the literature. For this study, the M1/M2 dichotomization was also used with the following justification. Sciatic pain is a complex phenomenon that is influenced by many variables amongst which: the type of disc herniation, the extent and type of macrophage infiltration, the inflammatory status of the endplate, and the severity of the nerve compression.

When it comes to understanding such complex clinical phenomena, this M1/M2 macrophage oversimplification may help to understand the bigger picture of the interactions between these many variables. Once research has provided a more solid basis for the theories on the role of macrophages that we have presented, the nuances in activation profiles become more important. For now, we feel confident to use the M1/M2 dichotomization, as long as we clearly define which expression factors/markers were used to identify M1 and M2 macrophages, in accordance with current expert guidelines [1].

Implications on disc herniations

A disc herniation is a traumatic event, in which other immune stimulators should not play a big role. Hence, at the time of surgery, the expected macrophage differentiation response would be towards M2, and is aimed at phagocytizing the herniated material without further immune recruitment and excessive damage. This effect was demonstrated in chapter 3, in which evidence was provided for disc resorption due to macrophages, accompanied by the observation that macrophage infiltration resulted in earlier recovery (chapter 2 and 4), and by the finding that the majority of macrophages was CD163 positive (M2 marker; chapter 6). In addition, evidence from the literature presented in chapter 2 showed that the cytokines (IL-4 and IL-10), which are expressed by M2 macrophages, also associate with a quicker recovery and relieve of pain symptoms. Collectively, this suggests that for these cases, macrophage infiltration is a beneficial event, and should not be inhibited, but rather stimulated with therapeutic strategies.

Unfortunately, macrophage infiltration is not beneficial in all cases. In chapter 4, it is shown that macrophage infiltration is not beneficial for patients that demonstrate MC in the adjacent vertebra. This is likely caused by an alteration in macrophage differentiation due to an alternative pro inflammatory environment, in which a treatment focusing on inhibition of inflammation may be more beneficial. Taken together, insight in the pathological process in herniated discs could impact the choice of treatment.

Therapeutic implications

Our current treatment approach for sciatica patients is conservative care for at least 8-12 weeks, during which the majority of patients recovers spontaneously [3]. However, in the subgroup of patients with persisting symptoms, surgery can be considered. Identifying this subgroup in an early disease stage, could advance the decision to operate, which would save them from an ineffective prolonged conservative approach. Based on our findings, assessing the inflammation status may provide such knowledge in an early stage. Because a hernia is likely to resolve spontaneously in the case of M2 infiltration, knowing whether M2 macrophages are present in the first weeks after the onset of symptoms could lead to a decision to treat conservatively for a longer period of time. Furthermore, if an absence of M2 macrophage infiltration can be assessed in the first weeks, spontaneous resorption is less likely, thereby making early surgery the preferential option. Saving patients from an ineffective conservative approach would not only reduce the duration of the disease, but also the cost for society since these patients can return to work quickly. When measuring macrophage differentiation in an early phase, it is vital to wait at least 5 days before M2 infiltration is assessed [4], as the inflammation profile on the first day of a normal wound healing response is mainly dominated by M1 macrophages [5]. In a normal wound healing process, M1 macrophages slowly differentiate into M2 during the following days [6]. Whether macrophage differentiation also occurs this quickly in the poorly vascularized intervertebral disc still has to be unraveled.

By contrast, if a M1 dominant infiltration is seen after the first week, neither decompression surgery nor conservative care may be the best option. For these patients, it is important to know if bacteria are present. If so, an antibiotic treatment approach can be considered. By contrast, in those with a M1 dominant infiltrate without a bacterial infection, the problem is more likely to be of degenerative nature, and arthrodesis or perhaps future regenerative disc therapies may be the preferable approach.

Alternatively, it could be that M1 and M2 differentiation is only a part of the picture, and that we should look at a more basic level and therefore consider vascular supply. With a poor vascular system, macrophages, other cells and essential nutrients may be unable to reach the herniated disc, which prevents a healthy wound healing response and thus disc resorption. For example, if a patient with poor blood supply due to hypercholesteremia, diabetes and/or extensive smoking has a small wound, the wound may take months to heal. In the worst case scenario, the wound may never fully heal. For a patient with a healthy vascular supply, such a wound can heal within a couple of weeks. Since resorption of a herniated disc can be seen as a wound in an area with little vascular supply, it is reasonable that in this location, a patients vascular status is even more important for recovery. Following this line of reasoning, recent studies showed that high LDL levels were a risk factor for disc herniations [7, 8], but whether it also contributes to the duration of symptoms still has to be elucidated. Nevertheless, the important role of macrophages and the likely role of the vascular system in the resorption of disc herniations indicate that we should look at a herniated disc as a wound, and focus our treatment strategies on optimization of the wound healing process. However, before we start developing new treatments, we first need to develop new biomarkers that help to identify which patients have altered macrophage differentiation and/or a poor vascular supply in the disc.

MRI features as biomarkers

Modic Changes as a biomarker

In this thesis, the predictive value of observed MC on MRI was investigated in multiple ways: In chapter 3 we did not find the presence of MC to be of influence on the association between the rate of disc resorption and macrophage infiltration. However, it should be noted that the original study population used in chapter 3 was insufficiently powered for this sub analysis. Hence, we recommend repeating this study with a larger sample size before a final conclusion can be drawn.

Regarding the proposed alternative macrophage differentiation in MC patients: in chapter 6 we illustrated that in MC patients, macrophages expressed CD163 (M2 marker) less frequently. Since M2 macrophages play an important role in down-regulation of the inflammation response, lower levels of CD163 are proposed to correspond to higher levels of pro-inflammatory cytokines in the local environment such as TNF-α [9], which in turn result in an increase in pain symptoms and a slower recovery rate as illustrated in chapter 2. In line with the decrease in M2 macrophage marker CD163, an increase in M1 markers was to be expected in MC patients. However, we did not find an increase in M1 markers CD40 or iNOS. Both M1 markers were only expressed in a small fraction of the total macrophage population (medians <3%), whereas CD163 was expressed in a moderate fraction (median 21%). With these markers, a large percentage of macrophages is neither marked as M1 nor as M2, which insinuates that additional markers are required to make a more complete categorization of macrophage differentiation. Hence additional M1 markers such as CD63, CD80 or CD86 could still reveal higher M1 differentiation status in MC patients [10, 11].

The underlying cause for the alternative macrophage differentiation found in patients with MC remains unknown. Literature suggests that this alternative differentiation may be caused by the presence of anaerobic bacteria in the disc in patients with MC in the adjacent vertebra, mostly Propionibacterium Acnes [12]. Alternatively, a degenerated disc or endplate with high levels of apoptosis and necrosis could also lead to alternative macrophage differentiation, without intervention of bacteria. Processes of apoptosis and necrosis, with or without anaerobic bacteria, are associated with an increase in oxidative stress, and high levels of reactive oxygen species (ROS) [13], which induce cell damage and result in an upregulation of pro-inflammatory gene expression and macrophage differentiation towards M1 [13]. In a normal inflammation response, high levels of ROS are eventually cleared by a process called 'detoxification', in which M2 macrophages play a pivotal role through expression of antioxidant enzymes such as catalase [14, 15]. In MC patients however, we found a decrease in the detoxification of ROS (chapter 7), which is likely leading to higher levels of ROS. This is in accordance with the low levels of M2 (CD163) macrophages demonstrated in patients with MC (chapter 6). Whether a lower number of M2 macrophages is the result of an increase in ROS or vice versa remains to be elucidated. Nevertheless, the two findings combined could very well explain the slow recovery of MC patients with inflammation that was demonstrated in chapter 3.

Taken together, MC may thus function as an indicator for a pro-inflammatory reaction aimed at either a bacterial infection or at a degenerated environment. However, not all patients with MC have a bacterial infection nor do they all have macrophage infiltration, and if they do, the distribution of M1 and M2 still varies from patient to patient. Hence, we must conclude that MC on its own is not a viable biomarker to predict a pro-inflammatory reaction/bacterial infection that could explain the persisting sciatica symptoms.

A possible reason why MC by itself are not so specific for a proinflammatory response, is the existence of multiple types of MC, of which type 1 and type 2 are the most prevalent. Even though MC type 1 and MC type 2 show similarities when their molecular signature is examined, they still differ in gene and protein expression profile [16]. MC type 1 is considered the active/acute inflammation type, and can be recognized by marrow-edema, whereas MC type 2 is more regarded as a chronic inflammation with pro-osteoclastic and fatty changes [16-18]. Hence, MC type 1 seems to be more relevant for identifying a pro inflammatory state in patients with a disc herniation. Unfortunately, solely focusing on MC type 1 is not always a viable option as MC type 1 and 2 are often mischaracterized.

Reliability of reporting the type of MC

The presence of MC can be reliably identified on MRI, as was illustrated by the substantial inter-observer agreement in chapter 3-5. Nevertheless, the distribution of MC type 1 and MC type 2 varies greatly amongst studies. In our study, < 10% of the patients with MC were characterized as MC type 1, whereas others have reported percentages as high as 78% [19]. Since MC type 1 is believed to be an acute process and MC type 2 a chronic inflammatory process, differences in distribution of MC type 1 vs MC type 2 could potentially be attributed to the timing of the MRI. However, the average duration of symptoms till baseline in our study was +/- 9 weeks, whereas the average duration of symptoms till baseline in the study from Matsuyama et al. who found 78% MC type 1 was +/-19 weeks [19]. Of course, when evaluating duration of symptom data, the influence of recall bias should not be underestimated. This makes the quality of the data less reliable and thus the conclusions drawn from it. Nevertheless, it seems that the duration of symptoms is not the only factor of influence on the distribution of MC type 1 and 2.

