BEYOND THE DRYNESS OF SJÖGREN's SYNDROME
RENAL COMPLICATIONS AND BONE METABOLISM

MEER DAN DROOGTE BIJ HET SYNDROOM VAN SJÖGREN
NIERCOMPLICATIES EN BOTMETABOLISME

Tim Both

The studies described in the thesis were performed at the Department of Immunology, Department of Internal Medicine, Erasmus MC, University Medical Center Rotterdam, the Netherlands

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MEER DAN DROOGTE BIJ HET SYNDROOM VAN SJÖGREN
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“Difficult roads
often lead to
beautiful
destinations”.

-author unknown

To my parents

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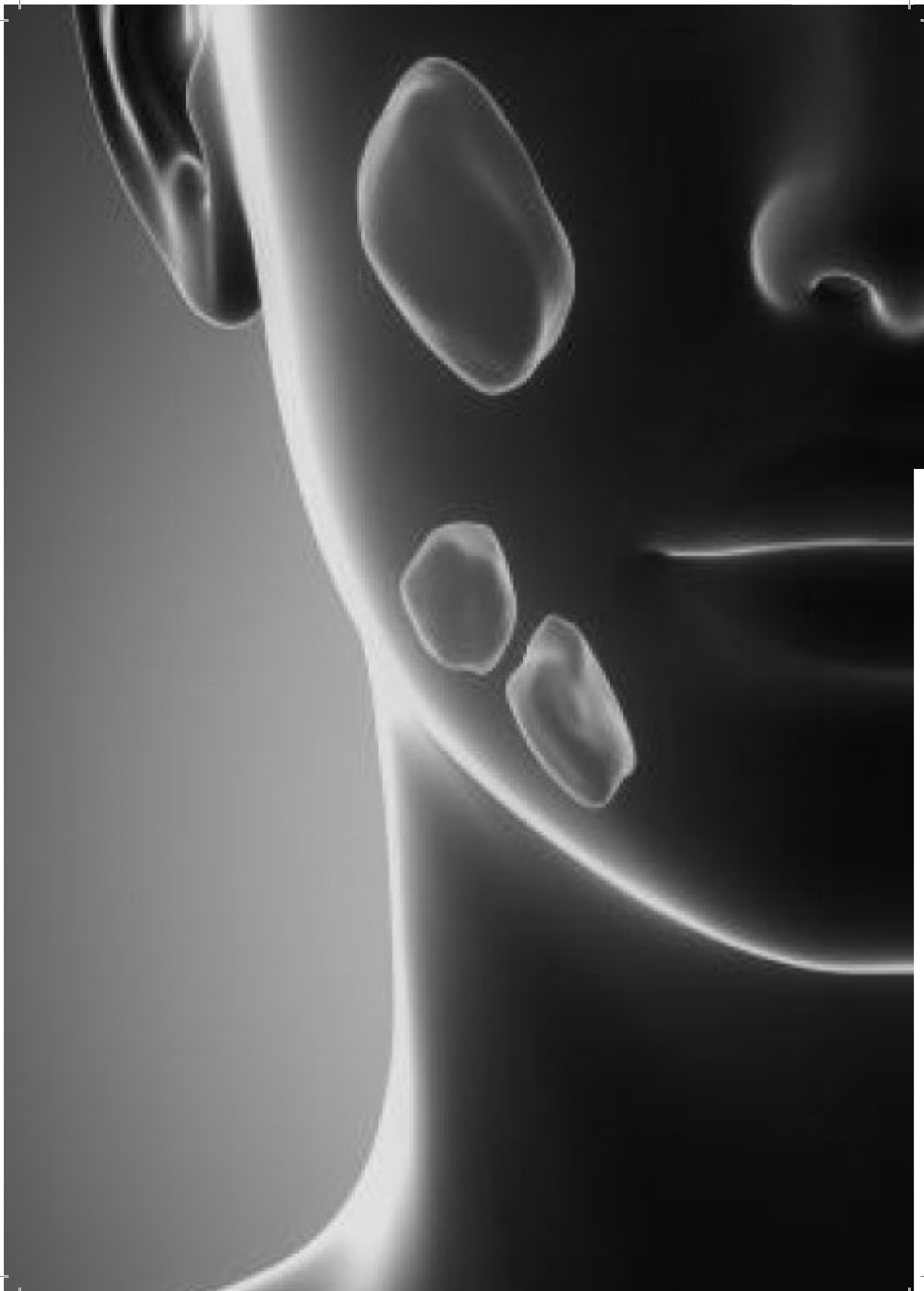
Introduction

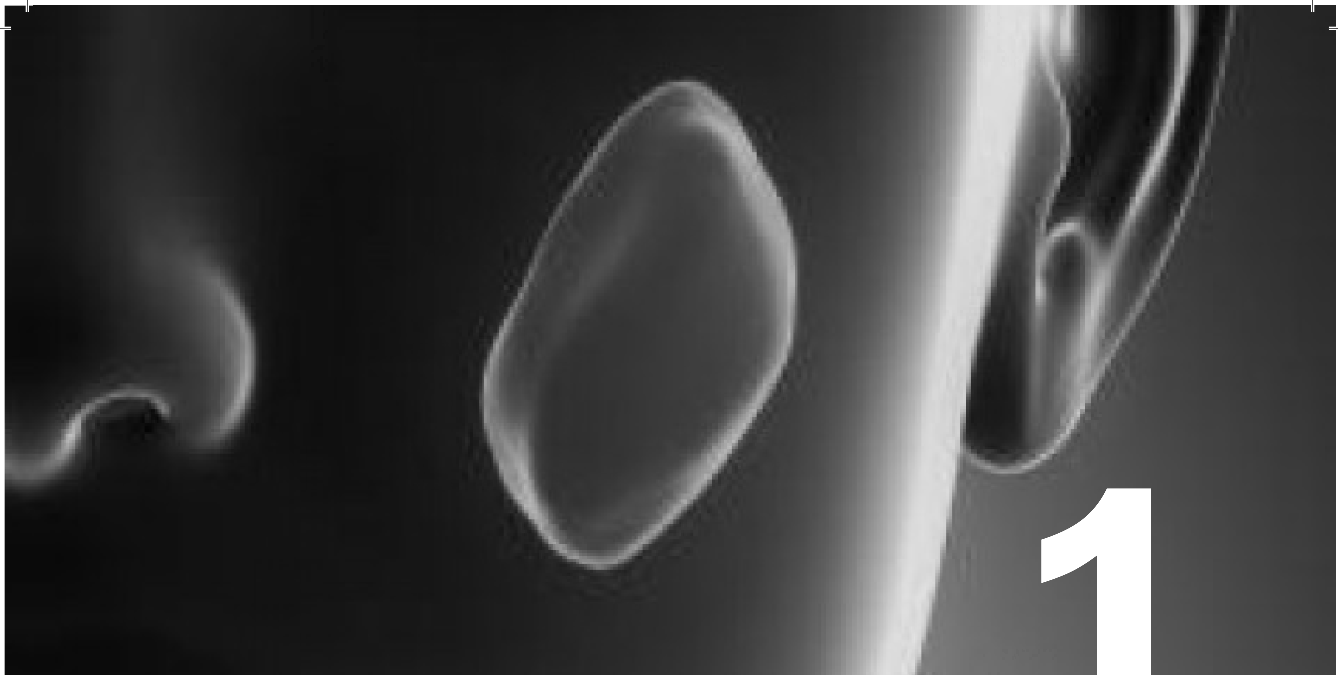
In this thesis, we present our research concerning metabolic disturbances in primary Sjögren syndrome (pSS). pSS is a systemic autoimmune disease, which is characterized by lymphocytic infiltration of the secretory glands. The symptoms of pSS include sicca syndrome (dry eyes and/or oral cavity) and systemic manifestations, such as pulmonary, articular, renal and neurological involvement. Systemic involvement is not always easy to recognize by the physician in a complex disease as pSS, since symptoms can be non-specific. Renal involvement is common in pSS with distal renal tubular acidosis (dRTA) as major manifestation, which is a known complication of pSS. dRTA is a urinary acidification defect leading to a metabolic acidosis and alkaline urine. Furthermore, dRTA is associated with decreased bone mineral density (BMD) leading to hypercalciuria and nephrolothiasis.

