A minimal-invasive method to retrieve and identify entheseal tissue from psoriatic arthritis patients

Eine minimal-invasive Methode zur Entnahme und Identifizierung von enthesialem Gewebe bei Psoriasis-Arthritis-Patienten

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1 Zusammenfassung

1.1 Hintergrund und Ziele

Die Enthese ist eine spezialisierte Grenzfläche, die aus dichten Bindegewebsfasern besteht und den Ansatz von Sehnen und Bändern am Knochen vermittelt. Die Enthesitis, definiert als Entzündung dieser Ansatzstellen, ist ein charakteristisches Merkmal der Spondyloarthritis (SpA), einschließlich der Psoriasis-Arthritis (PsA). Sie tritt bei etwa 30 % der PsA-Patienten auf und gilt als frühes primäres Ereignis in der Pathogenese der Krankheit. Daten zur Enthesitis bei PsA stammen aus MRT- oder Ultraschallstudien, die für die Formulierung der Diagnose, die Abschätzung der Gewebeschäden und die Beurteilung des Ansprechens auf die Behandlung von entscheidender Bedeutung sind. Trotz dieser Beiträge sind die molekularen Mechanismen, die der Enthesitis zugrunde liegen, noch immer nicht vollständig verstanden.

Eine qualitativ hochwertige Entnahme von menschlichem Gewebe hat entscheidend zum besseren Verständnis von Arthritiden wie der rheumatoiden Arthritis (RA) beigetragen. Tatsächlich wird die minimalinvasive, Ultraschall (US)-geführte Entnahme von Synovialgewebe routinemäßig sowohl für diagnostische Ansätze als auch für Forschungszwecke eingesetzt, um die pathophysiologischen Mechanismen der Krankheit zu klären und neue therapeutische Ziele zu ermitteln. Synovialgewebe ist jedoch leicht zugänglich und verfügt über eine gut definierte anatomische Struktur. Biopsien von Enthesen werden dagegen sehr selten durchgeführt und sind technisch anspruchsvoll. Dies liegt vor allem daran, dass ein vollständiges Präparat gewonnen werden muss und eine offene Operation erforderlich ist. Diese Einschränkungen sind der Grund für den derzeitigen Mangel an Studien, die sich auf enthesiales Gewebe bei Patienten mit PsA konzentrieren.

Hier schlagen wir einen minimalinvasiven, US-geführten Biopsieansatz vor, der keine gleichzeitige Entnahme von Knochengewebe erfordert und eine Second Harmonic Generation (SHG)-Mikroskopie beinhaltet, die auf dem Prinzip der Verdopplung der Schwingungsfrequenz des eingestrahlten Lichtes beruht, um das Vorhandensein von enthesialem Gewebe innerhalb der entnommenen Probe zu bestätigen.

1.2 Methoden (Patienten, Material und Untersuchungsmethoden)

Ellenbogen von fünf Körperspendern wurden aus dem Institut für Anatomie entnommen und zehn PsA-Patienten mit Ellenbogen-Enthesitis wurden rekrutiert. Die Biopsie (5 mm) aus den Strecksehnenansätzen des lateralen Epikondylus wurde mit einer Blakeslay-Zange unter Ultraschallkontrolle durchgeführt. Bei den Körperspendern wurde die gesamte Enthese einschließlich der angrenzenden Sehne, des Muskels und des Knochens nach der Biopsie für die weitere Analyse chirurgisch entfernt. Alle Materialien (Kadaverbiopsien, PsA-Biopsien, Kadaverresektionsmaterial) wurden mit 4 % Formalin fixiert, in Paraffin eingebettet und in 2-5 µm große Schnitte geschnitten. Das Material wurde mit Hämatoxylin/Eosin, Safranin O und Trichrom gefärbt oder ohne Färbung mit einem Multiphotonenmikroskop (Zeiss LSM 880 NL, Jena, Deutschland) zur Erfassung der SHG-Signale analysiert. Die Bildverarbeitung und die Quantifizierung der SHG-Intensität wurden mit Image J Software V.1.52 durchgeführt. Die statistischen Ergebnisse wurden mit R V.4.0.1 (R Foundation for Statistical Computing) und Prism V.8 (GraphPad Software) visualisiert und ausgewertet.

1.3 Ergebnisse und Beobachtungen

Die *B-Mode*-Ultraschalluntersuchung der lateralen Epikondylus-Enthese wurde an fünf Kadavern durchgeführt. Entlang der Linie zwischen dem lateralen Epikondylus und dem Radiuskopf wurde ein Einschnitt vorgenommen und durch den Einschnitt wurde eine Blakesley-Zange eingeführt. Eine 5 mm große Biopsie der Sehnenplatte der Streckmuskeln (Digitorum communis, Digitus minimus und Carpi radialis) wurde entnommen (= Kadaverbiopsieproben).

Die histologische Analyse der Kadaverresektionspräparate zeigte den angrenzenden Knochen (blau in Safranin O) und Muskel (rot in Trichrom) sowie die Enthese zwischen Knochen und Sehne. Die Induktion von SHG durch Multiphotonenmikroskopie ermöglicht die Visualisierung der Zusammensetzung von Strukturproteinen. Die SHG-Intensität (SHG-I) jedes Gewebes (Knochen, Enthese, Sehne, Muskel) wurde in verschiedenen Gewebsregionen (ROI) gemessen und auf den höchsten Intensitätswert (interne Kontrolle; =100%) normiert. Sehnen wiesen stets den höchsten SHG-I-Wert auf (Mittelwert±SD: 91±13%), gefolgt von Knochen (80±12%), während Muskeln den niedrigsten SHG-I-Wert (12±5%) aufwiesen. Enthesen zeigten ein einzigartiges intermediäres Signal (31±6%), das sich statistisch von den anderen Geweben unterschied.

Wir verwendeten SHG, um den enthesialen Anteil in Biopsieproben von Kadavern zu validieren. Wir konnten 68% der Biopsieproben als Enthesengewebe identifizieren. Derselbe Ansatz wurde dann zur Biopsie der lateralen Epikondylus-Enthese bei 10 PsA-Patienten verwendet. Die Standard-Histochemie erlaubte keine Unterscheidung zwischen Enthese Sehne und Muskel. Im Gegensatz dazu konnten wir anhand der vordefinierten Cutoffs zeigen, dass 65% der PsA-Gewebeprobe aus Enthesengewebe bestand. Dies stimmt mit unseren früheren Beobachtungen aus Kadaverbiopsien überein.

1.4 Schlussfolgerungen und Diskussion

Bislang ist sehr wenig über die zelluläre und molekulare Zusammensetzung menschlicher Enthesen bekannt. Nur wenige Studien haben Biopsien aus peripheren menschlichen Enthesen durchgeführt. Hier schlagen wir einen sicheren, gut verträglichen, minimalinvasiven, US-geführten Biopsieansatz der radialen Ellenbogenenthese vor, die auch eine der häufigsten Stellen für Enthesitis bei PsA ist. In dieser Studie haben wir uns die intrinsischen Eigenschaften der Kollagenfasern zunutze gemacht, die die Enthesen beherbergen, und wir haben einen Bereich von SHG-I beschrieben, der Enthesen identifiziert und von anderen umgebenden Geweben abgrenzt. Diese Ergebnisse ermöglichten es uns, Enthesengewebe in den Biopsien zu erkennen und zu bestätigen. Dieses neu etablierte, standardisierte Biopsieverfahren mit der angeschlossenen Methode zur Sicherstellung der Gewebequalität könnte nützlich sein, um die molekularen und zellulären Veränderungen menschlicher Enthesen bei Krankheiten und die spezifischen Auswirkungen entzündungshemmender Behandlungen bei Enthesitis zu untersuchen.

2 Introduction

2.1 Enthesis

2.1.1 Definition and function

The word enthesis originates from the ancient Greek adjective “enthetic” and the word “enthetikos” meaning: “introduced into the body from without” [1]. In the nineteenth century, the term was adopted to describe diseases that were “implanted into the body from external sources” [1]. The current terminology, as we use it nowadays, started in the twentieth century referring to the insertion sites of soft tissue into the bone surface [2]. Indeed, the term enthesis describes a specialized interface through which tendons and ligaments insert into the bone, facilitating the transition from “soft” to “hard” tissue [3]. This 500 μm-thick transition area has the highly sophisticated functions to transduce mechanical forces from the muscles to the skeletal system and to provide stability, both essential requirements for correct locomotion [3]. To satisfy these requirements, enthuses need to overcome high biomechanical stress, which in extreme situations could trigger an inflammatory response [3]. Investigations of the enthesis under pathological conditions showed that the inflammatory process does not limit itself to the entheseal area, but involves also the neighborhood environment, including the adjacent bone and soft tissues [4]. These changes, identified through magnetic resonance imaging (MRI) and ultrasound (US), supported the new definition of the enthesis as complex organ, constituted by a group of tissues, such as fibrocartilages, bursa, fat pad, adjacent trabecular bone, deeper fascia and the enthesis itself [4, 5]. The new definition of “enthesis organ complex” has a functional meaning, since only the whole assembly together is able to guarantee the dissipation of stress concentration away from the insertion site [5].