MC type 1 is identified by marrow edema, which is hyperintense on T2 and hypointense on T1, whereas MC type 2 is identified by fatty marrow changes, which is hyperintense on T1 and T2. The sensitivity of identifying hypo and hyperintensities on T1 depends on different MRI settings. For instance, when a low field strength is used (<1.0T), marrow inhomogeneities are less pronounced, which makes MC type 1 easier and MC type 2 harder to identify compared to 1.5T magnets. When comparing a 0.3T to a 1.5T scanner, the 1.5T resulted in twice as much identification of MC type 2 and reduced the identification of MC type 1 to 25-33% [20]. Moreover, an MRI with 3.0T or higher have superior conventional fat suppression, which increases the identification of MC type 1 as it reduces the chance that marrow edema is overlooked. In addition, another technique to increase the detection rate of MC type 1 is the usage of a fat suppression sequence. To what extent this increases the detection rate of MC type 1 is not properly studied [21]. Nevertheless, in order to prevent this overestimation of MC type 2 and underestimation of MC type 1, we recommend using a 3.0T scanner and or fat suppression techniques for future studies.

Mischaracterizing the MC type makes it challenging to distinguish the reported differences and associations regarding MC type 1 and MC type 2. since we used a 1.5T scanner and no fat suppression, our prevalence of MC type 1 is likely to be underestimated, whereas the prevalence of MC type 2 is probably overestimated. Thus, our conclusions regarding the altered macrophage behavior in patients with MC type 2 may actually be for MC type 2 and MC type 1.

With regards to future projects, it could be that accurately distinguishing MC type 1 from MC type 2, would result in MC type 1 being a useful predictor for an inflammatory environment due to a bacterial infection, and may consequently influence therapeutic strategies. Alternatively, as MC type 1 is characterized by marrow edema, it could simply only be a predictor for a poor vascular status with vascular malformation, which in turn could be associated with a higher occurrence of anaerobic bacterial infections and alternative differentiation of the infiltrated macrophages. In that case, MC type 1 would not be a sensitive predictor and may thus not be useful on its own.

By contrast, MC type 2 could also be the type to focus on, with the rationale that the inflammation response in MC type 2 is chronic, which could signify that the body was unable to clear a bacterial infection and that the infiltrated macrophages have remained in an ineffective M1 state and cause further damage. Such a process is unlikely to resolve spontaneously, which the acute MC type 1 response might.

In conclusion, this study showed that MC have a predictive value in the characterization of the inflammatory profile of herniated discs. However, its sensitivity and specificity seem to be insufficient to use in clinical practice on its own. Until the problem of mischaracterization of the MC types is solved, MC should only be used as an additional predicter combined with other parameters such as disc inflammation. Further, caution should be taken with comparing different types of MC and sticking to absence vs presence of MC may be the preferred approach if MRI parameters are not optimal and mischaracterization is likely.

Disc herniation features as a biomarker

Alternatively, considering that the presence of MC (both type 1 and 2) are associated with endplate avulsion, the limited predictive value could indicate that MC is a confounder for endplate avulsion. An avulsed endplate would provide a proper way of entry for both bacteria as well as macrophages to enter and could perhaps be a more accurate predictor. In chapter 7 we examined the effect of both endplate pathologies on the proteomic signature and showed that endplate avulsion was associated with an increase in the detoxification of ROS and an increase in immune activation and coagulation. Together, this illustrates a healthy traumatic immune response, making it less likely that a bacterial infection is present. Because, in contrast with endplate avulsion, MC showed a decrease in detoxification of ROS and immune system, it is unlikely that MC is a confounder for endplate avulsion. Instead, these two parameters should be treated independently. Nonetheless, still little is known regarding the effect of endplate avulsion on disc inflammation and recovery, and more research is needed in order to assess whether and how this parameter should be used.

A different feature to focus on is the extent of the disc herniation. In chapter 3, we found that extrusion is a predictor for macrophage infiltration and disc resorption after one year. However, these associations with disc extrusion were only moderate, which indicates that extrusion cannot be used as a reliable biomarker for disc resorption as their sensitivity and specificity would be too low to base clinical decisions on. Nonetheless, it could prove to be of adequate predictive value when combined with other predictive parameters.

Alternative Imaging techniques

A different promising technique that could be of use is Magnetic resonance spectroscopy (MRS), which is an imaging technique that can characterize metabolic features of the disc in vivo. For example, increases in Alanine and Lactate have been associated with increased pain symptoms and is regarded as a marker for inflammation [22]. However, whether MRS can also distinguish bacterial infection from macrophage infiltration and M1 from M2 differentiation, still has to be elucidated.

Studying inflammation in the disc

Immunohistochemistry: a qualitative, quantitative and illustrative technique
Of course, finding predictors for a pro-inflammatory environment such as MC is only useful if such an environment can be identified reliably. A suitable way to get information regarding the inflammatory status of the herniated disc is immunohistochemistry of the disc material. Immunohistochemical staining revealed that macrophages were seen abundantly in NP tissue but not in the cartilage endplate tissue, which was frequently present in the sample. Further, it was demonstrated that macrophages cluster around the edges of the collected tissue samples, and were barely seen in the central parts, which suggests that macrophages work their way inwards. When this last observation is combined with the finding that they were more prominent in extruded compared to bulging discs, it suggests that the observed macrophages were not resident macrophages, but that their presence is rather a consequence of the herniated part of the disc being exposed to the systemic circulation.

Immunohistochemistry: Distinguishing NP cells from macrophages
When compared to a purely quantitative technique such as ELISA or Western blotting, which provide a value for the protein expression of interest, immunohistochemistry can provide additional information that would otherwise have been overlooked. Besides, for many markers, there are multiple cells that express them: in chapter 7 it is shown that many resident cells of the disc (NP cells and chondrocytes) also express macrophage markers CD68, CD40, iNOS and Arg1, but that macrophages can be identified based on their morphology. Hence by using immunohistochemistry, quantification of protein expression could be restricted to macrophages, whereas with a purely quantitative technique, protein expression of all cell types would have been included and may have provided inadequate conclusions.

Interestingly, the expression intensity and expression by different cell types may vary greatly upon the specific brand of the antibody, buffer and pretreatment of the material. During the study, multiple antibodies for CD40 and CD163 were tested with various buffers and pretreatments and discarded if the results were unsatisfactory. This eventually resulted in the antibody panel that was used in chapter 7. This also shows, that if previous reports mention that a certain marker is specific for macrophages, this claim first has to be verified when not the exact same material and lab settings are used. In chapters 3 to 5, only CD68 was used because this marker is abundant in macrophages, and the samples used for this study were old, which may have compromised their quality and hence the identifiable protein expression that can be stained positively. For CD68, this resulted in reduced but still considerable amount of positively stained macrophages, thereby making CD68 useable as a parameter. Despite, the exact cell counts were likely to be less accurate, thus in order to maintain the validity of the data, macrophage infiltration was analyzed as a categorical variable with categories on a logarithmic scale. A positive consequence of the reduced staining intensity of CD68, was that NP cells and chondrocytes were not stained positively. Unfortunately, we were unable to include more advanced analysis with M1 or M2 distributions on this old tissue, as their corresponding antibodies were barely positive.

Immunohistochemistry: Cell counting

The downside of immunohistochemistry is that after finishing the lab procedure, there is no quantitative number that can be used directly. In order to quantify protein expression, a positive surface count or positive cell count can be performed. This is a labor intensive task that leaves room for subjectivity as it can be challenging to distinguish positive from negative cells when they are faintly stained. Hence it is advisable to use 2 raters in order to improve the quality of the data. Alternatively, in order to reduce the labor required and improve the objectivity and reproducibility of the cell counts, an algorithm can be developed that will count all tissue above a certain intensity threshold. The possible downside of the algorithm is that it does not distinguish between a positive macrophage and a positive chondrocyte, and that it tends to count double folded tissue as positive. Although some additional measures can be taken to combat these downsides, it does not work for all antibodies and tissues. In such cases, or if one is interested in the expression of a large number of proteins, other techniques such as proteomics can be a more attractive option.

Proteomics, a technique for biomarker screening

A different laboratory method that can be used to quantify protein expression is Proteomics, using Liquid Chromatography – Mass Spectrometry (LC-MS). Proteomics is a relatively new technique that quantifies all expressed proteins in the selected tissue. In short, it does so through first dissecting all proteins of the tissue into peptides, which are then possessed in the Mass Spectrometry machine, where all peptides are sorted based on their mass to charge ratio. Each mass to charge ratio corresponds to a peptide which is subsequently translated to its corresponding protein based on a database [23]. The interesting advantage of this exploratory technique is that it quantifies a large number of proteins at once, hence it is very suitable to select interesting targets for further research in an exploratory phase, and it is easy to retrieve information regarding the correlation between sets of proteins.

With regard to macrophage differentiation in disc herniations, proteomic seems remarkably beneficious when it comes to the identification of new biomarkers. At this point, we know that macrophage infiltration and their differentiation profile play a role in the etiology of disc herniations. However, how exactly the differentiation profile functions and what proper targets could be for future therapy still has to be elucidated. For example, we hypothesize that patients with MC tend to have more bacteria present, which in turn will likely alter macrophage differentiation from M2 towards M1. In chapter 6, we found that not all M1 markers (CD40 and iNOS) are suitable to study this question. This is exactly where proteomics proves its benefit. Instead of trying new markers one by one until one suffices, proteomics will reveal the full spectrum of proteins from which a proper candidate can be chosen. Besides, this spectrum of proteins is not limited to macrophage markers, so by using this approach, other important areas of interest can also be brought into view. This way, we can shed light on candidate targets and markers, instead of staying in the dark.

As of today, our knowledge on how MC relate to the inflammatory profile of the disc is still extremely limited, and even less is known for endplate avulsion, as this concept was only recently discovered by Rajasekaran et al. in 2013 [24]. Hence an exploratory technique seemed the most suitable for investigating these phenomena. Providing an overview of all abundantly expressed proteins in the disc could aid in selecting specific targets for future studies. Also, by focusing on the differences in expression profiles instead of individual markers, we could verify the immunohistochemistry results that illustrated a detrimental effect of inflammation in patients with MC.