In **chapter 1 and 2** we reviewed the current knowledge about pSS and dRTA, respectively. We evaluated in **chapter 3** the prevalence of dRTA in pSS patients by performing a urinary acidification test. Consequently, we evaluated in **chapter 4** the BMD in pSS patients and the effect of dRTA on BMD.

Studies in patients with systemic lupus erythematosus have reported a higher BMD in patients using HCQ compared to patients without HCQ treatment. In our cohort, 69% of the pSS patients was using HCQ. Therefore, we evaluated the effect of HCQ on bone cells *in vitro*.

In **chapter 5 and 6** we report the effects of HCQ on human osteoblasts and osteoclast *in vitro*, respectively. Also, we propose a mechanism of action for the effects of HCQ on both bone cells. Finally, in **chapter 7 and 8** we summarized all chapters and we will discuss our data more in depth. Also, we proposed new future research directions.





Reviewing primary Sjögren's syndrome: beyond the dryness

From pathophysiology to diagnosis and treatment

Tim Both, Virgil A.S.H. Dalm, P. Martin van Hagen,
Paul L.A. van Daele

Abstract

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, characterized by lymphocytic infiltration of the secretory glands. This process leads to sicca syndrome, which is the combination of dryness of the eyes, oral cavity, pharynx, larynx and/or vagina. Extraglandular manifestations may also be prevalent in patients with pSS, including cutaneous, musculoskeletal, pulmonary, renal, hematological and neurological involvement. The pathogenesis of pSS is currently not well understood, but increased activation of B cells followed by immune complex formation and autoantibody production are thought to play important roles. pSS is diagnosed using the American-European consensus group (AECG) classification criteria which include subjective symptoms and objective tests such as histopathology and serology. The treatment of pSS warrants an organ based approach, for which local treatment (teardrops, moistures) and systemic therapy (including non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying antirheumatic drugs (DMARDS) and biologicals) can be considered. Biologicals used in the treatment of pSS mainly affect the total numbers of B cells (B cell depletion (Rituximab)) or target proteins required for B cell proliferation and/or activation (e.g. B cell activating factor (BAFF)) resulting in decreased B cell activity.

The aim of this review is to provide physicians a general overview concerning the pathogenesis, diagnosis and management of pSS patients.

Introduction

Sjögren's syndrome (SS) is a relatively common systemic autoimmune disease characterized by lymphocytic infiltration of the secretory glands. This process leads to sicca syndrome, which is the combination of dryness of the eyes, oral cavity, pharynx, larynx and/or vagina [1]. Sicca syndrome is often accompanied by symptoms resulting from systemic involvement. SS can be present as a primary disease without any other accompanying symptoms (primary Sjögren syndrome, pSS). When SS presents as a secondary disease with other autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis, it is then called secondary SS (sSS) [2,3]. The prevalence of sSS is highest in RA patients and estimated to be around 20% [2,4]. In this review we will focus on pSS.

Epidemiology

A large Norwegian population based study estimated the prevalence of pSS in individuals aged 40–44 years at 0.44 (95% CI 0.34–0.57) using the European criteria [5]. This study also demonstrated that individuals aged 71–74 years compared to those aged 40–44 years had an 8.07 times higher prevalence rate of pSS using the European criteria. Two large cross-sectional population based surveys performed in the United Kingdom estimated the prevalence of pSS using the revised European criteria at 1.6 (95% CI 9–26) and 0.14 (95% CI 0.02–0.51), respectively [6,7]. A prospective study from Slovenia between 2000 and 2002 estimated the incidence of pSS at 3.9 per 100,000 (95% CI 1.1–10.2), in a total cohort of 599,589 subjects [8]. The incidence of pSS was noted to be ten times higher in women compared to men from Slovenia. Another prospective study performed in Greece identified 422 new cases of pSS in a population of 488,435 from 1982 to 2003, with a reported incidence of 5.3 per 100,000 (95% CI 4.5–6.1) [9]. In this population women were affected 20 times more likely than men. However, as many symptoms are non-specific, prevalence may be under- and overestimated.

Pathogenesis

The pathogenesis of pSS is incompletely understood but appears to be multifactorial. Although T cells were originally considered to be the key players in the autoimmune process, there is now growing evidence that B cells play at least an equally important role in the pathophysiology of

pSS **(Figure 1)**. In the next section we will discuss in more detail the known and potential roles of the different immune cells in the pathogenesis of pSS.

Genetics

The first studies reporting associations between gene polymorphisms in pSS were case–control studies based on data from studies performed in SLE. These candidate gene studies mainly reported positive associations between pSS and polymorphisms in *IRF5* (a crucial transcription factor of the type I IFN pathway) and polymorphisms in *STAT4* (a signal transducer and activator of transcription 4, a protein involved in the type II IFN pathway), which are both involved in the interferon pathways [10–14]. In 2013, two genome-wide association studies (GWAS) in pSS were published [15,16].