2.1.2 Structure of enthesis: macro and micro-anatomical characteristics

The ability of entheses to transmit contractile forces from muscles to bones and simultaneously to dissipate forces away from themselves is also possible thanks to their tissue properties [6]. In the last decades, the macroscopic and microscopic aspects of entheses have been largely investigated in order to gain knowledge on the anatomical characteristics and mechanical properties of these specialized interfaces. These transition zones are generally characterized by loss of the typical alignment and orientation of the tendon fibers and changes in the collagen content [3]. Despite some general and common observations, in our body there are different kind of entheses. The first classification was proposed by Biermann (1957) and Knese &

Biermann (1958) and was based on the localization of entheses on long bones: the chondral-apophyseal entheses are located at the end of long bones, whereas the periosteal-diaphyseal entheses at the shafts [6]. According to a more recent classification, the previous entheses can also be described as fibrocartilaginous and fibrous, respectively [7]. This new definition takes in consideration the microanatomy of the insertion sites.

2.1.2.1 Fibrous entheses

Fibrous entheses attach either directly to the bone or indirectly through the periosteum, allowing a further classification into "bony" and "periosteal" [8]. They are in both cases constituted by dense fibrous connective tissue, which inserts in the diaphysis of long bones. These entheses attach typically over a large area through mineralized collagen fibers, which represent a continuum between the ligaments/tendons and the bone [8]. Fibrous entheses have been less investigated so far, since they are rarely injured and less involved in inflammatory processes compar to the fibrocartilagineous one [6]. Indeed, this difference reflects the functional requirements to which different entheses have to respond. Fibrous entheses, such as the deltoid, have straight insertions, which allow a limitation in the tension applied for example during abduction of the arm, in comparison to the entheses of the rotator cuff tendons. The reduced mechanical stress affecting the fibrous entheses explains why these are structurally less complex than the fibrocartilaginous one and less prone to damage [9].

2.1.2.2 Fibrocartilaginous entheses

The majority of entheses in our body are typically localized on epiphyses or apophyses and are characterized by a fibrocartilage interface between tendons/ligaments and bone surface [9]. These kind of entheses are anatomically constituted by 4 different zones (Figure 1), which follow a continuous collagen gradient, according to the different biomechanical needs of each layer [9, 10].

Zone one:

The zone one is represented by pure tendinous or ligamentous tissue in linear aligned fibers, mainly constituted by type I collagen, some type III collagen as well as elastin and proteoglycans. This area is populated by tenocytes with elongated morphology [11, 12].

Zone two:

This area is characterized by an avascular uncalcified fibrocartilage with higher density of tenocytes, which start to acquire a more round shape and a "chondroid" morphology becoming

fibrochondrocytes [9, 10]. The matrix is also different compared to the zone one, with a higher amount of proteoglycan, in particular aggrecan. The collagen content is also qualitatively different, since a progressive transition takes place from type I collagen to type II and III [9, 10].

Zone three:

A tidemark signals the transition between zone two and three [9]. This is a basophilic line that separates uncalcified (zone two) from calcified fibrocartilage (zone three) and better defines the transition between soft and hard tissue. The calcified fibrocartilage is an avascular area also populated by fibrochondrocytes surrounded by a more predominant type II collagen. Here the regularity of the tendinous or ligamentous fibers alignment is lost. This zone identifies the real anchor to the bone, since the collagen fibers form a net together with the subchondral bone [11, 12].

Zone four:

Zone four is characterized by bone tissue, which is highly vascularized in order to provide nutrients and oxygen to the rest of the enthesis. Osteoclasts, osteoblasts and osteocytes populate the bone in a matrix constituted by type I collagen and carbonated apatite mineral [9].

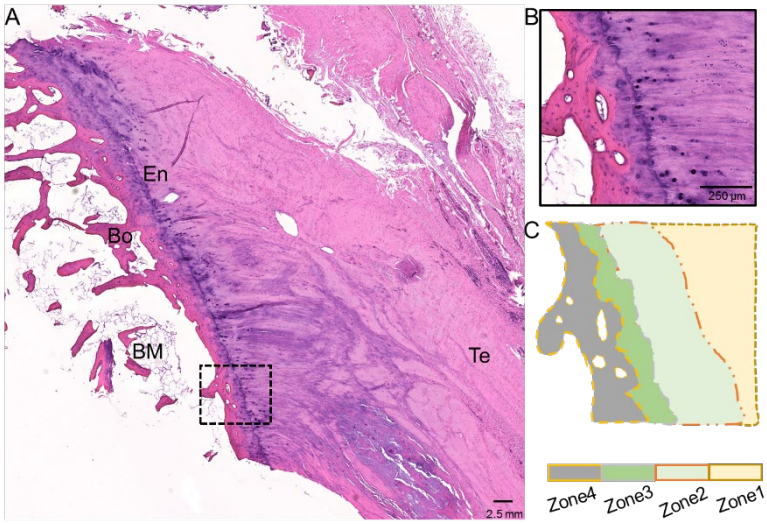

Figure 1. Enthesis of the triceps muscle.

(A) Hematoxylin & Eosin staining of the enthesis (BM = bone marrow; (B) Bo = bone; En = enthesis; Te = tendon); (B) 10x magnification of the enthesis and its cartoon (C) with spatial definition of the four zones.

2.1.3 Enthesitis: definition

High biomechanical stress can affect entheses triggering a local inflammatory response, called enthesitis [2]. Briefly, inflammatory cells, such as monocytes and lymphocytes infiltrate the tissues recruited after tissue damage, causing distraction of the superficial fibrocartilage and

subsequent neovascularization at sites where synovium, subchondral bone and bone marrow are close to each other [13]. In particular, formation of capillary-like vessels has been described between inflamed entheses and bone marrow [5, 14, 15]. On a later stage, the adjacent bone reacts causing ectopic bone formation, like surface spurs named enthesophytes [2]. Enthesitis can occur also in the context of autoimmune diseases, such as spondylarthritis (SpA), including psoriatic arthritis (PsA), as described in more details in the below sections [13].

2.2 Psoriatic arthritis

Psoriasis (PsO) is a common inflammatory disease, which affects around 2-3% of the population. Although the skin represents the main target of the disease, a variety of other clinical conditions can coexist at the same time [16]. Among these, the musculoskeletal involvement represents the most relevant and prevalent comorbidity, since it occurs in around 30% of the cases [16]. Indeed, psoriatic arthritis (PsA) is a complex inflammatory disease characterized by chronic inflammation of both the peripheral and axial skeleton, which leads to long-lasting functional disability and reduced quality of life [17]. In the context of PsA, entheses, defined as insertions of tendons and ligaments into the bone surface, are frequent targets of the inflammatory process and enthesitis, defined as inflammation of entheses, represents one of the hallmarks of the disease [18].

2.2.1 Epidemiology

The prevalence of PsA was thought to be rare before the introduction of the Classification Criteria for Psoriatic Arthritis (CASPAR) published in 2006 [19]. New data based on the CASPAR criteria suggested that PsA occurs in around 30% of patients with PsO with a prevalence between 30 to 100 cases per 10,000 in the U.S. population [20]. Skin manifestations generally precede joint involvement by an average of 10 years, although in around 15% of cases PsO and PsA can occur simultaneously or PsA can even anticipate the skin disease. In comparison to other autoimmune diseases, the male-to-female ratio is 1:1 and it is more prevalent in the caucasian population [17, 21]. PsA can also start in the childhood. However, prevalence and phenotype of PsA among children is quite different. According to the International League Association for Rheumatology (ILAR) two main clinical subtypes have been described: oligoarticular PsA which affects 1 to 2 years old children, with an involvement limited to a few number of joints, antinuclear antibody positivity and chronic uveitis [22]; oligo-

or polyarthritis affecting 6 to 12 years old children with a genetic association for the HLA-B27 [23].

2.2.2 Pathogenesis

PsA pathogenesis has not been completely understood, yet. PsO precedes PsA onset in about 70% of cases by an average of 7 years [24]. These data support the hypothesis of an existing crosstalk between the skin and joints, although the molecular mechanisms involved still need to be defined. Nevertheless, it is reasonable to believe that the transition from PsO to PsA is the result of a complex interplay between genetic predisposition, environmental factors and immune response dysregulation [18].

Genetic factors

Several studies addressed and supported the strong genetic contribution in both PsO and PsA pathogenesis. Evidence suggested that patients with PsO have an increased risk (recurrent risk ratio of about 40) of developing musculoskeletal symptoms, if they have first degree relatives affected by PsA. Moreover, patients without a history for skin disease, but with a first-degree familiarity for PsO have *per se* an increased chance to develop PsA [24, 25].