As this methodology provides a tremendous amount of data, it can be very overwhelming, which is why we used a bioinformatic approach to translate protein expression into pathways. By identifying alterations in pathways, which were a downregulation of detoxification of reactive oxygen species (ROS), complement system and immune system for MC, and an increase in coagulation and detoxification of ROS for EPA, we have provided a general overview of relevant pathways and identified new specific fields of interest for future research. For example, future research can focus on important players that modify these pathways. And since, LC-MS is unable to detect proteins in very low quantity, alterations of other relevant proteins in these pathways can be unravelled with different, more sensitive techniques.

Before the suggested personalized treatment strategies could be implemented in clinical practice, highly sensitive and specific biomarkers would have to be developed. In chapter 5, we evaluated gadolinium enhancement as a tool to assess macrophage infiltration, but found it to be inadequate. Hence, new non-invasive biomarkers are required that identify bacterial infection, macrophage infiltration and differentiation in the herniated disc.

Serology

Considering the infiltrated macrophages in the disc originate from the systemic circulation, analyzing patients' serum can provide information on the inflammatory status of the disc. In chapter two, this study discussed the serum levels IFN-γ, TNF-α, IL-6, IL-8, IL-1β, IL-10, and IL-4, some of which correlated with an increase in pain symptoms (TNF-α/IL-8/IL-6), while others resulted in decrease of pain symptoms (IL-4/IL-10). This indicates that even though the site of inflammation is small and relatively poorly vascularized, serology can still be used to search for biomarkers. Hence, we suggest future studies should focus on finding serological markers that correlate to bacterial infection and M1/M2 macrophage infiltration. This way, patients could receive personalized treatment strategies at minimal diagnostic cost.

Drug treatment strategies

Acquiring knowledge on the presence and type of macrophage infiltration will not only influence the choice to treat conservatively or surgically, but may also lead to new personalized drug treatment strategies. Knowing that a macrophage response will accelerate the resorption process, a possible new treatment approach is to stimulate macrophage proliferation and differentiation, locally, in the herniated disc. A possible example would be an epidural injection with Macrophage Colony Stimulating Factor (CSF-1), which can stimulate local proliferation, increases the cell number and skews macrophages towards M2 [25]. Besides, administration of CSF-1 has shown to accelerate wound healing in vitro [26]. Short term oral treatment with CSF-1 has been successfully administrated to patients with immune cell depletion due to chemotherapy, but side effects were not reported [27]. whether CSF-1 is also tolerated in non-immune-depleted radiculopathy patients remains unknown. If systemic therapy is not well tolerated due to systemic side effects, epidural injections can be considered.

Stimulating macrophage infiltration is likely to be beneficial in traumatic herniations, but in a disc with a M1 dominated response due to an infection or degenerative changes, increasing the number of macrophages may not have the desired effect, as these macrophages are more likely to differentiate towards M1. In these patients, an alternative strategy would be to skew macrophage differentiation towards M2, preferably combined with antibiotics in case of an infection. Possible ways of doing so are with steroid injections (methylprednisolone), which have shown to decrease M1 and increase M2 differentiation in a mice model with lung injury [28]. Furthermore, since M1 activation resulted in an increased expression of COX-2, but a decreased expression of COX-1, whereas M2 activation resulted in an increased expression of COX-1 [29]. Moreover, inhibition of COX-2 in an in vitro mice model resulted in an inhibition of macrophage differentiation towards M1 and promoted polarization towards M2 instead [30]. Hence, a different approach would be to use selective COX-2 inhibitors, such as Etoricoxib or Celecoxib.

A big advantage of these widely prescribed drugs is that they can safely be taken orally. However, the question remains whether oral administration will result in a sufficient concentration in the herniated disc, as the efficacy of these drugs for sciatic symptoms are still up for debate. Thus, we recommend studying different ways of administration and their corresponding efficacy, and targeting sciatica patients with a M1 dominant infiltrate in the herniated disc.

For those patients in which bacteria are present in the intervertebral disc, an antibiotic treatment strategy should be developed depending on the pathogen. With a pooled prevalence of 56.4%, the most common bacteria is Propionibacterium acnes [12], other reported bacteria are Staphylococci epidermidis, Corynebacterium propinquum, Pepto streptococci, Staphylococci aureus, Bacillus Cereus and Citrobacter braaki/freundi [31]. All these bacteria do well in an anaerobic environment [32-34], which makes a degenerated disc or endplate an ideal place to grow. Specific antibiotic strategies would have to be tailored to the resistance and susceptibility profile of each bacteria, although most (P. Acnes, S. epidermidis, S. Aureus and Pepto streptococci) will likely react to amoxicillin–clavulanate [35]. The efficacy of antibiotic treatment was demonstrated by Albert et al (2013) [36], who performed a RCT and randomized patients between conventional care and administering amoxicillin/clavulanate in a dose of 500/125 mg three times a day, at 8h intervals, for 100 days to long term radiculopathy patients with MC type 1. She and her group demonstrated a significant clinical improvement in the antibiotic treated group compared to control patients. Up till now this experiment was not repeated and the rationale for the outcome remains to be unraveled.

The other 3 bacteria are often resistant to Amoxicillin-clavulanate, but have reported susceptibility to Ceftriaxone (Corynebacterium) [37], imipenem/meropenem (Citrobacter) [32] and ciprofloxacin/ vancomycin (Bacillus) [38]. Nevertheless, the susceptibility of these bacteria should first be assessed using bacteria cultured from intervertebral discs, as these could be slightly different strains and their resistance profile could be altered. At last, duration and dosages of the antibiotics would have to be increased since the disc has a very limited vascular supply and is more dependent on diffusion. The extent of this increase depends on the pharmacokinetics of the respective antibiotics. Alternatively, the possibilities for local administration during surgery, or through epidural injection could also be explored.

To our knowledge, this study is the first to focus on macrophage differentiation and to integrate it with clinical outcomes and other inflammation parameters of the disc. This study has provided some insight on how macrophages play a crucial role in the course of disc resorption and its clinical implications, but has also created many new questions. These questions fuel the inspiration for new interesting studies that shall greatly improve our understanding of the pathology and will likely enlighten innovative ways to treat sciatica. For example, future research should focus on specifying the role of macrophages in relation to a bacterial infection and the inflammatory environment of MC, not to forget how these interactions may affect the clinical outcomes and whether they require different treatment strategies. On that account, a new study protocol was developed in chapter 9, which aims to answer these questions and search for biomarkers to integrate this material in clinical practice: the EIMICOR study (Effect of Infection, Modic and Inflammation on Clinical Outcomes in Radiculopathy). With the results of this new study protocol, a better understanding of the role of inflammation in sciatica will be created through evaluation of the disc material and blood samples. Bacterial status, M1/M2 differentiation and proteomic signature of the herniated disc and MC status of the adjacent endplate will all be associated with clinical outcomes. Subsequently, biomarkers that can predict the inflammation status of the disc will explored in blood. This way, differences in pathology between herniated discs can be determined and patient subgroups can be characterized with the help of non-invasive biomarkers. By doing so, we can work towards personalized treatment strategies and improve the quality of life in patients suffering with sciatica.

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Chapter 9

Study protocol: Effect of infection, Modic and Infl ammation on Clinical outcomes in surgery for radiculopathy (EIMICOR))

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Submitted

"If the challenge we face doesn't scare us, then it's probably not that important." (Tim Ferris)

Abstract

Background

Evidence indicates that inflammatory processes are involved in radicular pain as well as in resorption of herniated disc tissue. Furthermore there are indications that the presence of vertebral end plate pathology (Modic changes; MC) is associated with a negative effect of inflammation. It is hypothesized that in patients with MC, the (possibly bacterial induced) inflammation will be accompanied by pro inflammatory cytokines that worsen the outcome, and that in patients without MC, the inflammation is accompanied by cytokines that induce a resorption process to accelerate recovery.

Methods

This prospective-(longitudinal) observational cohort study will include 160 lumbar and 160 cervical patients (total of 320) that are scheduled for surgery for either a lumbar or cervical herniated disc with ages between 18 and 75. The effects and interactions of local bacterial infection (culture), inflammatory cells in disc material (immunohistology), MC (MRI), and blood biomarkers indicating inflammation or infection (blood sample evaluation) will be evaluated. Clinical parameters to be evaluated are leg pain on the 11 point NRS pain scale, Oswestry (lumbar spine) or Neck (cervical spine) Disability Index, Global Perceived Recovery, Womac Questionnaire, and medication status, at baseline, and after 6, 16, 26 and 52 weeks.

Discussion

Gaining insight in the aetiology of pain and discomfort in radiculopathy caused by a herniated disc could lead to more effective management of patients. If the type of inflammatory cells shows to be of major influence on the rate of recovery, new immunomodulating treatment strategies can be developed to decrease the duration and intensity of symptoms. Moreover, identifying a beneficial inflammatory response in the disc through a biomarker in blood could lead to early identification of patients whose herniations will resorb spontaneously versus those that require surgery.

Background

Radiculopathy is a clinical symptom that has its origin in irritation of the spinal nerve. In patients with a bulging or herniating disc, compression of the nerve is considered the main cause. In more recent studies on radiculopathy inflammatory processes appear to have a bigger role than originally thought. It is hypothesized that the disruptive process in the area of the spinal nerve starts with micro traumata in the vertebral endplate or disruption of the annulus fibrosis. Disruption of the annulus or micro breakage of the endplate at the location where the annulus is attached, leads to exposure of the nucleus pulposus to the epidural space [1]. In the epidural space, the nucleus pulposus may not only cause compression of the nerve, but is also exposed to the systemic circulation. This makes the disc prone to neovascularisation and macrophage infiltration, which is often seen in cervical and lumbar discs [2, 3]. Macrophage infiltration of the extruded material could promote a foreign body response and thereby worsen the symptoms [4]. However, they can also help to resorb the herniated material and thus alleviate the symptoms. This discrepancy could be explained by alternative macrophage differentiation: Macrophages can differentiate in many distinctive phenotypes with diverse functions [5], but they are often dichotomized in M1 and M2 macrophages [6]. M1 macrophages are considered pro-inflammatory, are characterized by expression of markers like CD40, CD80 and CD86 [6, 7], and produces pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, Tumor necrosis factor (TNF)-α, [5], all of which have been associated with and exacerbation of pain symptoms [8]. In contrast, M2 macrophages express markers such as CD163 and CD206 [6, 7], produce cytokines such as IL-4 and IL-10, and are believed to initiate resorption of the herniated disc material, which results in an amelioration of radicular pain and improvement of clinical outcomes [5, 8].