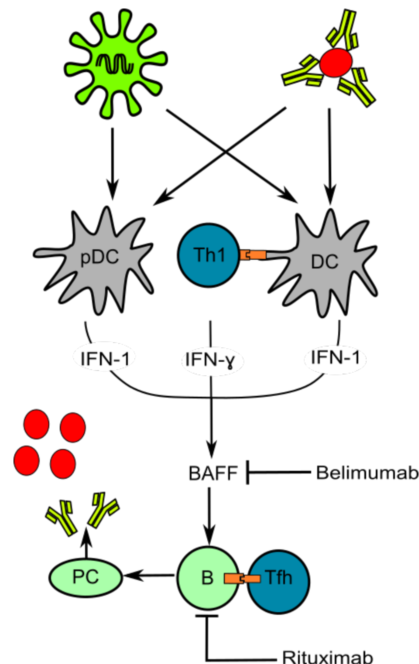


Figure 1 – A simplified overview of the pathogenesis of pSS with the targets for biologicals
An unknown cause (suggested to be a virus or immune complexes) may lead to activation of pDCs and DCs resulting in increased levels of interferons. Interferon-induced BAFF production leads to increased B cell proliferation and differentiation with autoantibody production as result.
Abbreviations: pDC, plasmacytoid dendritic cell; DC, myeloid dendritic cellTh1, T-helper 1 cell; IFN, interferon; B, B cell; PC, Plasma cell; Tfh, Follicular T-helper cell

GWAS is a powerful method offering the ability to screen thousands of regions of DNA. Lessard *et al.* analyzed over 10,000 subjects from Europe (controls and pSS patients) and identified seven genetic loci surpassing the statistical threshold [16]. These loci included MHC-II loci, *IRF5*, *STAT4*, *IL12A*, *BLK*, *CXCR5*, and *TNIP1*. The strongest association was with the HLA-II locus, *STAT4* and *IRF5*, which have all been previously identified by the candidate gene studies. In addition, this study reported three new genes (*IL12A*, *BLK*, and *CXCR5*) being significantly associated with pSS compared to controls and play important roles in immune signaling. The other GWAS in pSS was performed in China and included 1090 healthy controls and 597 pSS patients [15]. In addition to MHC class II genes, three other genes were identified. The most strongly associated gene in this Chinese cohort was *GTF2I*, which acts as a general transcription factor. Other significantly associated genes included MHC-II genes, *STAT4*, and *TNFAIP3*. Although additional confirmatory studies are needed, these results suggest that the Chinese and European pSS patients may have different risk associated genes.

T cells in pSS

The presence, and sometimes predominance, of CD4+ T cells in salivary gland infiltrates underlines their potential contribution to the pathogenesis of pSS [17]. A meta-analysis showed the association between pSS and several major histocompatibility complex class 2 (MHC2) alleles suggesting that autoantigen presentation is important in the pathogeneses of pSS [18]. Th1 cells are hypothesized to be the main subtype contributing to pathogenesis, since they interact with the MHC2 molecules initiating an immune response. In addition, pro-inflammatory Th1 cell cytokines (e.g. IL-1β, IL-6, tumor necrosis factor-α and interferon-γ) are increased in saliva of patients with pSS [19]. Furthermore, a study in 2009 reported a pSS-like syndrome in mice with IL-12 overexpression, which is known to induce Th1 cell differentiation [20]. Besides Th1 cells, the number of T helper 17 (Th17) cells are also increased at sites of inflammation in salivary gland biopsies of pSS patients [21]. IL-17, produced by Th17 cells, is increased in both serum and salivary glands of patients with pSS as compared to healthy controls [22]. Co-expression of IL-17 and IL-18 has been associated with increased severity of pSS, probably due to maintaining the inflammatory process [21,23]. Furthermore, regulatory T cells (Treg) have been identified in salivary glands of pSS patients and the increased presence of these cells has been associated with higher grade

of inflammation in the local lesions [24,25]. Tregs are known to have suppressive effects on the proliferation and function of effector T cells. It has been reported that the number of circulating Tregs are increased, while their function does not seem to be impaired in pSS, which suggests that Tregs are compensatory involved in pSS by lowering the autoimmune response [26].

B cells in pSS

B cells are adaptive immune cells that are responsible for antibody secretion and antigen presentation. B cells differentiate in the bone marrow. One of the key factors in this process is B cell activating factor (BAFF). BAFF is a cytokine that promotes B cell proliferation, maturation and survival and is primarily induced by type I and type II interferons [27,28]. Interferon I is mainly produced by plasmacytoid dendritic cells (pDCs) but also myloid dendritic cells can produce it. Type II interferon is produced by T cells [23,29]. It has been suggested that certain viruses (e.g. Epstein-Barr) and immune complex formation activate Toll-like receptors (TLRs) (e.g. TLR 3, 7 and 9), leading to activation of the innate immunity and interferon production. Although an increased activity in TLR pathway has been reported in pSS, a specific cause (virus or immune complex) has not yet been identified [30,31]. In pSS patients, 55% have an increased IFN type I activity versus 4.5% in healthy controls [32]. The presence of this so-called 'IFN type I signature' in monocytes of patients with pSS was shown to be associated with higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI), the presence of biological markers of activity (increased levels of IgG and/or hypocomplementia) and increased levels of BAFF mRNA in monocytes [32]. Circulating and salivary gland tissue levels of BAFF are significantly elevated in patients with pSS, which is associated with increased disease activity but also with higher risk of development of B cell lymphoma [33–35]. These findings support the hypothesis that innate immune system activation contributes to an autoimmune response by the adaptive immune system. Since BAFF is one of the links between innate and adaptive immune responses, it could be a potential target for therapy in pSS. The first results of studies on anti-BAFF therapy (belimumab) show a significant decrease in disease activity after twelve months of treatment as measured by the ESSDAI [36]. Upon antigen recognition in the germinal center, B cells proliferate and differentiate (e.g. class switching) into antigen specific B cell [37]. In pSS, germinal centers are reported in the epithelium of non-lymphoid tissues such as the salivary glands as well [38]. The formation of germinal centers

is probably important in the pathogenesis of pSS due to promotion of chronic stimulation and activation (by follicular T helper cells) of B cells. Phosphatidylinositol 3–kinase (PI3K) activity is increased in B cells suggesting that PI3K inhibitors may be new therapeutic agents in pSS [39]. Preliminary data from a mouse study showed positive effects of PI3K inhibitors on inflammation in salivary glands [40]. Currently, the first clinical trial with PI3K inhibitors has started in pSS. Patients with pSS often present with high levels of serum IgA and/or IgG [41]. Hyperglobulinemia may lead to the formation of immune complexes with the potential to precipitate in major organs leading to (irreversible) damage [42].

In addition, the presence of autoantibodies (anti-Ro52, anti-Ro60 and anti-La) are included in the diagnostic criteria set for pSS, but also other autoantibodies (e.g. anti-acetylcholinerecepter antibodies) are thought to play a role in the pathogenesis [43–46]. The presence of these autoantibodies is associated with early onset disease, parotid gland enlargement, extraglandular manifestations and lymphocytic glandular infiltration [23,47].

In sum, it remains unclear how these changes in the adaptive immune system lead to the clinical manifestations of pSS. The traditional view that chronic inflammation results in tissue destruction of the exocrine glands will only partially contribute to the pathogenesis of pSS. There is a poor correlation between the amount of damage observed in tissue biopsies and the measured decrease in fluid production, as the reduction in salivary production is often larger than expected from both clinical and histological appearance [43].

Clinical presentation

There is not a standard clinical presentation for pSS, as many patients have various degrees of systemic involvement at the time of presentation. The symptoms of pSS can be divided into three groups, 1) sicca syndrome, 2) systemic manifestations and 3) general symptoms.