Although, PsO and PsA showed a partial overlap between gene susceptibility, disease-specific loci have been identified, in both Human Leukocyte Antigen (HLA)-associated and non-HLA associated genes, as possible explanation of the different disease heterogeneity [18]. Among the HLA-associate genes, HLA-C*06:02 was found in around 60% of the PsO patients, whereas alleles of the HLA-B (B*08, B*27, B*38) were strongly associated with PsA [26]. The relevance of the HLA class I molecules in PsA pathogenesis has also been supported by several studies reporting a clonal expansion of CD8+ T cells, which correspond to the cytotoxic arm of the adaptive immune response, able to interact with the HLA class I [27]. Moreover, specific variations of the HLA genes seem to associate not only with susceptibility for the disease, but also with specific phenotypes. Indeed, HLA-B*27 is associated with a shorter interval between skin and joint manifestations, whereas HLA-C*06 with a longer one [28].

Gene associations and differences between the two diseases have also been described in the non-HLA loci. In particular, genome-wide association studies showed that polymorphisms in the genes encoding the interleukin 23 (IL-23) receptor (IL-23R) and tumor necrosis factor-induced protein 3 (TNFAIP3) were more stronly stronger associated with PsA than PsO [29, 30].

Environmental factors

Several environmental factors have been considered to play a role in PsA. Among these, infections, obesity, smoking and microtrauma seem to be involved in the autoimmune process [18]. The connection between PsO and infections has been largely investigated and so far a link between streptococcal infection and guttate psoriasis have been established [31]. Moreover, PsO and PsA tend to be more common and more severe in patients with HIV infection, where the CD4+ CD8+ T cells ratio decreases [32]. It is however still unclear if PsA is triggered by the HIV-infection or by the depletion of CD4+ T cells and the predominance of CD8+ T cells. Microbiota changes have also been investigated in both PsA and PsO. Specifically, the microbiota in new onset PsA compare to PsO patients or healthy individuals had a low intestinal diversity with a reduction of the commensal microbiota in PsA patients as compared with healthy individuals [17, 33]. In general, subclinical gut inflammation and dysbiosis have been described in PsA patients. As for trauma and obesity, several studies highlighted that both could contribute to an increase biomechanical stress at the entheseal sites and eventually work as a second hit for the development of enthesitis [3]. Data on smoking are currently controversial. Smoking seems to increase the risk of developing PsA in healthy controls, but seems to play a protective role in patients with PsO [34]. Further studies on this matter are needed.

Immune response

PsO and PsA are both chronic immune-mediated diseases, which share pathogenic pathways and overexpression of key pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-23/interleukin 17 (IL-17) [25]. One of the proposed models of PsO-PsA transition involves the spreading of the autoimmune process from the skin to the joint. Specifically, some evidence suggest that primed antigen-presenting cells localized in the skin could engage naive T cells leading to a local expansion of T helper and CD8+ cytotoxic T cells. A clonal expansion of CD8+ T cells has also been described in psoriatic synovium, where these cells express tissue-homing markers, including skin and gut-homing markers [35]. Moreover, circulatory skin-derived tissue-resident memory CCR10+CD8+ T cells were found to be increased in PsA patients compared to PsO patients [36]. The leukocyte migration hypothesis is also supported by the increased synovial angiogenesis described in patients with PsA as compared to RA. Indeed, the lining layer in PsA has increased number of blood vessels, with elongated and tortuous characteristics, compared to the thickened avascular synovial lining in RA [37-39]. Moreover, the increased sprouting of vessels with immature characteristics correlates with an

increased expression in the PsA patient's synovium of growth factors, such as vascular endothelial growth factor (VEGF) [39].

As mentioned before, also the cytokine milieu involved in PsO and PsA is overlapping. Specifically, IL-23 and IL-17 have been detected in the skin and synovial tissues of PsA patients, as well as in the skin of PsO patients, and the adoption of a treat-to target therapeutic strategy blocking these cytokines has been successfully engaged in the clinic to reduce the activity and the severity of both diseases [16]. Moreover, IL-17 producing cells, such as type 17 helper T cells (Th17) and type 3 innate lymphoid cells (ILC3s), have been found increased in the synovial fluid of PsA patients compared to RA patients [40, 41]. The importance of IL-23 in the PsO-to-PsA transition has also been addressed *in vivo*, where the skin specific overexpression of IL-23 could alone initiate psoriatic-like lesions and on a later stage lead to PsA features [42]. Concurrently, other data showed that the systemic overexpression of IL-23 *in vivo* could recreate PsA features [43]. Synovial fibroblasts in PsA play also a role in the disease pathogenesis [16]. Angiogenic growth factors, chemokines and adhesion molecules derived from fibroblast-like synoviocytes contribute to the recruitment and migration of inflammatory cells [44]. Moreover, these cells might contribute to the bone remodeling process, inducing osteoclastogenesis through the increased expression of the receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL), TNF and IL-7 [45]. RANKL binds to RANK expressed on osteoclast precursors inducing the differentiation towards mature resorbing cells [17]. Some evidence suggested that the precursors of osteoclasts in the entheseal sites derived from circulating CD14+ monocytes, which are found to be increased in active PsA patients as compared to healthy controls and tend to decrease upon TNF blocking treatments [46].

2.2.3 Diagnosis

Clinical Manifestation

As for other inflammatory joint diseases, PsA is characterized by fatigue, early morning stiffness for > 30 min, joint tenderness and/or swelling, pain aggravated by rest and ameliorated by movement and improvement under non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroid treatment [18]. The articular manifestations in PsA can be divided in peripheral and axial involvement [17]. Peripheral arthritis is the predominant feature. Here, the number of joints affected from the disease can vary, resembling either an oligoarthritis, with up to four joints involved, or a polyarthritis with five or more joints involved [17]. Typical features in PsA compared to other inflammatory joint diseases are: the involvement of the distal interphalangeal joints, present in around 30% of the patients [47]; the enthesitis, which is present in up to 67%

[47-49] of the patients on presentation, and dactylitis observed in 12-39% of the patients on presentation [47-50]. The latter affects mostly the third and fourth toes, can be acute or chronic and is associated with a more severe disease course [50]. The axial involvement in PsA is present in 5-28% of patients, but could be detected in up to 70% of patients with late-stage disease [47-49]. Moreover, the prevalence of an isolated axial involvement in patients with PsO is around 7-17% [51].

Laboratory testing and Imaging findings

PsA compared to rheumatoid arthritis (RA) does not have specific investigations, which can confirm the diagnosis. The absence of rheumatoid factor (RF) and anti-cyclin citrullinated peptide antibodies (anti-CCP) is observed in 95% of the patients [17]. However, the presence of a positive test does not completely exclude the diagnosis [18]. Systemic inflammatory markers such as C-reactive protein (CRP) could be found increased at presentation with a variety between 33 and 89% of the cases [47, 52]. The contribution of laboratory findings alone is limited in the diagnostic process, although the possibility to find biomarkers still remains an open task. A large contribution comes from the imaging evaluation of the articular and periarticular areas, which are also included in the classification criteria (see below). Radiologic findings in PsA with peripheral involvement include bone loss, eccentric erosions, new bone formation with periostitis, bony ankylosis and enthesophytes, which define the abnormal bone deposition at the attachment of tendons and ligaments [53]. This combination of bone and cartilage destruction occurring together with pathological bone formation constitutes one of the most distinctive aspects of PsA [17]. Damages in the cartilage and in the bone however mainly reflect a long lasting course of the disease, hence are generally absent at disease onset. Some data showed that around 27% of patients presented small erosions affecting a limited number of joints at the time of diagnosis [54]. Studies on MRI as well as Power Doppler US confirmed the utility of both techniques in the early stage of the disease, being able to identify synovitis, tenosynovitis, focal erosions, enthesophytes as well as bone marrow edema in the case of MRI and enhanced blood flow of US [55]. The axial involvement in PsA includes unilateral sacroiliitis as well as bulky paramarginal and vertical syndesmophytes [56]. Here, MRI evaluation represents so far the best method in exploring both bone as well as soft tissue involvement.

Classification criteria

Moll and Wright listed the first classification criteria of PsA, mainly based on clinical observations [57]. This was routinely used in the everyday clinic until 2006. According to this

classification, PsA could be divided in five different subtypes. The oligoarticular PsA is characterized by maximum four affected joints with an asymmetric distribution. In the polyarticular subtype the number of joints affected is equal or more than 5 with an involvement that can be symmetrical. The distal subtype describes an inflammation of distal interphalangeal joints of hands and or feet, which usually occurs with other subtypes and only rarely alone. Arthritis mutilans is a deforming and destructive form of arthritis, which gravely affects the bone with osteolysis. Finally, the axial subtype where the axial involvement predominates to the peripheral one [57]. In the Moll and Wright classification PsA diagnosis is fulfilled when an inflammatory arthritis (peripheral or axial) is identified with the presence of PsO and absence of RF [57]. This classification however focused on arthritis, giving less space to other articular manifestation such as enthesitis. In 2000, a large international consortium of rheumatologists gathered to redefine the classification criteria for PsA. The CASPAR criteria were then published in 2006 (Table 1) [19]. This new system centered the attention to patients with joint, spine and/or entheseal inflammation and extended the possibility to confirm the diagnosis in the 10% of patients without PsO (*sine* PsO) or in the 15% of cases with a positive RF [18].