In our previous work, we found that effect of macrophages on clinical outcome was dependent on the presence of Modic changes (MC), which are considered inflammatory vertebral endplate signal changes that occur frequently on cervical and lumbar endplates [9, 10]. We found that in patients with MC, a higher degree of macrophage infiltration, was accompanied by more radicular pain symptoms and less favourable clinical outcomes [4]. In addition, we also showed that in patients without MC, a higher degree of macrophage infiltration was associated with less radicular pain and a better clinical outcome [4]. Based on these findings, it is to be expected that patients with MC have a higher degree of M1 macrophages, which would be in line with their slower recovery, while in patients without MC, larger numbers of M2 are to be expected, thereby explaining their faster recovery.

A different possible scenario is that the exposure to the systemic circulation is accompanied by infiltration of Propionibacterium acnes or Staphylococcus epidermidis, both of which are opportunistic bacteria that were demonstrated in lumbar and cervical herniated disc material [11], and have been associated with MC [12]. In the herniated disc, they could induce a host immune response which increases inflammation and could aggravate radicular symptoms [13, 14].

For future perspectives, it is important to understand the interconnectivity and clinical relevance of inflammation, infection and MC. Until now, no studies have sought to explore the relationship of these three together and how their interconnectivity affects clinical outcomes. Therefore, we would like to investigate the interactions between MC, bacterial infection, inflammation, and their effect on the clinical symptoms of both sciatica and cervical radiculopathy patients. Explicating these mechanisms may lead to characterization of certain subgroups, of which some may benefit from antibiotic treatment, some from anti-inflammatory treatment, some from a conservative approach and some from (early) surgery. Hence, identifying these subgroups will allow future studies to focus on specific treatments for each subgroup. If this is accomplished, we could significantly decrease the disease burden of patients that suffer from herniated discs.

Methods

Objectives

Primary Objective(s):

1. In a group of lumbar and cervical radiculopathy patients undergoing disc surgery, this study will determine the effects of bacterial infiltration of the disc on patient reported pain scores, in the presence or absence of MC on MRI and histological defined disc inflammation.

Secondary Objective(s):

1. A secondary aim of this study is to assess whether patients that suffer from disc inflammation benefit more from anti-inflammatory drugs than those without inflammation.

2. Another secondary aim of this study is to further explore the inflammation process by characterizing different types of macrophages (M1 and M2).

3. At last this study aims to associate the presence of bacterial infection to the presence and type of MC.

Figure 1 illustrates the hypothesis of the present study.

Study design

This prospective-(longitudinal) observational cohort study is an imaging, histological, immunological and clinical study. Both the MRI scan and surgery are part of the usual care, in addition to these procedures, this study will draw 3 blood samples, use the rest material from surgery and ask the patient to fill in short pain related questionnaires.

The total duration of the study is approximately 2.5 years, of which 1.5 years for inclusion and 1 additional year for the follow up. Because Lumbar disc herniations occur more often than cervical ones, the inclusion of lumbar patients might be finished after 1 year, while the inclusion of the cervical patients will likely take 1.5 years. Patients will be recruited in from one of the three inclusion centers in which they are planned for surgery: the Spaarne Gasthuis Haarlem Zuid, Alrijne Hospital Leiderdorp, HMC the Hague and Haga Hospital the Hague. The analyses and coordination of the study will be done from the Leiden University Medical Center (LUMC).

Patient recruitment will take place during the first (pre-operative) visit with the neurosurgeon, where the patient will be informed about the study. Patients that want to participate will be asked to sign an Informed Consent Form (ICF) in the week prior to the surgery. Afterwards, the participant will fill in an online set of questionnaires in Castor EDC. This set of questionnaires will contain questionnaires that assess demographic data, pain scores (NRS back/leg pain for lumbar patients and NRS neck/arm pain for cervical patients), disability scores (ODI for lumbar patients and NDI for cervical patients), Osteoarthritis (Womac) and medication status. Furthermore, the patient will receive an MRI scan in the weeks before surgery which is part of the usual care. In addition, during surgery, the herniated part of the disc will be dissected and transferred to the LUMC for further analyses. The dissected material will be used for three different purposes: One part of the dissected material will be used for bacterial culture, one part for histological analyses, and one part will be snap frozen for later defined analyses. In addition to the frozen disc samples, this study will also collect blood samples for two purposes: the first is to assess the general inflammation (BSE, leukocyte differentiation) and vascular status (Cholesterol) and the second purpose is for future analysis, for which a sample will be stored in the freezer. Only if our hypotheses turn out to be true, the stored blood samples will be used to search for a predictive biomarker for a bacterial infection and/or inflammation of the disc. The exact analysis that will be used to identify the biomarker will defined in a later stage and will be based on our results from the histological analysis and bacterial cultures. Blood will be drawn in the waiting room for surgery from the canula that is placed for the purpose of the surgery. This means that only the donated blood is an additional aspect of the study, injecting the canula is part of the usual care.

All participants will be asked to co-operate during the entire follow-up. During follow-up patients will be asked to fill in questionnaires regarding clinical outcome (NRS, ODI/NDI & GPE) and medication status at 8 weeks, 16 weeks, 26 weeks and 52 weeks post-surgery. Patients will receive emails with a link to the follow up questionnaires around the above mentioned time points. A flow chart of the EIMICOR study is shown in figure 2.

Study population

Population

For this study, all patients (18-75 yr.) with 8 or more weeks of radicular pain symptoms, that are eligible for surgery according to a neurosurgeon in the participating hospital, will be asked to participate. Patients are eligible if they already planned to undergo surgery for herniated disc and meet the following in and exclusion criteria:

Inclusion criteria

Lumbar patients:

- Age 18-75
- a unilateral lumbosacral radicular syndrome, with at least the following criteria:
  - Radicular incitement: radiating pain from (a part of the) dermatome L4, L5 and/or S1
  - Present for at least 8 weeks
- MRI verified lumbosacral disc herniation that is corresponding to the side of the symptoms
- Indication for surgery
- Informed consent

Cervical patients:

- Age 18-75
- a unilateral cervical radicular syndrome, with at least the following criteria:
  - Radicular incitement: radiating pain from (a part of the) dermatome C4-C5, C5-C6, C6-C7 and/or C7-T1
  - Present for at least 8 weeks
- MRI verified cervical disc herniation that is corresponding to the side of the symptoms
- Indication for surgery
- Informed consent

Exclusion criteria

Lumbar patients:

- Previous lumbar spinal surgery or chemonucleolysis
- Paresis of MRC < 4
- History of spinal inflammatory disease
- Instability that requires surgical fixation
- Active infection at the time of surgery
- Usage of Anti-biotics in the past six months
- Epidural steroid injection in the past six months
- Pregnancy
- Inadequate knowledge of the Dutch language

Cervical patients:

- Previous cervical spinal surgery chemonucleolysis
- Paresis of MRC < 4
- Myelopathy as major complaint
- History of spinal inflammatory disease
- Instability that requires surgical fixation

- Active infection at the time of surgery
- Usage of Anti-biotics in the past six months
- Epidural steroid injection in the past six months
- Pregnancy
- Inadequate knowledge of the Dutch language

Study parameters/endpoints

Main study parameter/endpoint

The main study parameter will be the NRS leg pain for lumbar patients and NRS arm pain for cervical patients. A description of these endpoints and all other parameters used during the study are described below.

NRS pain scores

- The pain experienced by the patients will be measured by questionnaires that assess leg pain for lumbar patients and arm pain for cervical patients: NRS leg pain, and NRS arm pain. In these validated questionnaires, the patients will display the amount of pain they have experienced in respective locations during the week previously to the visit. The pain intensity will be determined on a scale of 0-10. 0 represents 'no pain' and 10 represents 'worst pain imaginable' [15]. All NRS pain scores will be measured during baseline (in the week before surgery) and at every follow-up moment (8, 16, 26, 52 weeks,). Previous test results will not be visible for the patient. In addition to the NRS leg pain, NRS back pain will also be used as an additional outcome measure. Also, the NRS arm pain we be accompanies by the NRS neck pain as an additional outcome measure.

Other patient reported outcome parameters

- Functionality
  For estimating functionality of the lumbar patient, the Oswestry Disability index (ODI) will be used. This validated questionnaire contains 10 topics related to the impact of the pain on the patient's life, with 5 grading's for each topic. The total will give a score between 0 (no disability) and 50 (maximum disability possible), which will be calculated to a 1-100% score [15]. For estimating functionality of the cervical patient, the Neck disability index (NDI) will be used, which is an adjusted version of the ODI focused on neck pain instead of back pain and is also a validated questionnaire. All functionality scores will be measured during baseline (in the week before surgery) and at every follow-up moment (8, 16, 26, 52 weeks,). Previous test results will not be visible for the patient.

- Recovery
  In order to estimate the perceived recovery of the patients, the Global Perceived Effect (GPE) questionnaire will be used. The GPE is a widely validated questionnaire in which patients can express their perceived recovery on a 7 point Likert scale. On this scale the numbers 1-7 are accompanied by an expression of a state such as 'Fully recovered'= 7, 'Same as before'= 4 or 'Very bad' = 1. All recovery scores will be measured at every follow-up moment (8, 16, 26, 52 weeks,). Previous test results will not be visible for the patient.

Predictive parameters

- Bacterial infection of the disc
  Bacterial infection in the disc will be verified by a bacterial culture protocol. Tissue necessary for the Bacterial culture will be harvested from herniated disc tissue that was taken out during surgery. The bacterial culture will be accompanied by a gram stain and methyl blue stain. infection can be distinguished from a contamination by the quantity of colonies on the culture (0-10 is regarded as contamination).