1) Sicca syndrome

Sicca syndrome is the combination of dryness of the eyes (xeropthalmia), oral cavity (xerostomia), pharynx and/or larynx, which are the classical symptoms of pSS. In woman, also vaginal dryness is a common feature of pSS [48]. These symptoms are part of the American-European

classification criteria (AECG) of 2002 for the diagnosis pSS and occur in more than 95% of patients (43). The positive predictive value of the AECG criteria is between 54-77% and the negative predictive value is between 94-98% as compared to the classification criteria of 1986 [43]. Xerostomia may lead to secondary problems like oral candidiasis (33%), dental carries (65%) and periodontal disease [49,50]. Xerophtalmia may result in chronic irritation and destruction of the corneal epithelium and ocular infections. Additionally, sicca syndrome also includes hoarseness, non-productive cough, skin dryness and, in woman, dyspareunia [51,52]. Patients with pSS experience a significantly decreased quality of life compared to subjects with sicca syndrome without autoimmune features as measured by the SF-36 depression scale [53,54].

2) Systemic manifestations

Approximately 71% of the patients with pSS present with extraglandular manifestations **(Table 1)** [55]. Of those, malignant lymphoma has the highest mortality [56]. A large cohort study reported a nearly 5-fold higher relative risk in pSS patients with a life-time risk of approximately 10% [57,58]. The most common subtype is mucosa-associated lymphoid tissue (MALT) lymphoma often seen in the parotid glands, which is usually a low-grade indolent neoplasm [59,60]. Clinical risk factors include persistent, unilateral salivary gland enlargement, lymphadenopathy, splenomegaly, skin vasculitis, cryoglobulinemia and the development of glomerulonephritis [61,62]. Laboratory-assessed biological risk factors for lymphoma in pSS include cryoglobulinemia, lymphopenia (especially low total numbers of CD4+ T cells), hypocomplementia, increased serum BAFF and the presence of a monoclonal component in serum [61,63]. Articular involvement in pSS predominantly consists of symmetric, intermittent, nonerosive arthropathy [64,65]. Arthritis is less common and occurs in approximately 16% of pSS patients and mostly involves proximal interphalangeal joints (35%), metacarpal-phalangeal joints (35%) and wrists (30%) [65,66]. Approximately 10-20% of pSS patients develop interstitial lung disease (ILD) [67]. In general, patients will have evidence of both airway disease and ILD by radiographs (plain X-ray and/or CT-scan) and pulmonary biopsy [67].

Table 1 − Systemic manifestations in primary Sjögren Syndrome

Domain	Prevalence (%)	Clinical manifestations	Investigations
Lymphadenopathy [58,59]	10	persistent, unilateral salivary gland enlargement; lymphadenopathy; splenomegaly; skin vasculitis	Serology, biological markers, biopsy
Glandular [112]	30-50	firm, diffuse, nontender, swelling of mostly the parotid gland	-
Articular [64,66]	50	Arthralgia; arthritis	Radiography
Skin [114–116]	23-67	Xerosis; Raynaud phenomenon; annular erythema, erythema nodosum; livedo reticularis; lichen planus; vitiligo; granuloma annulare; vasculitis	Biopsy (if required)
Lungs [67,69,70]	10-20	dry cough; nasal dryness; dyspnea,; interstitial lung disease	Radiography, CT, pulmonary function
Kidneys [73,74,117]	30	Distal renal tubular acidosis; nephrogenic diabetes insipidus; proximal tubular acidosis; hypokalemia	Systematic renal tests, acid loading test, biopsy
Muscles [118,119]	44	Myalgia; muscle weakness; myositis	Biopsy
Peripheral nervous system [81,120,121]	10	painful, burning dysesthesias in the distal extremities; sensory ataxic neuropathy; axonal sensorimotor polyneuropathy; mononeuritis multiplex; cranial neuropathies; radiculoneuropathy; autonomic neuropathy	EMG
Central nervous system [80,122]	20-25	motor or sensory deficits; seizures or cerebellar syndromes; psychiatric abnormalities; dementia and spinal cord involvement; subacute aseptic meningitis; chorea; optic neuritis; cognitive dysfunction	EMG, MRI, CSF investigation, psychiatric analysis
Haematological [41]	20	Normochromic, normocytic anemia; thrombocytopenia; mild leukopenia; lymphopenia	Biochemical tests, bone marrow
Biological [41,123]	36-62	Hypergammaglobulinemia; hypogammaglobulinemia; hypocomplementia; cryoglobulinemia	Serology and biological tests, bone marrow

Abbreviations: EMG, Electromyography; CSF, cerebrospinal fluid; CT, Computed tomography; MRI, Magnetic resonance imaging;

Another study reported that patients with pSS who do not have pulmonary symptoms already may have radiographic or computed tomography (CT) scan abnormalities (22%) or an impaired pulmonary function test [68]. The most frequently observed CT patterns consist of interstitial pneumonia, centrilobular abnormalities and lymphoproliferative disease [68]. This emphasizes that a frequent pulmonary function test or a high-resolution CT-scan should be performed in the follow-up of pSS patients with and without pulmonary complaints. The most common histopathological phenotype of ILD in pSS is nonspecific interstitial pneumonia (NSIP), which has been reported in approximately 45% of the pSS patients with ILD [69,70]. ILD is difficult to treat and results in an increase of dry cough and dyspnea, leading to significantly decreased quality of life. ILD is usually treated with glucocorticoids but other immunosuppressive drugs are also available, such as azathioprine, mycophenolate mofetil, cyclophosphamide and cyclosporine [71,72]. Furthermore, renal involvement is common and includes a wide spectrum of manifestations, of which interstitial nephritis is the most prevalent followed by distal renal tubular acidosis (dRTA) [73,74]. Consistent screening for renal function is important since renal failure (defined as a glomerular filtration rate < 60 ml/min) occurs in approximately 24% of the pSS patients [75]. Additionally, a urinary acidification test should be considered in pSS patients given the high prevalence and non-specific symptoms of dRTA. There is no standardized treatment of renal involvement in pSS. Glucocorticoids are the treatment of first choice in tubulointerstitial nephritis, whereas other immunosuppressive drugs are only shown effective in a small study (mycophenolate mofetil) or not effective at all during the induction phase (cyclophosphamide) [76,77]. dRTA can effectively be treated with potassium citrate for both the symptoms and complications of dRTA, by restoring acid-base balance. It is unknown whether treatment with corticosteroids in autoimmune disease has a positive effect on dRTA. Neurological involvement in pSS includes both the peripheral and central nervous systems and shows many comparisons with the clinical course of multiple sclerosis (MS) [78]. There are similar immunologic mechanisms underlying the pathogenesis of pSS and MS [79]. In many patients, neurologic symptoms precede the onset of other signs and symptoms of pSS [80,81]. In general, intravenous corticosteroids are first-line therapy for patients with pSS associated neuropathy. Cyclophosphamide or intravenous immunoglobulins can be used in patients who do not improve with corticosteroids [81–83].

By performing the ESSDAI in pSS patients on a regular basis, all the above discussed systemic

manifestations can be recognized. pSS is also associated with hepatitis C (12%), autoimmune thyroid disease (10%), autoimmune chronic active hepatitis (2%) and primary biliary cirrhosis (5%), but the ESSDAI does not include these diseases [84,85].