Finally, the current definition of PsA is based on the exploration of six clinical domains - PsO, nail disease, enthesitis, dactylitis, peripheral joint disease and axial disease - which should be weighted in the treatment decision [58].

Table 1 Classification Criteria for Psoriatic Arthritis (CASPAR)

Patients with inflammatory disease involving joints, spine and/or entheses meeting at least three points of the following five criteria [18]	
Criterion	**Points**
Psoriasis	
Current Psoriasis	2
Personal history of Psoriasis	1
Family history of Psoriasis	1
Psoriatic nail distrophy	1
Negativ test for rheumatoid factor	1
Dactylitis	
Current dactylitis	1
History of dactylitis	1
Radiographic evidence of juxtaarticular new bone formation	1

2.2.4 Therapy

The treatment approach in PsA was changed after the year 2000, thanks to the introduction of numerous immunologically targeted biological disease-modifying anti-rheumatic drugs (bDMARDs) and targeted synthetic drugs (tsDMARDs). These new pharmaceutical options were originally proposed for the treatment of RA and later on showed efficacy in other conditions, including PsA [18]. Up to date, treatment in PsA remains a challenge, due to the heterogeneity of the disease, which often causes a delay in the diagnosis. Indeed, on the first evaluation of a PsA patient the disease activity should be measured in respective to each domain [58]. The domain with the highest activity will weigh the most in the treatment decision. Four international organizations have published and updated the PsA treatment recommendations: the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA), the European League Against Rheumatism (EULAR), the American College of Rheumatology (ACR) in collaboration with the National Psoriasis Foundation (ACR-NPF). The GRAPPA guidelines provide recommendations based on the domains [58]. The EULAR guidelines are arranged in an algorithm according to the stage of the disease and the different disease activity [59]. The ACR-NFP adopted a strict Grades of Recommendation Assessment, Development and Evaluation (GRADE) approach [60]. The different guidelines are overall similar, except for some decisions regarding the use of one class or group of drugs before another, depending on concomitant aspect, like the severity of skin disease. In general, in case of patients with a mild disease activity and oligoarticular presentation, NSAIDs combined with intra-articular steroid injections can be effective [58-60]. In case of patients with a more severe disease presentation and polyarticular involvement conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) represent the first therapy strategy [58-60]. Among these, Methotrexate (MTX) has been one of the most used medications of PsA [17]. However, very few studies have addressed the efficacy of MTX in PsA. In the Methotexate in Psoriatic Arthritis trial the endpoint was not different between MTX and placebo group [61]. However, the study may have been underpowered and the oral doses of MTX were lower than what is normally prescribed [17]. Other subset analysis showed that MTX was effective in PsA patients with similar disease activity to RA patients [18]. Leflunomide was found to be effective in treating the peripheral arthritis, but less effective in treating the skin [62]. Sulfasalazine has modest efficacy for both arthritis and PsO [63], whereas cyclosporine showed greater benefit in skin PsO [64]. In case csDMARDs are not able to overcome the diseases, bDMARDs or tsDMARDs can be adopted alone or in combination with csDAMRDs. Among the bDMARDs five classes can be identified. TNF inhibitors showed efficacy across all PsA domains, including

enthesitis and can retard the radiographic progression of the disease [65]. Antibody directed against the p40 shared subunit between interleukin 12 (IL-12) and IL-23 (ustekinumab) has been introduced as therapy for both PsO and PsA, although several data showed a higher efficacy in the skin disease compare to the joint manifestations [66, 67]. More recently, the novel IL-23p19 inhibitor guselkumab has been adopted for PsA treatment, showing similar efficacy in the peripheral joint involvement to the IL-17 inhibitors [68, 69]. The efficacy on the axial feature still needs to be determined. The class of IL-17 inhibitors includes antibodies against the receptor for the IL-17 (secukinumab) or against the IL-17A (ixekizumab). Both medications reported good efficacy across all domains, including the axial involvement [70, 71]. Abatacept is a human fusion protein that binds CD80/CD86 on antigen-presenting cells, preventing the interaction with the CD28 expressed on T cells. So far, abatacept showed modest efficacy in arthritis and minimal efficacy in PsO compared with placebo [72]. Another therapeutic option, includes the tsDMARDs and specifically the oral phosphodiesterase 4 (PDE4) inhibitor apremilast and a variety of Janus kinase (JAK)-inhibitors. Studies have showed modest efficacy of apremilast in skin lesions, arthritis, enthesitis and dactylitis [73]. As for the JAK-inhibitors evidence suggested bigger efficacy against the articular inflammation, as compare to the skin [74]. Whether different JAK-inhibitors with different selectivity for specific JAK isoforms have different efficacy profile across the PsA domains remains to be determined.

2.3 Enthesitis in Psoriatic arthritis

Enthesitis, defined as inflammation of tendon and ligament insertion into the bone, represents one of the hallmarks of PsA [3]. Its importance was acknowledged in the CASPAR criteria, becoming later on one of the disease domains according to the GRAPPA [19, 58]. More importantly, several evidence suggested enthesitis as an early primary event in PsA pathogenesis [3]. Hence, the increasing attention over the years in understanding the micro-anatomical structure as well as the pathophysiological mechanisms behind the inflammatory response in this transition zone. Entheses are typically located outside the joint capsule, however in some specific joints, including the sacroiliac, sternoclavicular and distal interphalangeal joints the fibrocartilage constitutes part of the joint itself [3]. These are often involved in PsA. As mentioned above, enthesitis has been described in ca. 30-67% of PsA patients, with Achilles tendon, plantar fascia and lateral epicondyle insertion as the most common area involved [75].

2.3.1 Pathogenesis

The pathogenesis of enthesitis is so far not completely understood, due to the difficulties in harvesting biological material from this area and, as consequence, to the lack of studies specifically focusing on this domain. However, combination of studies performed on retrieved material from healthy subjects and *in vivo* data, support the current knowledge on enthesitis pathogenesis (Figure 2).

In healthy individuals enthesitis often appears after mechanical stress leading to repetitive microtrauma. One very common condition is the "tennis elbow", describing the lateral epincodylitis after repetitive movement during the sport activity [3]. As mentioned above, mechanical stress can also trigger a chronic inflammatory response in PsA [76]. Cambre *et al.* showed that mechanical stress can induce the expression of CXCL1, CCL2 and other chemokines facilitating the recruitment of immune cells [77]. A key mediator of inflammation in enthesitis is the prostaglandin E2 (PEG2), which begins the inflammation in the entheseal sites, inducing vasodilatation and further recruitment of immune cells either from the circulation or from the bone marrow [15, 78]. Indeed, McGonagle *et al.* showed that entheseal tissue in patients with SpA had abnormal architecture with increased vascularization and immune cell infiltration [13]. PEG2 plays also an important role in the T cells production of IL-17, which is together with IL-23 one of the key mediator in PsO and PsA pathogenesis [78]. IL-23 itself is instead produced by macrophage and dendritic cells and its relevance in enthesitis has been proved by *in vivo* studies [43, 79]. Indeed, the systemic overexpression of this cytokine could induce entheseal inflammation followed by arthritis and bone remodeling, through the production of other mediators, such as TNF and IL-17 [79]. Recent data on entheseal tissue from healthy individual described a CD14+ myeloid population that produces most of the inducible IL-23, IL-1β, TNF and CCL20 mediators [80]. This population showed a similar gene expression profile to the matched blood CD14+ population [80]. Sherlock *et al.* described CD4-CD8- T cells expressing IL-23R (likely γδ T cells) as local resident cells harbored in the entheses, which are able to release IL-17 upon IL-23 stimulation [43]. Nevertheless, the role of T cells in enthesitis needs still to be clarified since insertion site inflammation can also occur in a model of mice deficient for T cells [77]. Recently, IL-17 producing ILC3s have been described as immune resident cells in the entheses of healthy individuals [40]. Moreover, ILC3s have been found increased in the circulation of PsA patients with active disease and in the skin lesions of PsO patients, suggesting their role in both diseases pathogenesis [81, 82]. According to the current data, it seems that a major source of IL-17 in the enthesis are ILC3s and γδT cells,

which are harbored in the enthesis and enable the site-specific attraction of immune cells, including neutrophils, which release protease and reactive oxygen species aggravating the pain [83]. Other important mediators in the enthesitis process are TNF and IL-22, which have also been involved in the process of new bone formation [76]. Mechanistic studies with transgenic mice overexpressing TNF showed, that TNF can induce enthesitis together with other spondylathritis features [84]. Late stage of entheseal inflammation results in local bony overgrowth and development of enthesophytes in the peripheral joints or syndesmophytes in the spine. The process of new bone formation is thought to be the result of mesenchymal stromal resident cells, which differentiate into chondroblasts and osteoblasts [76]. In particular, hedgehog proteins activate a specific cell population in the enthesis, which express the transcription factor GLI1 [85, 86]. These cells are very important for the mineralization of the fibrocartilage and their activity seems to respond to the muscle loading. Moreover, some *in vivo* data imply the involvement of bone morphogenic proteins (BMPs) and Wnt proteins in development of enthesophytes in SpA [87]. PGE2 is also a strong inducer of osteoblast differentiation as well as IL-22 [88, 89]. Whereas, IL-23 and IL-17 seems to have osteoclastogenesis properties together with TNF [3]. In particular, TNF seems to induce Dickkopf-related protein 1 (DKK1) and sclerostin, which work against bone formation [90].