- Disc inflammation
  Disc material harvested during surgery will be stained for the presence of macrophages, for M1 and M2 macrophages separately, and for B and T cells. Evaluation will be done through counting cells and subsequently categorizing them.

- Modic changes
  Type (1,2 or 3) and severity (<50% & >50%) of MC will be scored at baseline on MRI. Type 1 shows an hypointense endplate on T1 MRI and a hyperintense endplate on T2 MRI, Type 2 shows an hyperintense endplate on both T1 and T2 MRI and Type 3 shows a hypointense endplate on both T1 and T2 MRI.

Secondary study parameters/endpoints
A possible mediator in this study is the amount and type of pain medication that patients take, either in self-care or on prescription from the neurosurgeon or GP as part of the usual care. This could potentially alter the inflammation profile (NSAID's) or could lead to lower perceived pain scores. In addition, it could be that patients with severe inflammation benefit more from anti-inflammatory drugs. Therefore this study will measure participants usage of pain medication as follows:

- Pharmacological data
  Participants will be asked to fill in a form regarding the frequency, type and dosage of pain medication and anti-inflammatory drug usage. This form will be given to the participant at baseline (in week before surgery) and will also be given during the one-year follow-up at 8, 16, 26 and 52 weeks.

- Osteoarthritis
  In order to investigate to what extent MC are related to clinical features of osteoarthritis, participants will be asked to fill in the Womac questionnaire. The Western Ontario and McMaster Universities Osteoarthritis (Womac) index questionnaire evaluates pain and physical function, containing 24 questions about daily functioning and stiffness. The questionnaire will be given to participants at baseline and after one year follow-up.

Other study parameters
Furthermore, some additional study parameters that may cause confounding will be measured:

- Demographic data
  From all patients, general information will be collected: age, gender, BMI, ASA, Diabetes (due to possible polyneuropathy symptoms), smoking habits, duration of symptoms.

- MRI data
  Nerve root compression (no or mild, moderate, severe) will be scored on MRI. (compression is believed to lead to more severe pain symptoms).

Study procedures

Surgical techniques
Surgery techniques lumbar surgery
A unilateral transflaval discectomy will be performed according to usual care patients are placed in the knee-elbow position. Using anatomical landmarks and fluoroscopy the level of incision is determined. A small midline incision in the lumbosacral region is made. Muscles are unilateral detached from the spinous process. After spreading the wound a very small partial resection of the rostral lamen is executed, followed by a flavectomy with unilateral opening of the lateral recess. The nerve root is identified as well as the bulging disc. A discectomy is performed and the tissue taken out is assembled in a jar, identified with patient name and study number. If the nerve root is compressed by a sequester, only a sequesterectomy is performed if deemed necessary by the surgeon. After decompressing the nerve and performing a discectomy, the wound is closed in layers.

Surgery will be performed by a qualified neurosurgeon. Postoperative care will consist of an admission period of 2 days (day of surgery and day after) and one postoperative visit to the physiotherapist. From there on, the general practitioner will take care of the postoperative care.

Surgery techniques cervical surgery
For anterior discectomy, the level of surgery is verified by fluoroscopy. The operation will be carried out by a qualified neurosurgeon. Most surgeons operate using loupe magnification. The platysma muscle is separated or cleaved at the right side of the midline (less frequently on the left side), and the prevertebral space is reached by an approach medial to the sternocleidomastoid muscle and the carotid artery, and lateral to the trachea and oesophagus. The disc is incised and the corpora are distracted. Discectomy is performed as thorough as possible. Regularly the posterior ligament is cut and the spinal root is decompressed. If necessary, spondylarthrotic rims are removed. To the preference of the surgeon bone graft or an intervertebral fusion device is left behind.

MRI protocol
The MRI imaging process, which are part of the usual care, will be done according to the standard protocol of each participating hospital. Every patient will receive a series of images performed by a 9.0 Tesla scanner:

- Sagittal T1SE (turbo spin echo)
- Sagittal T2TSE
- Transversal T1-TSE
- Transversal T2-TSE

Evaluation of the MRI's will be done by two independent researchers, both experienced in evaluating spine MRI scans , by using two evaluators, this study can perform a intra agreement analyses and provide a kappa value to put the accuracy of the evaluators in perspective. The evaluators will describe the disc characteristics (bulging, herniated or sequestrate) and the severity of nerve root compression. In addition, the images will be scored on the presence, severity and type of MC. The Type will be scored according to criteria from Modic et al [16, 17]. Type 1 shows an hypointense endplate on T1 MRI and a hyperintense endplate on T2 MRI, Type 1 shows an hyperintense endplate on both T1 and T2 MRI and Type 3 shows a hypointense endplate on both T1 and T2 MRI. Severity of MC will be categorized as >50% and <50%.

Disc sample
During surgery, the neurosurgeon will collect a sample of the herniated disc tissue that was removed during the procedure, which will be used for further histological analysis.

All harvested discs will be fixed in a 4% formaldehyde solution for 3-7 days. Tissue will subsequently embedded in paraffin blocks and 5-µm thick slices shall be taken from the middle of the block for haematoxylin staining, HE stained coupes will be evaluated under the microscope for clear signs of infiltrating inflammatory cells, if tissue from one sample exceeded the capacity of 1 paraffin block, multiple blocks will be formed and a slides of each block will be evaluated. one slide of each disc that contained inflammatory cells was submitted to further analysis using immunohistochemistry.

Presence of M1 macrophages will be characterized by the co-presence of CD68 (DAKO, Denmark), with CD40 (Sanbio, Netherlands) [6]. Presence of M2 macrophages on the other hand, will be verified by co-presence CD68 and CD163 (Abcam, Netherlands). In order to be certain that the selected anti-bodies are a valid tool to assess the presence of M1 and M2 macrophages, the panel has been tested in a pilot study In this pilot study, T-cells (CD3) and Neutrophils (CD15) were present in very low quantities and hence not included in the study protocol.

For the staining procedure, 5-µm paraffin slices will be rinsed in ethanol and methanol solutions and prepared for the expression of CD68, CD40, CD163. Immunohistochemistry will be performed using a three-step indirect method. Antibodies will be cooked in EDTA pH 9.0 buffer as a pre-treatment. Subsequently, an avidin-biotin complex technique will be performed with the Vectastain ABC-Elite Kit (Vector Lab. USA) and the appropriate biotinylated antibodies. Visualization of the peroxidase reaction will be done with DAB solution (Sigma). Moreover, samples will be counterstained with Harris haematoxylin. All of these samples will be accompanied by a positive control. In control samples, primary antibodies will be omitted, which results in the expected absence of any cellular labelling. In order to standardize the evaluation of the samples, all samples were photographed under the microscope before they were evaluated. Since previous studies have reported the expression of CD68, CD40 by nucleus pulposus cells/chondrocytes [18, 19], cells were analysed based on morphological features and only macrophages were photographed and evaluated. The same approach was used for CD163.

In order to assess and characterize the bacterial infiltration of the herniated discs, additional samples will be extracted from the resected tissues. Disc samples will be used for anaerobic and aerobic bacterial cultivation, gram staining and methyl blue staining, to verify and characterize a bacterial infection according to standard clinical protocol. Samples with > 10 colonies per species per culture will be scored as infection, In contrast, samples with 0-10 colonies per species will be scored as contamination. At last, a third disc sample will be collected from the herniated excised tissue, and will be snap frozen for future analysis.

Blood samples

Blood samples will be collected from the canula directly after it has been inserted on the holding (OR preparation room). By doing so, the blood sample for the study will be drawn before the patient receives prophylactic antibiotic treatment according to the usual care. A total of 3 blood samples will be collected: the first sample (4ml EDTA) will be used for a general blood count, the second (3ml Heparin) for cholesterol, these samples will be send directly to the laboratory in respective hospitals, the 3th and 4th samples will be collected in a 4ml EDTA for future purposes and will be transferred to the LUMC at the end of the day. At the LUMC, the 3th sample will be deposited at the microbiology department where the plasma will be subtracted according to standard protocol and stored in the freezer. Since the plasma samples will only be analyzed if the hypotheses turn out to be true, the exact analysis protocol will be defined at a later stage.

Statistical analysis

Sample size calculation

Distinguishing between the presence of MC (yes/no), bacterial infection (yes/no) and inflammation (yes/no) we have 2x2x2=8 groups. We expect the mean during follow-up of the primary outcome (NRS pain score) and the distribution of patients among the 8 groups to be as in Figure 3. The standard deviation of the NRS is expected to be 1. We will primarily test for *any* effect of bacterial infection by comparing the full factorial model to a model without bacterial infection by means of an F-test on 4 degrees of freedom. We implemented a Monte-Carlo simulation in the statistical software R to compute the appropriate sample size. We found that a total of 160 patients suffices to have about 90% power to detect any effect of bacterial infection. To be able to address the secondary goals of the study, we intend to include 160 lumbar and 160 cervical subjects.

For the statistical analysis, a linear mixed model will be used in which bacterial infection, MC and disc inflammation are used as a fixed factor and the NRS-scores of all time points (baseline, 8, 16, 26, 52 weeks) are used as an outcome measure. The model will be full factorial (assess all main and interaction effects). In addition, age, sex, nerve root compression and pain medication will be used as a covariate.

All secondary study parameters will be analysed by multiple tests including a Pearson/ Spearman correlation test, linear mixed model and a Chi-square test for categorical variables.