3) General symptoms

The most prevalent general symptom is fatigue, occurring in up to 70-80% of pSS patients [86]. Fatigue in pSS has been well studied using the multidimensional fatigue inventory (MFI) on which pSS patients scores were two-fold worse on all dimensions as compared to healthy controls [87,88]. In addition, chronic pain is often seen in pSS due to accompanying fibromyalgia and/or polyarthralgia [89]. Depression and anxiety are also more common in pSS patients compared to healthy controls [90]. A study showed that 47% of the working age pSS patients received disability compensation, because they were considered to be (partially) unfit for work [91]. The same study also reports that significantly more patients with the following demographic/disease characteristics receive disability compensation: male patients, patients with a high educational level, an increasing number of systemic manifestations and/or the use of artificial saliva and/or HCQ [53,54,91].

pSS treatment requires a patient-specific approach that accounts for disease severity. In the Erasmus MC, we evaluate every pSS patient at least 1-2 times a year. In addition to recording the patient's self-reported symptoms and conducting a standard physical examination, we perform blood tests (including total blood count, liver and renal function, C3, C4 and IgG) and urinalysis to screen for organ involvement. In the case of mild disease activity (as measured by disease activity scores, ESSDAI), we do not perform additional invasive tests such as scans or functional tests (e.g. EMG, pulmonary function). In the case of self-reported symptoms or abnormal physical and/or laboratory examinations, additional testing for the presence (or change) of organ involvement is required. Also, patients with systemic immunosuppressive treatment or with increased organ involvement should be seen more frequently at the outpatient clinic (at least once every 3 months) to evaluate whether treatment is effective and potential side effects are tolerated.

Diagnosis

The diagnosis of pSS is based on the American-European consensus group (AECG) classification criteria for Sjögren syndrome [43]. These criteria include: 1) subjective presence of ocular dryness, 2) subjective presence of oral dryness, 3) objective measures of ocular dryness by Schirmer's test or corneal staining, 4) focus score > 2 in a salivary gland biopsy, 5) salivary scintigraphy showing reduced salivary flow (1.5 mL in 15 minutes) and/or diffuse sialectasias and 6) positive autoantibodies against SS-A and/or SS-B. SS is diagnosed when 4 out of 6 items are present; either salivary gland pathology or the presence of autoantibodies against SS-A/SS-B is mandatory.

The specific questions (criteria 1 and 2) should reveal whether eye and mouth symptoms are characteristic for pSS and additional tests should be performed. If pSS is suspected, laboratory investigations should be performed (e.g. markers for inflammation, systemic biochemical tests, serology and haematology) and the patient should be referred to an ophthalmologist for evaluation of ocular dryness. Recently, the American Group of Rheumatology (ACR) has developed new diagnostic criteria for pSS since the increasing use of (expensive) biologic agents should be based on more objective rather than subjective criteria [92]. The newly proposed criteria by the ACR differ from the AECG criteria by focussing more on objective measurements. Therefore, ocular and oral dryness are no longer part of the classification criteria. It remains unclear whether the new criteria are more sensitive than the AECG criteria. Based on a comparison study in 646 subjects, the AECG criteria had an overall sensitivity in the general population of 88% compared to 83% of the ACR criteria. On all test characteristics (sensitivity, specificity etc.) the AECG criteria scores better compared to the ACR criteria, however, the results are not significantly different [93]. In conclusion, there is no clear evidence for increased value of the new ACR criteria over the old and familiar AECG criteria from the clinical or biological perspective [93]. Currently, the AECG criteria are still the most frequently used in clinical practice and research protocols. In **Table 2**, we summarize both sets of classification criteria.

Treatment

Patients with pSS should be managed by a multidisciplinary team including at least a clinical immunologist/rheumatologist, ophthalmologist and dentist. Extensive clinical trials concerning the treatment of pSS are limited and thus, guidelines are lacking. Nowadays, multiple drugs are used in the treatment of pSS which can be divided in local and systemic therapy **(Table 3)**.

Table 2 – Comparison of the Revised American-European Consensus Group (AECG) Classification criteria and the American College of Rheumatology (ACR) Classification criteria for Sjögren's syndrome.

Criteria (#)	AECG	ACR
1	Ocular symptoms: a positive response to at least one of the following questions: - Have you had daily, persistent, troublesome dry eyes for more than 3 months? - Do you have a recurrent sensation of sand or gravel in the eyes? - Do you use tear substitutes more than 3 times a day?	
2	Oral symptoms: a positive response to at least one of the following questions: - Have you had a daily feeling of dry mouth for more than 3 months? - Have you had recurrently or persistently swollen salivary glands as an adult? - Do you frequently drink liquids to aid in swallowing dry food?	
3	Objective ocular signs - a positive result for at least one of the following two tests: - Schirmer's test, (≤5 mm in 5 minutes) - Rose Bengal score or other dye (≥4 according to van Bijsterveld's scoring system)	Keratoconjunctivitis sicca with ocular staining score ≥3
4	Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm^2	Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm^2
5	Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following tests: 1. Unstimulated whole salivary flow (≤1.5 ml in 15 min) 2. Parotid sialography showing diffuse sialectasias, without obstruction in major ducts 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer	
6	Autoantibodies: presence in the serum of the following autoantibodies: 1. Antibodies to Ro (SSA) and/or La (SSB) antigens - Have you had a daily feeling of dry mouth for more than 3 months? - Have you had recurrently or persistently swollen salivary glands as an adult? - Do you frequently drink liquids to aid in swallowing dry food?	Autoantibodies: presence in the serum of the following autoantibodies: 1. Antibodies to Ro (SSA) and/or La (SSB) antigens
3	Objective ocular signs - a positive result for at least one of the following two tests:	Keratoconjunctivitis sicca with ocular staining score ≥3

Table 3 - ***Overview of treatment options in primary Sjögren Syndrome.***

Drug	Usual dose	Main indications	Main contra-indications	Main side-effects	Monitoring needed
Systemic					
Pilocarpin [97,98]	20-30mg/day orally	Dryness of oral cavity	Untreated cardiovascular condition, untreated asthma	Headache, transpiration, frequent miction	
NSAID [124]	100-150 mg/day, orally	General symptoms (mainly arthralgia)	Peptic ulcer, GI-bleeding, IBD, CHF, CVA, liver- or renal failure.	GI effects, dizziness, rash, elevated liver enzyme test	*Six monthly:* Blood count, systemic liver and kidney test. Cardiovascular risk profile
Immunomodulatory					
Hydroxychloroquine [100,125]	200-400 mg/day orally	General symptoms (sicca, arthralgia and pain)	Retinopathy, breastfeeding	GI effects, rash, retinopathy, neuromyopathy	*Six monthly:* blood count and muscular strength *Yearly:* complete eye examination by ophthalmologist
Methotrexate [102]	10-20mg/ week orally or intramuscular	Insufficient effect of HCQ on chronic complaints	Liver and severe renal failure, severe respiratory failure, alcohol abuse, pregnant or lactating women	GI effects, neutropenia, liver and renal toxicity, interstitial pneumonitis, alopecia	*3 monthly:* blood count with differentiation, systemic liver and kidney test. *Yearly:* pulmonary function
Glucocorticoids [71,126]	20-40mg/day orally or intravenous 1g/day max. 3 days	Active systemic involvement (renal, pulmonary, neurological, muscular)	Active infections (viral, fungal), ulcus ventriculi / duodeni	Weight gain, hypertension, osteoporosis, diabetes, infection, neuropsychiatric reactions	*Next outpatient visit:* Weight, arterial blood pressure, glycaemia, bone density
Rituximab [36,127]	1000mg intravenous; repeat after 2 weeks. 30 minutes in prior: 100 mg methylprednisolone	Active systemic involvement not responsive to non-biologic immunosuppressive drugs	Pregnant or lactating women, active severe infection, severe CHF	Infections, allergic reaction	*Next outpatient visit:* Blood count with differentiation, systemic liver and kidney test