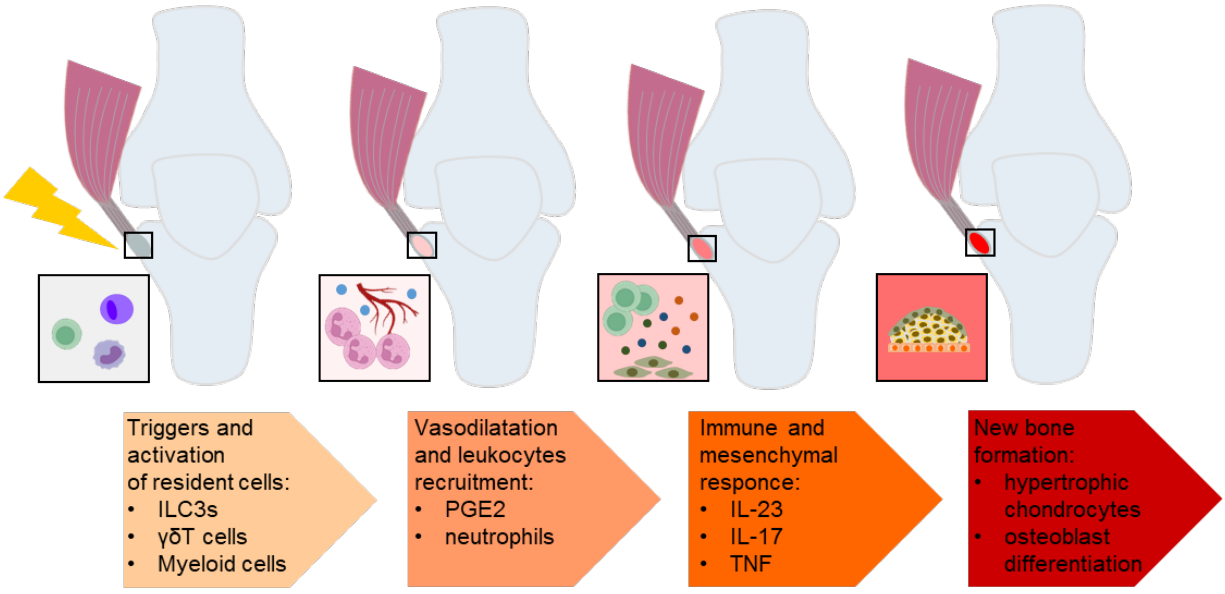

Figure 2. Pathogenesis of enthesitis in PsA

Schematic representation of enthesitis pathogenesis according to the current knowledge. Type 3 innate lymphoid cells (ILC3s), prostaglandin E2 (PEG2)

2.3.2 Clinical manifestations, imaging and diagnosis

Enthesitis is clinically perceived as tenderness in the insertion-sites. In the contest of a chronic inflammatory disease as PsA, the pain has inflammatory characteristics, improving with the movements [91]. So far, the clinical assessment of enthesitis has been based on a combination of clinical scores and imaging evaluation. Several indices have been proposed to quantify and recognize enthesitis in clinical trials as well as in the everyday clinic, such as the Mander/Newcastle Enthesitis Score (MASES) index, the Spondyloarthritis Research Consortium of Canada (SPARCC) index, or the Leeds Enthesitis Index (LEI) [91]. Plain radiography is a useful tool on a late stage of the disease, when articular and periarticular features of PsA already took place, such as erosions and new bone formation [92]. High-resolution peripheral quantitative CT (HRCT) scan can visualize bone microstructure in detail and has been adopted to address the remodeling changes in PsA [92, 93]. Different erosions have been described between PsA and RA patients [93]. Another study comparing PsA and osteoarthritis described a similar number of bony spurs in the two diseases, which however differ in the localization. In PsA the new bone formation was mostly detected at the radial side of the second metacarpophalangeal joint, whereas in osteoarthritis it was more dorsal and palmar. Moreover, bone apposition was more common in the entheseal region in PsA patients [94]. According to another study addressing PsO patients, new bone formation was found in this group compare to the healthy donors, although the bony spur were present before the start of symptoms [95]. Radiography and HRCT can be adopted to assess the damage in PsA, however cannot provide information regarding the actual activity of the disease, neither can they quantify the inflammation in the periarticular area. Musculoskeletal US as well MRI have been largely adopted with this purpose, being able to provide details regarding the inflammatory status of the entheses and the neighboring tissues [92]. Among them, US can provide further details, including the enhanced vascularity in the enthesis, but it cannot image below the bony cortex. Moreover, US imaging might be difficult to interpret in patient with body mass index > 30, who often experience enthesitis due to the mechanical load. MRI offers a better understanding of inflammatory and structural tissue abnormalities in both peripheral and axial involvement [92]. However, it has also some limitations. Indeed, in order to better evaluate the entheses, high-resolution images are required, as for the natural disposition of entheses it is technically challenging to capture the transition areas without creating artefacts [92].

2.3.3 Therapy

The current knowledge on enthesis treatment is also limited, due to the lack of studies specifically design to evaluate this domain. To date, DMARDs have not been studied and among the clinical trials performed on biologic treatment the power of the study did not take in account the enthesitis response [3].

Most of the data available come from the clinical practice. US-based studies have confirmed the efficacy of NSAIDs treatment in reducing the vascular influx and inflammation in the insertion sites [3]. However, in a chronic inflammatory disease such as PsA, NSAIDs might not be enough. CsDMARDs including MTX showed no efficacy in blocking entheseal inflammation [91]. Among the tsDMARDs, apremilast showed encouraging data, being able to induce remission of the enthesitis in around 50% of PsA patients after 1 year treatment according to the MASES index [96]. Based on several clinical trials on PsA, TNF inhibitors resulted to be efficacious in controlling peripheral enthesitis. This has been observed among all TNF inhibitors, including infliximab, which showed around 50% of improvement, as well as adalimumab, certolizumab, golimumab and etanercept [97]. Moreover, imaging data through MRI supported the TNF inhibitors efficacy also in controlling the axial enthesitis [98]. Among the other class of bDMARDs, encouraging results come from inhibitors of IL-23. Interestingly, ustekinumab was efficacious in treating enthesitis in ca. 50% of the patients, whereas similar data have not been achieved in the joint involvement *per se*, suggesting the importance of IL-23 in driving the pathogenesis of enthesitis in PsA [66]. Similar data have been also observed under IL-17 inhibitors. Indeed, secukinumab showed resolution of enthesis in ca. 50% of the patients [99], whereas ixekizumab in 30-40% [100, 101]. Among the JAK-inhibitors, tofacitinib demonstrated a significant improvement compared to placebo at 3 months of treatment in the 63-75% of the patients, who had enthesitis at baseline. Only few studies have addressed the enthesophyte progression in response to therapeutic approaches and no effect have been reported in both patients treated with TNF inhibitors or with MTX [92].

2.4 Second harmonic generation microscopy

Multiphoton microscopy is a laser-based microscopy, which adopts non-linear excitation of fluorescent probes to generate signal within a thin raster-scanned plane [102]. This technique has become a standard approach for non-invasive imaging of thick specimens with cellular resolution [102]. Multiphoton microscopy can generate both fluorescence and second harmonic generation (SHG) as contrast mechanisms, to provide information regarding tissue structure as

well as orientation and polarization of chiral proteins [103, 104]. SHG is a coherent nonlinear optical process, during which two photons combine and emit a single photon with visible light. SHG is a label free tool, since it generates its intrinsic contrast from the interaction of light with non-centrosymmetric (piezoelectric) structures, such as type I collagen [105]. Because of that, this technique is commonly used simultaneously with two photon fluorescence microscopy because both are using the same experimental setup [106]. The SHG signal is easily distinguished and isolated from the fluorescence, since it is exactly centered at twice the frequency of the excitation laser source and does not require electronic excitation. This is a major advantage compared to fluorescence as electronic excitation results in phototoxicity and photobleaching. Additionally, SHG can offer many of the same benefits of traditional multiphoton microscopy, such as high resolution, deep imaging tissue evaluation with a penetration up to ca. 500μm [106].