Discussion

At present, the guideline for radiculopathy is the same for all patients: a wait and see approach, and surgery is only offered to those with persevering symptoms. Even after surgery, for some patients the symptoms persist or return after a short period of relief. The great variety in how radiculopathy patients recover indicates that our 'one size fits all' model for treating radiculopathy requires refinement. Previous research has already indicated that inflammatory cells such as macrophages play a crucial role in recovery and that the extend of this inflammation response varies from patient to patient [2]. Moreover, the presence of inflammation is not always a beneficial sign, as recent studies have indicated that presence of MC seem to indicate a chronically irritating inflammation response [4], and others have found bacteria in herniated discs [13]. Such findings strongly indicate that refinement of our current treatment approach is needed and that radiculopathy patients should be further sub characterized based on their inflammation status. Up till now, the evidence for inflammation subgroups within radiculopathy is not convincing and most studies focus on only one aspect without incorporating the rest of the inflammation status. For example, many recent studies have focussed on proving bacterial presence in herniated discs, but none have assessed whether this has any effect on clinical outcome [13]. Other studies have tried to treat patients with anti-inflammatory drugs but failed to assess whether inflammation was present in these patients [20, 21]. Some studies have associated presence of MC with poor clinical outcome but failed to incorporate inflammation status [22]. Further, many studies required information regarding the inflammation status from analyzing the disc material from surgery and don't look for biomarkers in blood [23-26]. Other by contrast focus on drawing blood without verifying whether the blood results correspond to the status of the disc [27-29]. In order to create a more personalized treatment approach it is first essential to understand how which patients are affected by what kind of inflammation and how this affects clinical outcomes. Moreover, it is important to explore biomarkers in blood that reliably resemble these different inflammation statuses, so personalized clinical decisions can be taken in an early disease stage without invasive and costly diagnostic procedures. Therefore, the EIMICOR will be the first study to incorporate all various types of data on tissue and blood level and assess their relevance concerning clinical outcome. By doing so we aim to connect the dots and elucidate the complex role of inflammation in sciatica.

Possible operational issues

The design of the present study is aimed at reducing the amount of time and effort that is required from the participants and surgeons as much as possible. At the same time we focus on saving unnecessary costs by postponing additional analyses until our initial hypothesis is confirmed and additional analysis are more likely to reveal critical information. Unfortunately, this design comes with some practical issues that have to be dealt with. For instance, transferring the samples to the LUMC takes time and if surgery is scheduled late in the afternoon, it could mean that the samples cannot be processed in the LUMC the same day which may impact the quality of the data. Therefore it is crucial for the quality of our data that the inclusions are tailored to the OR schedules.

Moreover, as participants fill in the follow-up questionnaires through an email link, it makes it harder to control if they fill in the questionnaires, which may lead to missing data. In order to manage this issue, the status of the questionnaire will be monitored frequently and participants that forgot to fill in the questionnaires will be reminded to do so by email and phone.

List of abbreviations
MC = Modic Changes
LPS = Lipopolysaccharide
IFN-γ = Interferon-gamma
TNF = tumor necrosis factor (TNF), and expression of
GM-CSF = granulocyte macrophage colony-stimulating factor
M-CSF = macrophage colony-stimulating factor
IL = Interleukin
ICF = Informed consent form
NRS = Numerical Rating Scale
ODI = Oswestry Disability Index
NDI = Neck Disability Index
GPE = Global Perceived Effect
LUMC = Leiden University Medical Center

Declarations

Ethics approval and consent to participate
The protocol was approved by the Medical ethical committee METC-LDD

Consent for publication
All authors have read the final draft of the protocol and have approved it for publication

Availability of data and materials
Data and materials will be stored for 15 years after study completion.

Competing interests
None of the authors have a conflict of interest

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Authors' contributions
Niek Djuric: protocol design and writing, receiving EANS funding
Geraldine Lafeber: protocol design
Sjoerd van Duinen: coordination of the immunohistochemistry protocol and supervision
Sandra Bernards: coordination of the bacterial culture protocol and supervision
Wilco Peul: Supervision
Carmen Vleggeert-Lankamp: protocol design, recieving Imke Meyer fonds funding, Supervision.

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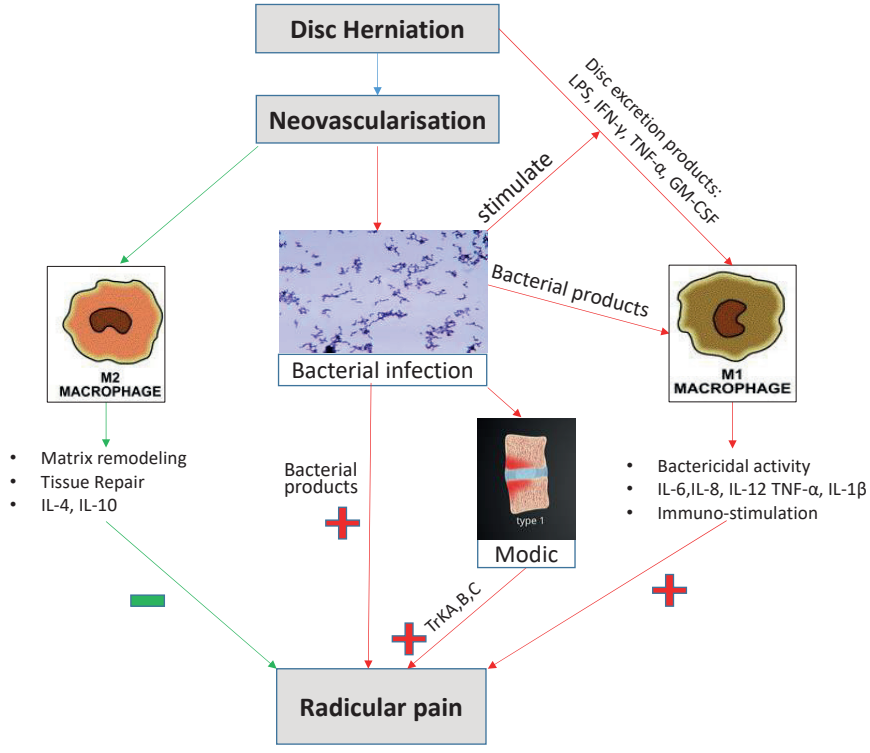
Appendix

Figure 1. Illustration of the hypothesis:



After the disc is herniated, neovascularisation can be formed, which could lead to a couple of scenario's. Firstly, in healthy discs, macrophages can enter and likely predominantly differentiate towards M2, which will help with disc resorption and reduce radicular pain. Secondly, likely in a degenerated disc, neovascularisation can be accompanied by infiltrating bacteria, which by itself may irritate the nerve and cause pain symptoms, can stimulate the disc to excrete pro inflammatory factors which worsen pain, may stimulate macrophages to differentiate towards M1. This could result in more M1 and less M2 macrophages and results in more radicular pain symptoms. In addition, the adjacent endplate might also get involved, which could lead to more irritation of the adjacent nerve.

Figure 2. Flow chart of the EIMICOR study

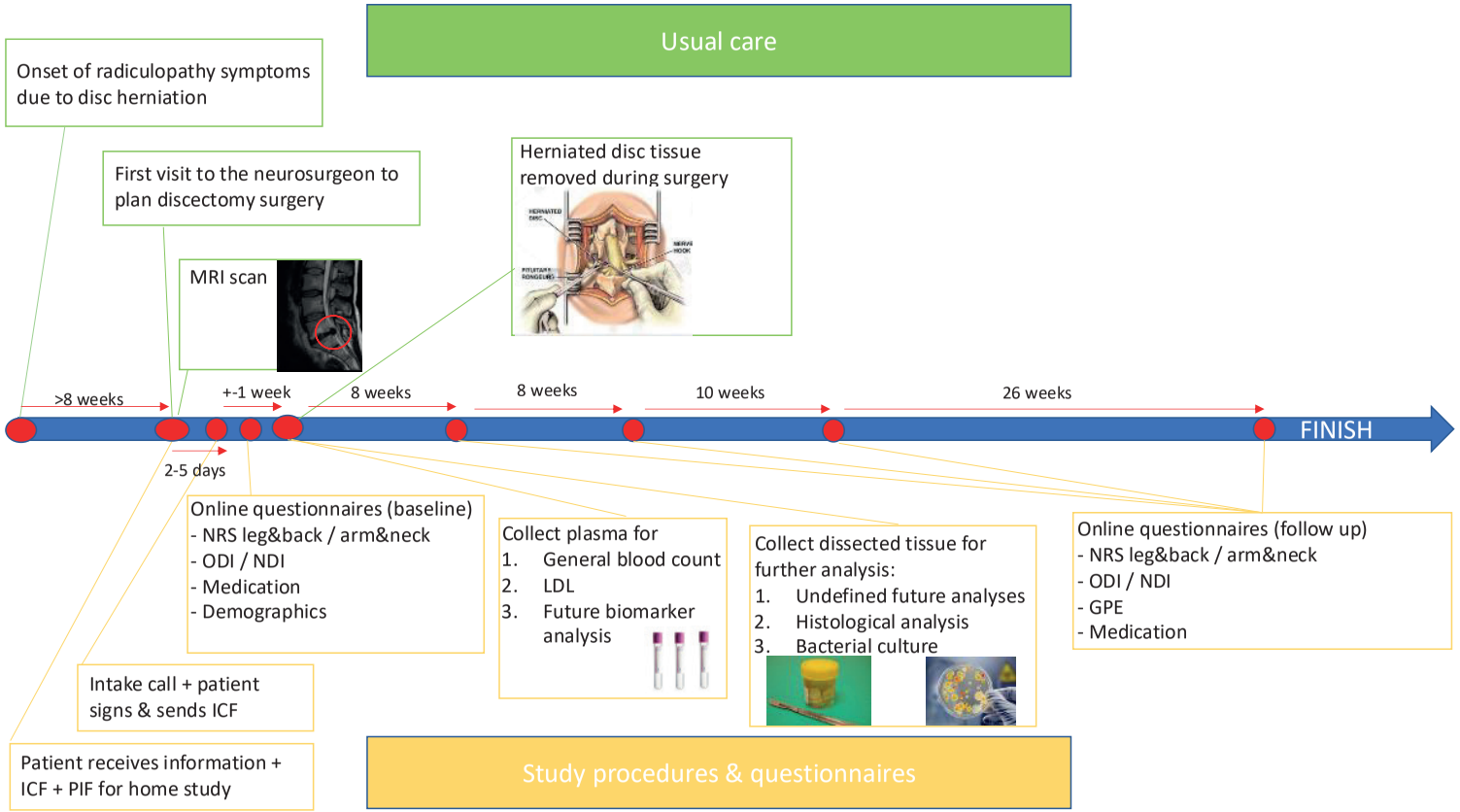


Figure 3. Subgroups with hypothesized average NRS scores during the one year follow-up