Preventive and local therapy

Alcohol and smoking should be avoided and thorough oral hygiene is essential [94,95]. Xeropthalmia can be managed with preservative-free teardrops and ocular lubricating ointments. Severe refractory dryness of the eyes can be treated with cyclosporin 0.05% [96]. Patients with xerostomia can manage the dry mouth by doing gustatory stimulation (chewing gum) and moisture replacement.

Systemic therapies

The majority of patients use pilocarpine, a muscarinic receptor agonist, which stimulates residual salivary gland function [97,98]. Systemic treatment is indicated when: 1) general symptoms (e.g. arthralgia) cannot be managed with local treatment or adjustment of the patient's lifestyle and 2) in case of organ involvement. Non-steroidal anti-inflammatory drugs (NSAIDS) have beneficial effects on general symptoms, like arthralgia. When general symptoms become more chronic, hydroxychloroquine (HCQ) is indicated [99,100]. It has been reported that patients with arthralgia benefit from HCQ [99]. A recent study shows, however, that fatigue does not improve by HCQ treatment [101]. In case of more severe organ involvement, other DMARDS or glucocorticoids should be added. Since methothrexate (MTX) is effective in RA, MTX is also used in the treatment of arthritis in pSS patients [102]. Glucocorticoid treatment is predominantly indicated when (severe) cutaneous, pulmonary, renal, musculoskeletal and/or neurological involvement occurs [103]. In case of insufficient effect of glucocorticoid therapy, glucocorticoid intolerance due to side effects and/or to reduce glucocorticoid dose, adding or switching of a DMARD (mycophenolate mofetil, cyclosporine A, azathioprine) should be considered. Therapy resistant pSS with proven organ damage is an indication to start biologicals, with the B cell as the most promising target based on the aetiology of pSS.

Biological therapies

Rituximab is a monoclonal antibody targeting the CD20 molecule (human B lymphocyte-restricted differentiation antigen) expressed on the surface of most B cells, including pre-B and mature B lymphocytes leading to B cell depletion [104]. Several studies have demonstrated a favourable effect of rituximab in pSS. Two studies combining 274 pSS patients reported that the

severity of glandular, articular, renal, neurological, pulmonary and haematological involvement was significantly decreased in approximately 60% of the patients after six months [34,105]. As a consequence of rituximab treatment, serum BAFF levels are increasing in order to stimulate B cell maturation, which can be countered by anti-BAFF treatment (belimumab) to achieve a longer B cell depletion and associated longer treatment effect [36]. Based on these findings and the pathophysiology of pSS, combination therapy with belimumab and rituximab would be (an expensive) but promising option [36]. The combination of rituximab and belimumab may be effective since this combination leads to an effective depletion of both the tissue and circulating B cells as well as a depletion of one of the stimulators (BAFF) required for B cell differentiation. Currently, new potential anti B cell therapies are being evaluated in (pre)clinical trials including anti-CD40 (decreases antigen presentation by B cells), anti-BAFF receptor (inhibits the effects of BAFF), anti-inducible costimulatory ligand (ICOSL, decreases activation of T-cells) and phosphoinositide 3-kinase delta inhibitor (PI3Kδ, inhibition of B cell development and activation) [106–109].

Prognosis

Patients with pSS should be closely monitored to evaluate the development of systemic manifestations and the effects of treatment. Compared to the general population, pSS patients have an increased mortality risk. The standardized mortality ratio (ratio of observed deaths in the study group to expected deaths in the general population, SMR) of pSS patients is on average 2.86, showing that pSS has an impact on patients' survival [110,111]. The leading cause of mortality in pSS is lymphoma with a lymphoma-specific SMR of 7.89, associating lymphoproliferative disorders directly with death in pSS [56]. However, once lymphoma is diagnosed, the prognosis is relatively favourable with a 15-year survival of almost 80% [112]. Other causes of death in pSS include vasculitis, renal failure due to glomerulonephritis and infections after the administration of immunosuppressive medication [77,113]. Morbidity in pSS is mainly due to extreme fatigue and the presence of systemic manifestations and should be evaluated for each patient individually. Patients with systemic complications and lymphoma development have an increased mortality risk. Therefore, risk factors (clinical and biological) for lymphoma and other organ involvement (e.g. pulmonary function, renal function, neurological evaluation) should be assessed frequently.

Conclusion

We summarized the clinical aspects of pSS. Physicians should be aware of pSS in patients presenting with sicca or general symptoms since the systemic manifestations are severe and are associated with increased morbidity and mortality. The treatment of pSS is effective and includes both local and systemic therapy. New therapies were developed, such as biologic treatment, of which the effectiveness in pSS should be evaluated. Further research should also focus on revealing new aetiological targets for therapy.