2.4.1 Second harmonic generation and collagen

Extracellular matrix (ECM), or non-cellular component of tissue, represents the basilar structure of each tissue sustaining and supporting the cellular content. More than a biomechanical and biochemical function, the ECM plays an active physiological role in cell communication, migration, adhesion and proliferation [107, 108]. ECM is composed of approximately 300 proteins in different proportions according to different biomechanical requirements in different tissues [109]. Among them, fibrous proteins, such as collagen, elastin, fibronectin and laminin constitute the major components of ECM [110]. In particular, collagen is the most abundant protein, reaching ca. 90% of ECM and 30% of the total protein weight in the human body [111], thus representing the major constituent of connective tissue, muscle, bones, etc. The basic structure of collagen fibers is tropocollagen, a triple helix molecule (ca. 1.5nm in diameter and ca. 300 nm in length) composed of three protein chains [110]. Tropocollagen molecules spontaneously self-assemble to form microfibrils (ca. 3.6 nm in diameter), which together constitute collagen fibrils (10-500 nm in diameter and a length up to tenths of microns). Fibrils further aggregate to form fibers (1-20 μm diameter and a length up to few millimeters) [112]. The most typical tissue reflecting the collagen microstructure is the tendon, which is composed of highly aligned collagen fibrils[102]. Several studies have been performed to address the molecular process behind collagen fiber constitution, starting from collagen monomers. Concentration, temperature and pH of the collagen solution during the growth process are all essential parameters in the resulting structure of collagen, in terms of diameter, length and scaffolding [112]. Tropocollagen is produced within the cells and

transported to the extracellular space [113]. Here, the long axis of the triple helix molecules assume a parallel orientation to the long axis of the fibrils, creating a specific orientation of the fibres [113]. The relative intensity of SHG signal is dependent on the orientation of collagen relative to the polarized excitation light. A higher intensity is obtained when the excitation light is oriented to highlight the molecular and fiber direction [105]. The structure of the collagen fibers intrinsically responsible for the production of a strong SHG signal. On a molecular level, the chemical moieties of the polypeptide constitute a non-centrosymmetric arrangement rising a dipolar contribution of the fibrillar collagens, this leads to the generation of SHG from individual collagen fibrils of around 50 nm of diameter. Microscopically, the collagen architecture drives the coherent nature of SHG, which means that the signal not only depends on the density of scatterers, but also on the overall structural organization within the focal volume of the microscope objective [106]. Several studies validate the possibility to study qualitative and quantitative changes in the collagen compartment comparing SHG staining to anti-collagen immune-labeling [114]. These data confirmed that the SHG signals arise from fibrillar collagen, whereas no SHG signals were observed from other type of collagen, for example type IV [114].

In the last decades, SGH signal has been used to visualize and characterize the collagen architecture in several tissues, such as skin, tendon, bone and cornea, in both physiological and pathological settings [102, 112]. Properties of collagen fiber organization have been identified as candidate imaging biomarkers in a number of pathological conditions, including cancer, aging, wound healing, atherosclerosis and diabetes [114]. In this contest, studies on the organization of fibrillar collagen, such as fiber density, distribution, and alignment became important for the definition of collagen signatures, which can be adopted as a scoring system as well as prognostic factors in context of diseases. In cancer, Tumor Associated Collagen Signatures (TACS) were described as alterations in collagen reorientation and deposition during mouse mammary tumor progression [115]. Subsequently, it was observed that the presence of highly aligned collagen fibers oriented perpendicular to the tumor boundary (TACS-3), is a negative prognostic indicator in human breast cancer. Similar correlations were found in other cancer types such as skin, ovarian, prostate, pancreas, and others [115]. Other studies, instead focus the attention on SHG signal from musculoskeletal structures, such as bone, to address the collagen content and its architectural structure [105]. The potential use of SHG in studying and characterizing the enthesis and enthesitis has not been addressed yet.

3 Original publication

As part of this thesis, the original publication, result of the scientific project.

TRANSLATIONAL SCIENCE

Concise report: a minimal-invasive method to retrieve and identify entheseal tissue from psoriatic arthritis patients

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ABSTRACT

Objectives To establish a minimally invasive biopsy technique for the analysis of entheseal tissue in patients with psoriatic arthritis (PsA).

Methods Human cadavers were used for establishing the technique to retrieve tissue from the lateral humeral epicondyle enthesis (cadaveric biopsies). After biopsy, the entire enthesis was surgically resected (cadaveric resections). Biopsies and resections were assessed by label-free second harmonic generation (SHG) microscopy. The same technique was then applied in patients with PsA with definition of entheseal tissue by SHG, staining of CD45+immune cells and RNA extraction.

Results Entheseal biopsies from five cadavers allowed the retrieval of entheseal tissue as validated by the analysis of resection material. Microscopy of biopsy and resection sections allowed differentiation of entheseal, tendon and muscle tissue by SHG and definition of specific intensity thresholds for entheseal tissue. In subsequent entheseal biopsies of 10 PsA patients: the fraction of entheseal tissue was high (65%) and comparable to cadaveric biopsies (68%) as assessed by SHG microscopy. Furthermore, PsA biopsies showed immune cell infiltration and sufficient retrieval of RNA for further molecular analysis.

Conclusion Entheseal biopsy of the lateral epicondyle is feasible in patients with PsA allowing reliable retrieval of entheseal tissue and its identification by SHG microscopy.

Key messages

What is already known about this subject?

⇒ Enthesitis is a hallmark of psoriatic arthritis, however, direct assessment of entheseal tissue in humans has been challenging to date. Second harmonic generation (SHG) microscopy allows to define differences in collagen structure in tissues.

What does this study add?

⇒ This study describes a new reliable and well-tolerated biopsy approach for harvesting entheseal tissue in humans in vivo by performing biopsies of the lateral epicondyle enthesis. Furthermore, SHG assessment of the retrieved tissue allows to define the quality of the biopsy by visualising the content of entheseal tissue in the sample.

How might this impact on clinical practice or future developments?

⇒ This method allows to perform reliable molecular and cellular analyses of entheseal tissue in humans, which is needed for a better understanding of diseases such as psoriatic arthritis.

INTRODUCTION

Enthesis is a specialised interface tissue that connects tendons and ligaments with bone.[1 2] Enthesitis, the inflammation of these insertion sites, represents a hallmark feature of spondyloarthritis, including psoriatic arthritis (PsA). Enthesitis has been suggested as an early event in PsA, with the Achilles tendon, the plantar fascia and the lateral epicondyle being the most commonly involved sites.[3] So far, most of the data on enthesitis in PsA are based on clinical assessment of tenderness as well as MRI or ultrasound examinations.[4] These approaches, however, do not allow molecular analysis of entheses, which will ultimately require acquisition of entheseal tissue.

Good quality sampling of human synovial tissues has been instrumental for enhancing the understanding of rheumatoid arthritis.[5 6] While synovial tissue is rather easily accessible and based on well-defined anatomical structures, assessment of entheses is technically challenging. Hence, it is currently unknown, which entheseal structure in humans would qualify for a feasible biopsy and how correct sampling of entheseal structures could be ascertained within such biopsy material. These technical challenges have led to substantial lack of knowledge on human entheseal tissues. To overcome these hurdles, we developed a guided biopsy approach of entheses, which does not require the concurrent sampling of bone tissue and includes a second harmonic generation (SHG) microscopy-based sampling control to identify and confirm the presence of entheseal tissue within the collected sample.

Table 1 PsA patient characteristics

Patient characteristics	
	Patients (n=10)
Female sex; n (%)	4 (40)
Age (years); mean±SD	53.7±9
Tender joint count mean±SD	7.7±6.7
Swollen joint count mean±SD	2.4±3.6
DAPSA mean±SD	22±11
LEI mean±SD	1.5±1
SPARCC mean±SD	4.1±2
PD signal before biopsy; n (%)	10 (100)
Treatment before biopsy	
Non-steroidal anti-inflammatory drugs; n (%)	4 (40)
Prednisolon; n (%)	1 (10)
Methotrexate; n (%)	5 (50)
VAS after biopsy; mean±SD	2±1
Haematoma after biopsy; n (%)	2 (20)
Wound infection; n (%)	0 (0)
Normal function after 14 days; n (%)	10 (100)

DAPSA, Disease Activity Psoriatic Arthritis; LEI, Leeds Enthesitis Index; PD, Power Doppler; PsA, psoriatic arthritis; SPARCC, Spondyloarthritis Consortium Canada; VAS, visual analogue scale.

MATERIALS AND METHODS

Minimally invasive entheseal biopsy

We obtained elbows from five cadavers from the Institute of Anatomy and recruited 10 PsA patients who fulfilled the classification criteria for PsA.[7] All patients with PsA showed clinical signs of enthesitis at the elbow (without synovitis), including stiffness and pain elicited on local pressure and/or exercise and had a positive Power Doppler (PD) signal in the ultrasound (table 1).

All patients with PsA were naïve to biological disease-modifying anti-rheumatic drugs (bDMARDs) and had active enthesitis under treatment with conventional (c)DMARDs. Extent of enthesitis was assessed by Leeds Enthesitis Index and Spondyloarthritis Research Consortium of Canada. Biopsy was done by Blakesley forceps in 60° flexed position after identifying the lateral epicondyle, the olecranon and the radial head by ultrasound. In patients with PsA, 2 mL of 1% mepivacaine was injected prior to biopsy while skin incision was closed by two stitches with non-absorbable suture material after biopsy. The instrument was inserted until bone contact, then slightly withdrawn to obtain the biopsy (5 mm) from the extensor tendon insertions of the lateral epicondyle. In cadavers, the whole enthesis including adjacent tendon, muscle and bone was surgically resected after the biopsy for further analysis. Ethical approval from the local institutional review board (University of Erlangen-Nürnberg, #30_19B) and written informed consent was obtained from all participants. Further methodological information is provided in online supplemental file.