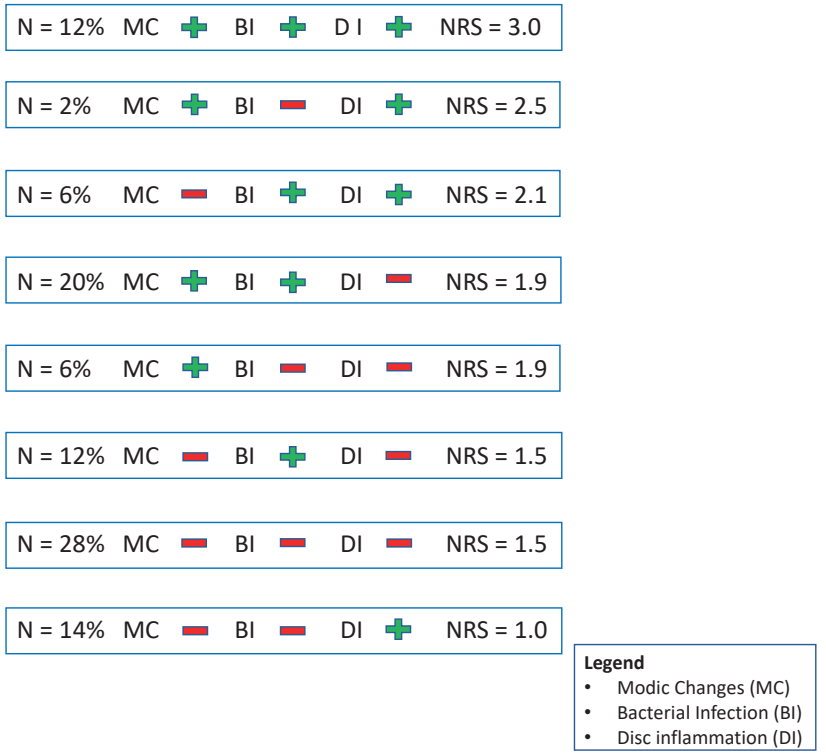


Figure 3 displays the hypothesized groups with predicted NRS scores. The first column shows the expected percentage of patients in that subgroup, the second shows the MC status, the third whether a bacteria in present, the fourth if inflammatory cells are present and the fifth what the expected average NRS score is during the one year follow-up.

Summary

It is highly likely that sciatica symptoms due to lumbar disc herniation are not only caused by mechanical compression of the nerve root, but also by pain inducing elements from inflammatory processes. This inflammatory response can be induced by macrophages that infiltrate the disc as a consequence of the herniation. By contrast, macrophages are also thought to influence recovery through resorption of the disc material. Hence, the presence and behavior of macrophages can have a strong influence on pain and recovery. Macrophage behavior depends on their differentiation, which can be polarized into either M1 or M2 macrophages: the M1 type being associated with pro-inflammatory processes and M2 with anti-inflammatory processes. In a systematic review, we demonstrated that high levels of M1 related pro-inflammatory cytokines (TNF-α, TNFR1, IL-6, IL-8, IFN- γ) were all associated with higher pain scores. In contrast, we showed that high levels of M2 related cytokines IL-4 and IL-10 associated with lower VAS scores. No associations were established for TGF-β. Moreover, presence of macrophages in general (CD68+) was associated with lower pain scores during follow-up.

Now that it is clear that macrophage infiltration in the disc matters for clinical recovery, we further verified their importance in recovery with radiological evidence. In a retrospective histological study, we assessed how macrophage infiltration affected the rate of resorption on MRI between baseline and one year post surgery and found that the degree of macrophage infiltration was not associated with herniation size at baseline, but significantly associated with reduction in size of the herniated disc at one-year post surgery.

Next, we proceeded with finding markers that predict the extent of macrophage infiltration and their behavior. The first marker of interest was the type of the herniation, complete extrusion versus disc bulging. The former was expected to result in more macrophage infiltration due to a higher exposure to the systemic circulation. The second was the presence of Modic changes (MC), which symbolizes fibrotic and/or inflammatory changes in the endplate. MC are believed to correlate with a pro-inflammatory environment, therefore a higher percentage of M1 macrophages is to be expected and thus inferior clinical outcomes. Firstly, we found that the degree of macrophage infiltration was higher in extrusion in comparison to bulging (protrusion) discs. Secondly, with regard to the clinical outcomes: we found the presence of MC to be predictable for macrophage behavior: For those without MC, macrophage infiltration (CD68) resulted in reduced leg pain during follow-up after surgery, but in patients with MC2 the opposite was seen. In this group, patients with considerable inflammation were significantly more disabled compared to patients with mild inflammation. When data was subsequently split for extruded and bulging discs, similar effects on clinical outcome were seen for extruded discs, but no significant effects in bulging discs. Taken together, this shows that macrophage infiltration most often occurs in extruded discs, and that, for many patients, macrophage infiltration means a quicker resorption of the disc and a quicker relief of pain symptoms. However, for patients with MC, detrimental macrophage behavior was observed, which resulted in poorer recovery after surgery.

In search of a more sensitive marker for inflammation than disc extrusion, we evaluated the reliability of gadolinium enhancement on MRI as a marker for macrophage infiltration. Degree of inflammation by macrophages was not associated with gadolinium enhancement of nerve roots or herniated discs. Therefore, gadolinium enhancement was regarded as an unreliable indicator for inflammation of disc herniation or nerve root in patients with sciatica.

Because MC are associated with a detrimental effect of macrophage infiltration on clinical outcomes, we proceeded by exploring the pathophysiology of herniated discs with MC in the adjacent endplate. Moreover, since MC involves the endplate, we also evaluated the effect of endplate avulsion, which is a mechanism of herniation in which the integrity of the disc is lost due to a tear in the endplate instead of a tear in the annulus fibrosus. In our proteomic and bioinformatic study, we were able to identify that MC were associated with an altered protein expression that signified a decrease in the pathway 'detoxification of reactive oxygen species (ROS)' (a decrease in the elimination of toxic products) and a decrease in the complement system and immune system. In contrast, compared to an annular tear, avulsion of the endplate was associated with an increase in coagulation and 'detoxification of ROS'. Together, this signifies that endplate avulsion is a traumatic injury of the endplate which is followed by a wound healing response. In contrast, MC illustrates a decrease in a healthy immune response, either through a decrease in quantity or an altered functionality/differentiation of factors involved in the immune response. When this result is combined with our previous findings that MC predicted a detrimental effect on the clinical outcome if macrophages had infiltrated, an altered differentiation seems to be the most likely cause for the 'decrease in a healthy immune response'.

This altered differentiation profile was further assessed in a histological pilot study. This study showed, in line with the previous findings that the main type of macrophage infiltration was M2 (CD163+), and that the percentage of M2 was lower in patients with MC as compared to those without.

In this study, we demonstrated that macrophages play a crucial role in lumbar disc herniations; in most cases, macrophages differentiate towards M2 and thereby speed up resorption of the disc to relieve patients from their sciatic symptoms. However, patients with MC are less likely to differentiate towards M2, which results in increased disability and reduces the rate of recovery. The pathophysiology behind the detrimental effects of MC is still not completely understood. Recent studies have found that MC are associated with a bacterial infection of the disc, which is known to shift macrophage differentiation away from M2 and towards M1, and thus may function as an explanation for the altered differentiation pattern in MC patients. Everything considered, we provide 3 subgroups of disc herniation patients that may benefit from different treatment approaches: firstly, the patients without macrophage infiltration, who have a herniated disc that will likely resorb slowly and may benefit from early surgery; secondly, patients with a M2-dominant inflammation response, who most likely have a herniated disc that will resorb spontaneously and will benefit from prolonged conservative care; thirdly, patients with a detrimental M1-dominant inflammation response, who's herniated discs are unlikely to resorb quickly, but decompression surgery will also not completely satisfy these patients as there is still an inflammatory response that may irritate the nerve root. This third type is likely to require additional antibiotic treatment, in case of a bacterial infection, or, if the inflammation in the disc has an autologous nature, may require arthrodesis surgery. In order to implement such personalized treatment strategies, we first need to discover non-invasive biomarkers, using MRI or blood samples, that can recognize macrophage infiltration and differentiation with high sensitivity and specificity. Moreover, role of bacteria and other causes that may influence macrophage differentiation in the disc should be further explored. Hence, in the last chapter, we outlined a new study protocol that aims at finding these biomarkers and unravelling the causes for alterations in macrophage differentiation: Effect of Infection, Modic and Inflammation on Clinical Outcomes in Radiculopathy (EIMICOR). Hopefully, with the results of this new trial, we will be able to implement personalized treatment strategies that will significantly improve recovery rate and reduce disease burden in all sciatica patients.

Summary in Dutch

Samenvatting

Ischias ten gevolge van een lumbale hernia wordt hoogstwaarschijnlijk niet alleen veroorzaakt door een mechanische compressie van de zenuwwortel, maar ook door pijn inducerende componenten van een ontstekingsproces. Deze ontstekingsreactie kan worden geïnitieerd door macrofagen wanneer zij de gehernieerde discus infiltreren. Echter kunnen macrofagen ook een gunstig effect hebben op het herstel, middels het resorberen van het gehernieerde discus materiaal. Hiermee speelt de aanwezigheid en het gedrag van macrofagen dus een cruciale rol in zowel de pijn beleving als het herstel proces. Het gedrag van de macrofagen is afhankelijk van hun differentiatie, welke kan worden gepolariseerd tot M1 en M2 macrofagen: M1 wordt geassocieerd met een pro-inflammatoir proces terwijl M2 met een anti-inflammatoir proces wordt geassocieerd. In een systematisch literatuur onderzoek demonstreerde we dat hoge concentraties van M1 gerelateerde pro-inflammatoire cytokines (TNF-α, TNFR1, IL-6, IL-8, IFN- γ) associeerde met hogere pijn scores. Daarnaast lieten we ook zien dat hoge concentraties van M2 gerelateerde anti-inflammatoire cytokines IL-4 en IL-10 associeerde met lagere pijnscores. Voor TGF-β werden geen associaties gevonden. Tevens werd de aanwezigheid van macrofagen (CD68) geassocieerd met lagere pijn scores tijdens de follow-up periode.