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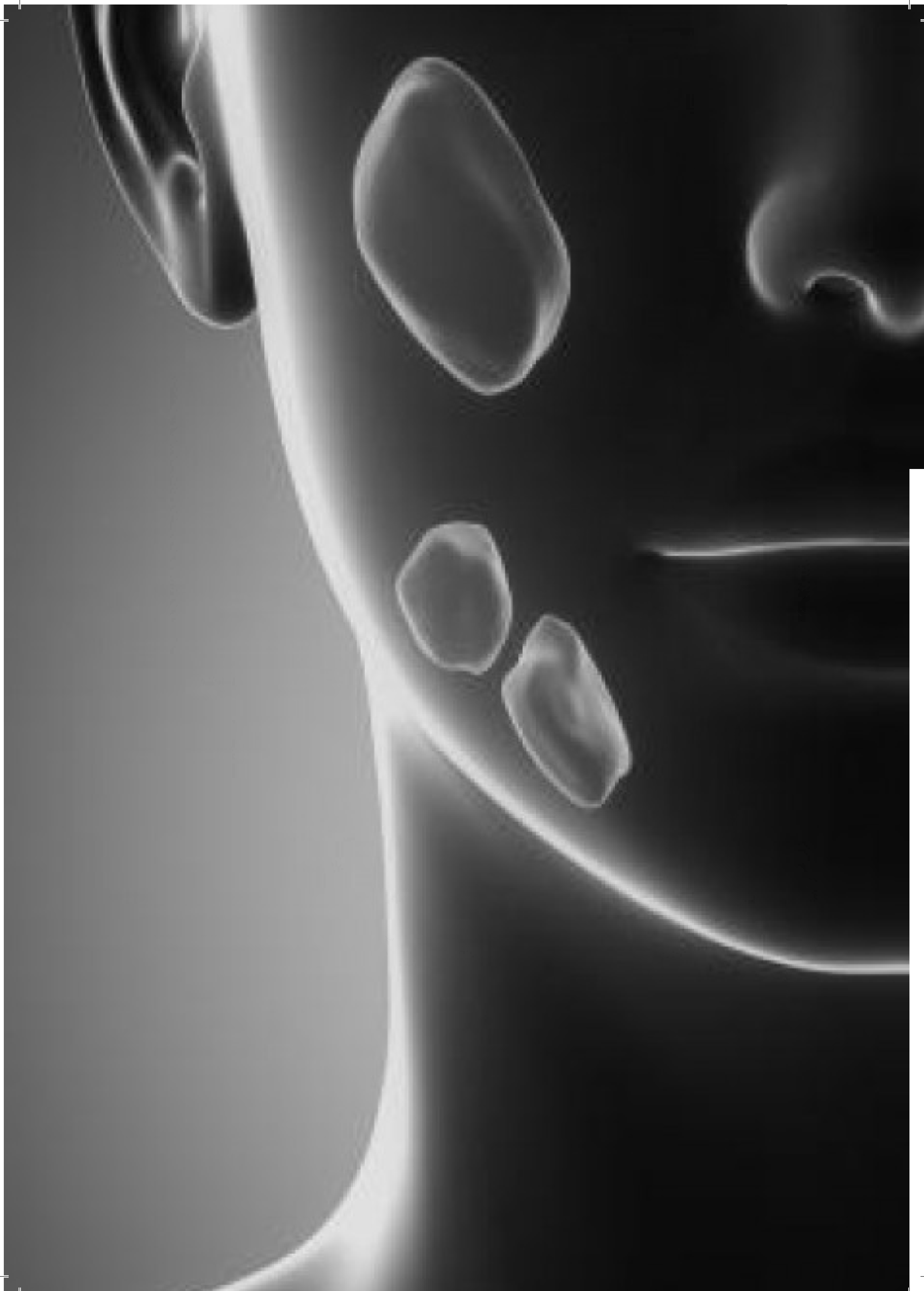

Everything you need to know about distal renal tubular acidosis in autoimmune disease

Distal renal tubular acidosis: from pathophysiology to diagnosis and treatment

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Abstract

Renal acid-base homeostasis is a complex process, effectuated by bicarbonate reabsorption and acid secretion. Impairment of urinary acidification is called renal tubular acidosis (RTA). Distal renal tubular acidosis (dRTA) is the most common form of the RTA syndromes. Multiple pathophysiologic mechanisms, each associated with various etiologies, can lead to dRTA. The most important consequence of dRTA is (recurrent) nephrolithiasis. The diagnosis is based on a urinary acidification test. Potassium citrate is the treatment of choice.

Introduction

Distal renal tubular acidosis (dRTA) is characterized by an impairment of normal urinary acidification process in the distal part of the nephron in the presence of a normal glomerular filtration rate. The term "distal" implies that acidification by the distal parts of the nephron (connecting tubule and collecting duct) are disturbed in contrast to proximal tubular acidosis, in which the reabsorption of bicarbonate by the proximal tubule is impaired. The prevalence and incidence of dRTA in the population are not known. dRTA is associated with auto-immune diseases such as primary Sjögren syndrome and systemic lupus erythematosus [1–3]. Prevalence of dRTA in primary Sjögren syndrome is estimated to be 5-25% [4–7]. Recurrent nephrolithiasis and/or chronic metabolic acidosis with a randomly measured high urinary pH suggest the presence of dRTA. Of patients with dRTA approximately 5% develops nephrolithiasis (mainly calcium phosphate stones), while 56% of dRTA patients has significant nephrocalcinosis [8,9]. Vice versa, in 41% of the patients with calcium phosphate stones dRTA is the underlying condition [10]. The availability of an effective treatment for dRTA should lower the threshold for testing suspected patients [11,12]. To confirm the diagnosis dRTA an urinary acidification test is recommended using either the well-known ammonium chloride test or a recently proposed combination of furosemide and fludrocortisone [13].

The aim of this review is to make physicians aware of a disorder in urinary acidification in patients presenting with a chronic metabolic acidosis and/or nephrolithiasis, especially in case of calcium phosphate stones. Both the physiology of renal acid-base regulation and the clinical aspects of dRTA will be reviewed.

Acid-base homeostasis

Our basal metabolic reactions and daily food intake lead to acid excess. Carbon dioxide (CO_2) originating from the oxidation of carbohydrates, fats, amino acids and proteins is by far the largest potential source of acid (15.000 mmol/day). CO_2 is a volatile acid that is removed by pulmonary ventilation, preventing CO_2 to react with H_2O to form protons [14].

Human metabolism also produces nonvolatile acids (e.g. phosphate, sulfate) and nonvolatile bases (e.g. bicarbonate), that cannot be excreted by the lungs. Together with acid from our diet

and intestinal base loss, the body is exposed to approximately 70-100 mmol of nonvolatile acids per day [15]. The role of the kidney is to excrete this acid excess as well as to monitor arterial pH to maintain a normal acid-base balance.

The kidney can maintain the arterial pH between 7.35-7.45 by preventing loss of filtered bicarbonate (4320 mmol/day HCO_3^-) and by net secretion of H^+ (70-100 mmol/day). The kidney cannot simply secrete this amount of acid, because this would require urinary pH to decrease to approximately 1.3. Due to the energetic maximum of H^+-ATPase, urinary pH can be maximally decreased to 4.2, which is not sufficient to clear the acid excess. In order to get rid of the acid excess, secreted protons will 1) be titrated by filtered bicarbonate resulting in bicarbonate reabsorption, 2) excreted by titratable acids, 3) titrated and excreted by ammonium and 4) excretion of free protons.

Proton secretion

The secretion of protons over the apical membrane is for 90% achieved by the so-called Na^+-H^+ exchanger isoform 3 (NHE3), that exchanges sodium for protons over the apical membrane. This transporter is present in the proximal tubule, thick ascending limb and distal convoluted tubule and is dependent on the basolateral Na^+/K^+ pump activity. A second mechanism to secrete protons is carried out by the vacuolar H^+-ATPase located in the distal tubule (10%). The vacuolar H^+-ATPase is limited to create a chemical gradient of 10^3 of H^+ over the apical membrane. This limitation is caused by a lack of ATP to keep the transporter functioning at a higher gradient. The maximally reached gradient over the apical membrane is reflected by a decrease of urinary pH from 7.5 to 4.5 [16].

Titration of bicarbonate

The kidney filters about 4320 mmol/day of bicarbonate, of which 99.9% is reabsorbed. The proximal convoluted tubule is responsible for the reabsorption of 80-85% of filtered HCO_3^- [17]. Remaining HCO_3^- is reabsorbed further downstream in the nephron. All intraluminal bicarbonate can be protonated and subsequently reabsorbed. This means that the complete reabsorption of filtered HCO_3^- requires 4320 mmol/day of secreted protons, which is considerably more than the 70-100 mmol/day of proton secretion required for neutralizing of nonvolatile acids.