RESULTS

Ultrasound-guided biopsy of the lateral epicondyle enthesis in cadavers

We first established ultrasound-guided biopsy of the lateral epicondyle in five human cadaveric specimens (online supplemental file). Ultrasound examination using B-mode of the region defined the lateral epicondyle enthesis (figure 1A,B). Anatomical landmarks (lateral humeral epicondyle, radial head and olecranon) were marked on the skin (figure 1C). An incision (5 mm) was made along the line between the lateral epicondyle and the radial head followed by preparation of subcutaneous tissue and fascia (figure 1D). A Blakesley forceps was inserted through the incision (figure 1E) and one 5 mm biopsy of the tendon plate of the extensor muscles (digitorum communis, digitus minimus and carpi radialis) was taken (figure 1F). Retrieved tissue was later examined by histochemistry and SHG (=cadaveric biopsy specimens).

Definition of the anatomic environment of the entheses

After biopsy, the subcutaneous tissue and the fascia were split up until the enthesis (figure 1G), making the biopsy site clearly visible (figure 1H,I). The joint capsule was not affected indicating extra-articular localisation (figure 1I). For histologic validation, the entire entheseal region including adjacent bone, tendon and muscle was then removed (=cadaveric resection specimens).

Identification of entheseal tissue by SHG

Histological analysis of cadaveric resection specimens showed the adjacent bone (blue in Safranin O) and muscle (red in Trichrome) and the enthesis between bone and tendon (figure 1J). Induction of SHG by multiphoton microscopy allows visualising the composition of structural proteins, that is, collagens.[8] SHG intensity (SHG-I) of each tissue (bone, enthesis, tendon and muscle) was measured in different regions of interests and normalised to the highest intensity value (internal control=100%) (figure 1K,M). Tendons always had the highest SHG-I (mean±SD: 91%±13%) followed by bone (80%±12%), while muscles emitted the lowest SHG-I (12%±5%). Entheses showed a unique intermediate signal (31%±6%), which was statistically different to other tissues (figure 1L,M). No difference was observed between calcified and uncalcified entheseal areas (figure 1N). As SHG allows identifying entheseal tissue, we used SHG to validate the content of entheseal tissue in cadaveric biopsy specimen (figure 1O-Q-O-Q). To identify optimal cut-offs to differentiate enthesis from muscle and tendon, we used 16-bit pixel depth images with a high resolution and a dynamic intensity range from 0 till 65 535 (=2^{16} units on a grey scale). Based on the observed data, we were able to identify optimal cut-offs to differentiate muscle from enthesis (5507 95% CI 4227 to 6962 units on the 16-bit grey scale) and enthesis from tendon (12 318, 95% CI 11 159 to 13 817 units on the 16-bit grey scale) (figure 1P) with high accuracy (0.93 and 0.95, respectively). We identified 68% of cadaveric biopsy sample being entheseal tissue (figure 1Q).

Biopsies of the lateral epicondyle enthesis in patients with PsA

Based on the data retrieved from the biopsies of the entheses in cadavers, we addressed the feasibility to biopsy lateral epicondyle enthesis in 10 patients with PsA (table 1) using the same approach (figure 2A–F). Prior to biopsy, diagnosis of enthesitis was confirmed by clinical examination and PD Ultrasound. The aforementioned anatomical structures were defined by ultrasound. After incision, a 5 mm biopsy of the tendon plate of the extensor muscles was taken (figure 2E). All incisions healed well without complications and stitches could be removed after 7–10 days. Range of motion of the elbow was examined 14 days after biopsy with normal function in all participants. Only two patients developed mild haematoma, with no functional limitations. Standard histochemistry did

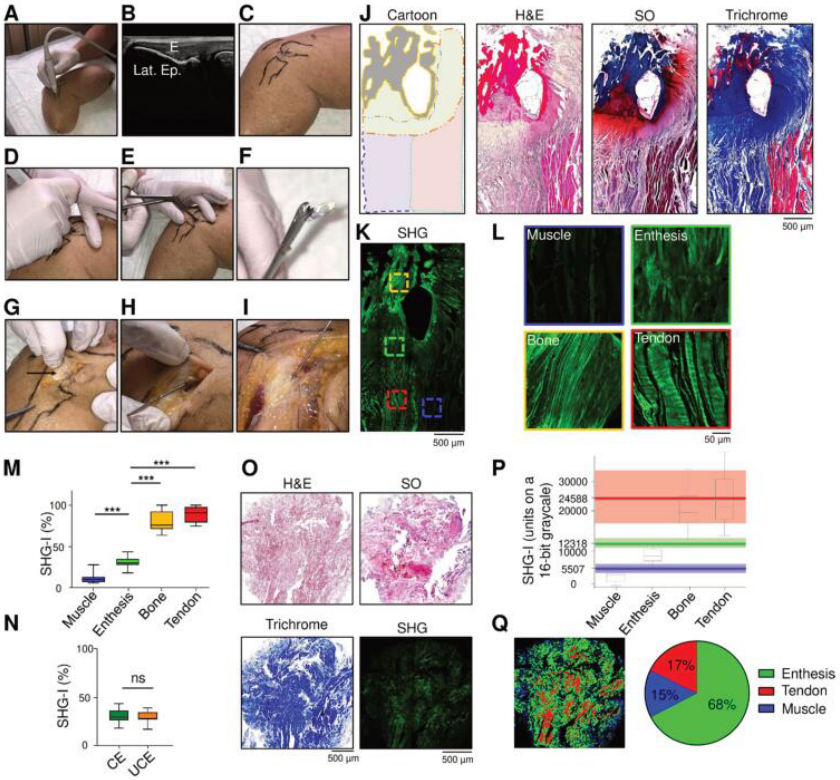

Figure 1 Minimally invasive ultrasound-guided biopsy. (A) Ultrasound (US) identification of the extensor tendon enthesis. (B) B-mode US image of the lateral epicondyle enthesis (E) of the lateral epicondyle (Lat. Ep.). (C) Anatomical landmarks (lateral humeral epicondyle, radial head and olecranon) on the skin. (D) Skin incision (5 mm) between the radial epicondyle and the radial head. (E) Insertion of Blakesley forceps. (F) Biopsy of the enthesis. (G) Longitudinal skin incision to directly visualise the biopsy area and for later resection of the entire enthesis. (H) Identification of the percutaneous biopsy site. (I) Identification of the percutaneous biopsy site outside the joint capsule. (J) Evaluation of a representative entheseal resection sample showing the complete enthesis: a cartoon illustrating the different tissues and their extent (B: bone; BM: bone marrow; CE: calcified enthesis; UCE: uncalcified enthesis; T: tendon; M: muscle); H&E, safranin O and trichrome stains.[18] (K–L) Evaluation of a entheseal resection sample by second harmonic generation (SHG) microscopy and corresponding magnifications for each tissue (muscle, enthesis, bone and tendon). (M) Quantification of SHG intensity in entheseal resection samples: for each sample, the signal intensities of muscle, enthesis, bone and tendon were separately measured (five cadaveric samples; three assessments/subregion each) and normalised to the highest value within the sample (=100%). Data are shown as mean±SEM, ***p<0.0001 was determined by ordinary one-way analysis of variance (ANOVA) test. (N) SHG intensity quantifications of calcified (CE) versus uncalcified (UCE) areas of the enthesis. Data are shown as mean±SEM, ns (p>0.05) was determined by Student's t-test. (O) Staining of a representative cadaveric entheseal biopsy with H&E, safranin O and trichrome stains and evaluation of a cadaveric entheseal biopsy by SHG microscopy. (P) Observed intensity values and estimated thresholds with 95% bootstrap confidence intervals to discriminate different tissue types: (Red) cut-off and range for bones and tendons (24588, 95% CI 16 776 to 31905), (green) range for entheses (12318, 95% CI 11 159 to 13817), (blue) range for muscle (5507 95% CI, 4227 to 6962)[19] . (Q) Fractions of tissues in cadaveric entheseal biopsies expressed as percentages. ***p<0.0001 was determined by ordinary one-way ANOVA test.

not allow differentiating entheseal, tendonal and muscular components (figure 2G). In contrast, substantial differences in SHG-I corresponding to muscular (lowest), entheseal (medium) and tendonal (highest SHG) tissues were found (figure 2G,H). Based on the defined cut-offs, we could show that 65% of the tissue sample consisted of entheseal tissue (figure 2I), which was in line with our previous observations from cadaveric biopsies. In contrast to cadaveric biopsies, all PsA specimens showed infiltration with CD45+ immune cells (figure 2J,K). In addition, samples were validated with respect to their suitability to retrieve enough RNA for molecular analysis. In situ hybridisation of deparaffinised sections as well as whole specimen digestion and subsequent measurements of expression levels of target genes by real-time PCR revealed a high RNA integrity (figure 2L,M).