Nu het duidelijk is dat macrofaag infiltratie in de disc van belang is voor het klinische herstel, hebben we hun relevantie voor het herstel verder gevalideerd door middel van radiologisch bewijs. Met behulp van een retrospectieve histologische studie hebben we bekeken hoe macrofaag infiltratie de resorptie snelheid op MRI beïnvloedde tussen baseline en 1 jaar na de operatie.

Daarna zijn we, met dezelfde dataset, doorgegaan met het vinden van markers die de omvang van de macrofaag infiltratie en hun gedrag kunnen voorspellen. De eerste marker waar het oog op viel was het type hernia, waarbij complete uitdrijving (extrusie) werd vergeleken met uitpuiling (protrusie). Hierbij werd de hypothese gesteld dat extrusie zou resulteren in een omvangrijkere macrofaag infiltratie; aangezien dit type een grotere blootstelling heeft aan de systemische circulatie. De tweede marker was de aanwezigheid van Modic Changes (MC), die symbool staan voor fibrotische / inflammatoire veranderingen in de eindplaat. Van MC word geloofd dat ze correleren met een pro-inflammatoire omgeving, waardoor er een hoger percentage M1 macrofagen wordt verwacht en dus slechtere klinische uitkomsten bij een grotere hoeveelheid macrofagen.

De uitkomsten van dit onderzoek toonden ten eerste dat de mate van macrofaag infiltratie groter was in extrusie in vergelijking met protrusie disci. Ten tweede, met betrekking tot de klinische uitkomsten, vonden we dat de aanwezigheid van MC bepalend was voor het gedrag van macrofagen: Bij degene zonder MC resulteerde macrofaag infiltratie (CD68) in een vermindering van been pijn tijdens de follow-up na de operatie, Het tegenovergestelde effect was te zien bij patiënten met MC2, waar patiënten met macrofaag infiltratie significant meer door pijn werden belemmerd in hun dagelijkse leven in verlegijking met patiënten zonder inflammatie.

Wanneer de data vervolgens werd gesplit voor extrusies en protrusies, werd hetzelfde effect gezien op de klinische uitkomsten bij extrusies, terwijl bij protrusies geen significante resultaten werden gevonden. Samen genomen laat dit zien dat macrofaag infiltratie het meest voorkomt bij extrusies en dat dit voor de meeste patiënten betekent dat de hernia sneller resorbeert en de pijn sneller afneemt. Aan de andere kant wordt bij patiënten met MC een nadelig effect van macrofagen vastgesteld, wat resulteert in een langzamer herstel na de operatie.

Zoekende naar een gevoeligere marker voor ontstekingen dan extrusie van de discus, evalueerde we de betrouwbaarheid van gadolinium aankleuring op MRI als een marker voor macrofaag infiltratie. De mate van ontsteking, gemeteten als hoeveelheid macrofagen, werd niet geassocieerd met gadolinium aankleuring van de zenuw of discus. Gadolinium werd dus beschouwd als een onbetrouwbare indicator om ontstekingen van de discus of zenuw wortel vast te stellen in patiënten met sciatica. Omdat MC associeerden met een nadelig effect van macrofaag infiltratie op de klinische uitkomsten, hebben we ons verder verdiept in de pathofysiologie van hernia's met MC in de aanliggende eindplaat. Aangezien MC een pathologie is van de eindplaat, zijn de effecten van eindplaat avulsie ook nader onderzocht. Eindplaat avulsie is een mechanisme van herniatie waarbij de integriteit van de disc verloren gaat door een scheur in de eindplaat in plaats van een scheur in de annulus fibrosus. In onze Proteomic en bioinformatica studie vonden we dat MC associeerden met een verandering in eiwit expressie in de nucleus pulposus, deze verandering symboliseerde een vermindering in de 'detoxificatie van reactive oxygen species (ROS)' (een vermindering in het onschadelijk maken van schadelijke producten) en een vermindering in de aanwezigheid van het complement systeem en immuun systeem.

Daarentegen werd avulsie van de eindplaat, in vergelijking met een annulus scheur, geassocieerd met een toename van coagulatie en detoxificatie van ROS. Samengenomen kan avulsie van de eindplaat gezien worden als een traumatisch letsel van de eindplaat waarna een wondgenezing proces van het lichaam volgt, terwijl MC een afname van een gezond immuunsysteem symboliseert, welke wordt veroorzaakt door een afname in kwantiteit of door een verandering in functionaliteit/differentiatie. Wanneer deze bevinding wordt gecombineerd met de eerdere bevindingen waarbij MC associeerde met een negatief voorspellende waarde van macrofaag infiltratie op de klinische uitkomsten, is een verandering in differentiatie profiel de meest aannemelijke oorzaak voor de gevonden 'afname van een gezond immuun systeem'. Dit differentiatie profiel is nader uitgezocht in een histologische pilot studie. In lijn met de verwachtingen toonde de resultaten van deze studie dat M2 (CD163+) het dominerende type macrofaag was in discus samples en dat het percentage M2 lager lag in patiënten met MC in vergelijking met degene zonder MC.

In deze studie hebben we gedemonstreerd dat macrofagen een cruciale rol spelen in lumbale discus hernia's, in de meeste gevallen differentiëren macrofagen naar M2 waardoor ze het resorptieproces van de discus bevorderen en de patiënten sneller van hun ischias klachten af zijn. Maar bij patiënten met MC differentiëren ze minder naar M2, wat leidt tot een toename in belemmeringen in het dagelijkse leven en een langzamer herstel. De pathofysiologie die ten gronde ligt aan de nadelige effecten van MC wordt nog steeds niet helemaal begrepen. Recente studies toonden aan dat MC geassocieerd zijn met bacteriële infecties van de discus, hetgeen een bekende trigger is voor een shift in differentiatie profiel van M2 naar M1 en dus een verklaring zou kunnen zijn voor het afwijkende differentiatieprofiel bij MC patiënten. Samen genomen zijn er 3 subgroepen hernia patiënten, die elk een eigen behandelingsstrategie nodig hebben: Ten eerste, de groep met hernia's zonder macrofaag infiltratie, waarbij de hernia waarschijnlijk langzaam spontaan zal resorberen en de patiënt zal baten bij een vroege operatie. Ten tweede, een groep met een M2 dominante inflammatie response, waarbij de hernia waarschijnlijk spontaan zal resorberen waardoor deze groep het meeste baat zal hebben bij een conservatief beleid. Ten derde, patiënten met een nadelige M1 gedomineerde inflammatie response, waarbij het onwaarschijnlijk is dat de hernia spontaan resorbeert en een operatie ook de klachten niet volledig weg zal halen; aangezien er dan een ontstekingsreactie achter kan blijven die de zenuw blijft irriteren. Deze derde groep heeft wellicht een additionele antibiotica behandeling nodig indien er sprake is van een infectie; wanneer de inflammatie daarentegen een autologe aard heeft, is een arthrodese operatie wellicht aan de orde.

Voordat zulke gepersonaliseerde behandelstrategieën kunnen worden geïmplementeerd, dienen we eerst niet-invasieve biomarkers te identificeren middels MRI of bloed samples, die macrofaag infiltratie en differentiatie kunnen herkennen met hoge sensitiviteit en specificiteit. Verder zal ook de invloed van bacteriën en andere oorzaken op macrofaag differentiatie in de discus verder moeten worden onderzocht. Daarom bevat het laatste hoofdstuk een nieuw studie protocol dat gericht is op het vinden van deze biomarkers en het ontrafelen van de factoren die macrofaag differentiatie beïnvloeden: "Effect of Infection, Modic and Inflammation on Clinical Outcomes in Radiculopathy" (EIMICOR). Hopelijk kunnen we met de resultaten van deze nieuwe trial gepersonaliseerde behandelingsstrategieën gaan implementeren die de hersteltijd en de ziektelast van alle patiënten significant zullen verbeteren.

Curriculum Vitae

Niek Djuric was born in Hilversum, the Netherlands on the 21st of February 1995. In 2013 he obtained his high school diploma from the 'Gemeentelijk Gymnasium Hilversum'. That same year, he won the 'EGERIA fund for young entrepreneurs' for his idea to provide personal training and diet advice online. After he finished high school he started studying medicine at the University of Leiden. In 2014, whilst continuing his medicine study, he started a second bachelor, biomedical sciences at the VU University Amsterdam. He finished his bachelor in medicine in 2016 and biomedical sciences in 2017. During the last year of biomedical sciences he began with the MD-PhD track at the neurosurgery department in the Leiden University Medical Center (supervisors Prof. dr. W.C. Peul and Dr. C.L.A. Vleggeert-Lankamp), during which he focused on the role of inflammation in sciatica. In 2017, after being inspired by the neurosurgery department, he continued his studies with a master in Neuroscience at the VU university, during which he went to Ganga Hospital in Coimbatore, a city in the south of India for a 5 month fellowship in the beginning of 2019. At Ganga hospital, he worked as a researcher in a lab specialized in proteomics. He subsequently finished his master's degree in early 2020, which was around the same time that he started his masters in Medicine. During his days as a student, he worked as an independent contractor in multiple pharmacies where he consulted patients with multiple medications that often suffered from side effects. As a PhD student, he won the 'EANS research fund' for the EIMICOR study protocol, and got to present his work at various congresses: the 19th European congress of Neurosurgery in Dublin, 21st EUROSPINE meeting in Helsinki, Dutch Spine Society annual meeting in Rotterdam and the 47th ISSLS annual meeting.

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