DISCUSSION

To date, very little is known about the cellular and molecular composition of human entheses. McGonagle and colleagues were the only who have undertaken the effort to perform biopsies from peripheral human entheses in patients with SpA.[9] They performed Yamshidi needle-based biopsies of plantar and patellar entheses in five patients with SpA and showed infiltration by T cells and macrophages as well as increased vascularisation.[10] Other, even more invasive studies were done in surgery material from the spine of patients with non-SpA and SpA, showing infiltration by immune cells, fat deposition and expression of inflammatory cytokines, such as IL-23, IL-17 and tumour necrosis factor alpha (TNFα).[9 11–13] While these studies indicate that access to entheseal tissues might improve our

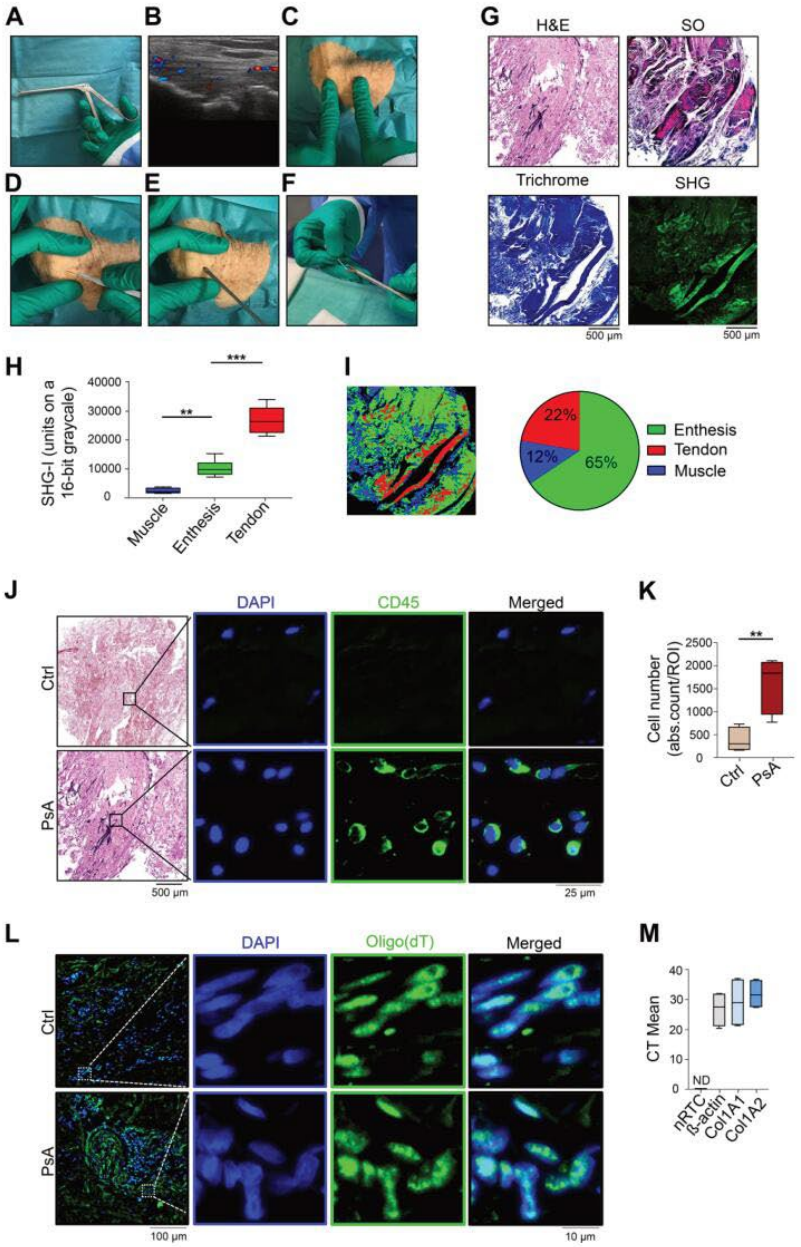

Figure 2 Evaluation of entheseal biopsies from psoriatic arthritis patients. (A) Blakesley forceps adopted for the biopsy retrieval; (B) Power Doppler ultradsound image showing enthesitis of the lateral humeral epicondyle; (C) palpatory identification of the anatomical structures (lateral humeral epicondyle, radial head and olecranon); (D) skin incision (5 mm) between the radial epicondyle and the radial head. (E) Insertion of Blakesley forceps. (F) Biopsy of the enthesis. (G) Staining of entheseal biopsies from patients with psoriatic arthritis (PsA) by H&E, safranin O and trichrome and evaluation of PsA entheseal biopsies by SHG microscopy. (H) Individual intensity values for each tissue in an established intensity range between 0 and 65 535 units. (I) Fractions of tissues in PsA entheseal biopsies expressed as percentages. Data are shown as mean±SEM, ***p<0.0001 was determined by ordinary one-way analysis of variance (ANOVA) test. (J) Representative H&E stainings from cadaveric (Ctrl) and PsA entheseal biopsies; inserts show the region for fluorescence stainings for leukocytes (anti-CD45, green) and total cells (DAPI, blue). (K) Cellular numbers in cadaveric biopsy specimens (Ctrl) and PsA patients; (L) in situ hybridization from cadaveric (Ctrl) and PsA patient biopsy specimen; inserts show the region for mRNA (Oligo-dT, green) and nuclei (DAPI, blue) detection. (M) RNA expression of target genes expressed as CT values in PsA patient biopsy specimen, NRT (non-RT control).

current understanding of SpA and PsA, the lack of a standardised and feasible biopsy procedure for peripheral entheses and the absence of a quality control procedure to confirm presence of entheseal tissue in the biopsy represent serious complications.

Herein, we present a safe, well-tolerated, minimally invasive, US-guided biopsy approach to retrieve entheseal material from the lateral epicondyle, which is one of the most common sites of enthesitis in PsA.[3] While conventional histology is often used for

evaluation of ex vivo biopsies, visual distinction of fibrocartilage from tendon is challenging if not impossible if the available tissue volume is low. In addition, entheses lack tissue-specific markers separating them from tendon and cartilage. Of note, entheses display a specific feature of collagen assembly, where type-II collagen fibres display a distinct fibrous organisation.[14 15] SHG can visualise fibrillar collagen assembly, which intrinsically emits a strong SHG signal.[16] Therefore, SHG has been used for qualitative and quantitative analyses of collagen in various diseases, such as cancer.[17] In this study, we defined SHG-I signatures that allow to differentiate entheseal from tendon and muscle tissue and, therefore, allow identification of entheseal tissue in small biopsies. Furthermore, in vivo biopsies are useful for the immunohistochemical detection of immune cells as well as for extraction of sufficient amount of RNA for expression studies.

In summary, this standardised entheseal biopsy procedure with the adjoined method to assure tissue quality can be carried out by rheumatologists and is useful to study the molecular and cellular changes of human entheses in diseases and the specific effects of anti-inflammatory treatments in enthesitis.

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Ethics approval This study involves human participants and was approved by Ethical Committee of the Friedrich-Alexander-University (FAU) Erlangen-Nürnberg. Participants gave informed consent to participate in the study before taking part.

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5 Abbreviations

ACR	American College of Rheumatology
anti-CCP	anti-cyclin citrullinated peptide antibodies
bDMARDs	biological disease-modifying anti-rheumatic drugs
BMPs	bone morphogenic proteins
CRP	C-reactive protein
csDMARDs	conventional synthetic disease-modifying anti-rheumatic drugs
DKK1	Dickkopf-related protein 1
EULAR	European League Against Rheumatism
GRAPPA	Group for Research and Assessment of Psoriasis and Psoriatic Arthritis
HLA	Human Leukocyte Antigen
ILC3s	type 3 innate lymphoid cells
IL-7	Interleukin 7
IL-12	interleukin 12
IL-17	interleukin 17
IL-23	interleukin 23
IL-23R	interleukin 23 receptor
JAK	Janus kinase
MRI	magnetic resonance imaging
MTX	Methotrexate
NPF	National Psoriasis Foundation
NSAIDs	non-steroidal anti-inflammatory drugs
PDE4	phosphodiesterase 4
PEG2	prostaglandin E2
PsO	Psoriasis
PsA	psoriatic arthritis
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RF	rheumatoid factor
SHG	second harmonic generation
SpA	spondylarthritis
Th17	type 17 helper T cells
TNFAIP3	tumor necrosis factor-induced protein 3
TNF	tumor necrosis factor

tsDMARDs	targeted synthetic disease-modifying anti-rheumatic drugs
US	ultrasound
VEGF	vascular endothelial growth factor

6 List of publications

PUBLICATIONS (COMPLETE OVERVIEW, STARTING WITH THE MOST RECENT PUBLICATION)

Original articles

1. Pachowsky M*, **Raimondo MG***, Xu C*, Rauber S, Tascilar K, Labinsky H, Vogg M, Saad MSA, Soare A, Bräuer L, Rech J, Simon D, Kleyer A, Schett G, Ramming A *Concise Report: A minimal-invasive method to retrieve and identify entheseal tissue from psoriatic arthritis patients.* Ann Rheum Dis, 2022. **81**(8): p. 1131-1135.
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Reviews

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