However, the process of HCO_3^- reabsorption is not accompanied by net H^+ excretion.

Titratable acid excretion

Secreted protons will also interact with buffers other than HCO_3^-. These buffers originate from metabolic reactions. The most significant buffers are phosphate (pKa = 6.8), urate (pKa = 5.8) and creatinine (pKa = 5.0). With a lower urinary pH a higher percentage of the buffer will be protonated, regardless of the pKa of each buffer.

In the proximal convoluted tubule are the so-called sodium-phosphate cotransporters (NaPi) located, that are responsible for phosphate reabsorption. Early studies already showed that these transporters are down-regulated in periods of metabolic acidosis [18]. Recent studies indicate that these transporters are directly inhibited by protons resulting in hyperphosphaturia [19]. Because of its relative high pKa and the pH-dependent reabsorption of phosphate, phosphate is an important buffer. The amount of buffer that is ultimately excreted in the urine is largely dependent on the GFR and the plasma concentration of the buffer. For example, an average individual with a normal plasma phosphate concentration and normal GFR will excrete approximately 30 mmol/day of phosphate

Regulation of ammonia secretion

Ammonia (NH_3) is extremely important as urinary buffer, because of its high pKa of 9, which means that almost all the ammonia will be protonated to ammonium (NH_4^+). NH_4^+ is in equilibrium with NH_3 and H^+ in both the intra- and extracellular space of the nephron. Ammonia is produced in every segment of the nephron, but predominantly in the proximal tubule by the metabolism of mitochondrial glutamine **(Figure 1)** [20]. Produced ammonium is secreted by the proximal tubule by NHE3 mediated Na^+/H^+ exchange and Ba^{2+}-sensitive K^+ channels (ROMK) [21,22]. Additionally, NH_3 is transported over the apical membrane by still undefined channels. Secreted ammonium will be reabsorbed in the thick ascending limb of Henle's loop either via the $K^+/H^+(NH_4^+)$ exchanger, or by the Ba^{2+}-sensitive K^+ channels (ROMK) or by the Na^+-K^+-($2Cl^-$) cotransporter (NKCC2) [23]. Electroneutral K^+/NH_4^+ exchange and diffusive NH_3 transport across the apical plasma membrane by undefined channels take also place, but are less important. Cytosolic NH_4^+ will mainly exit the tubulus cell via the basolateral NHE4 transporter [24]. A second

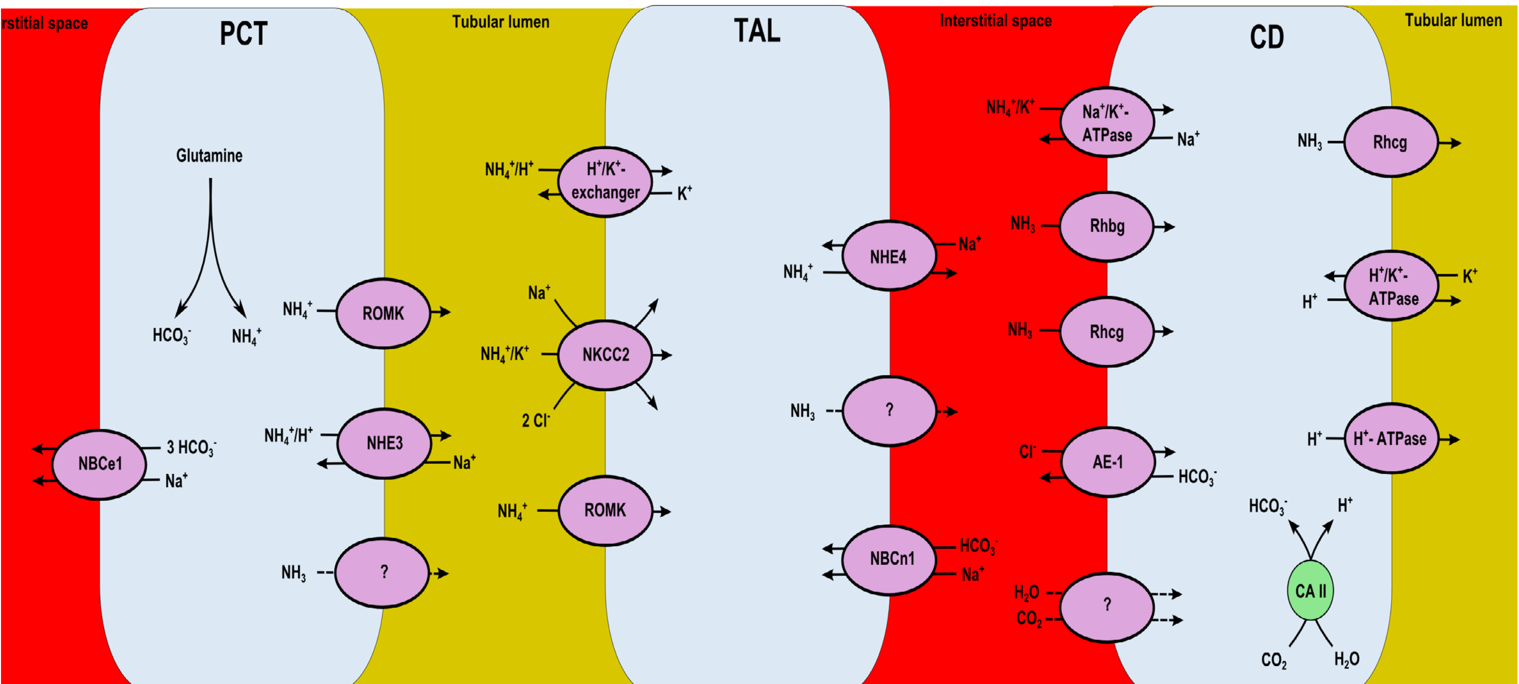

Figure 1 – An overview of ammonium transport through the nephron

Ammoniagenesis takes place in the proximal convulated tubule cells and ammonium is subsequently secreted. The thick ascending limb reabsorbs intraluminal ammonia in order to create a chemical gradient. The collecting duct utilizes this gradient to secrete ammonia over the apical membrane to buffer the simultaneously secreted protons.

Abbreviations: PCT proximal convulated tubule; TAL, thick ascending limb; CD, collecting duct; NBCe-1, Na^+-HCO_3^- cotransporter; NHE-3, Na^+-H^+ exchanger isoform 3; ROMK, Ba^{2+}-sensitive K^+ channel; NKCC2, Na^+-K^+-($2Cl^-$) cotransporter; NBCn-1, sodium-bicarbonate cotransporter; Rhbg, Rhesus glycoprotein type B; Rhcg, Rhesus glycoprotein type C; AE-1, chloride-bicarbonate cotransporter

mechanism of basolateral NH_4^+ exit may involve dissociation of NH_4^+ to NH_3 and H^+. Transport of NH_3 over the basolateral membrane in the thick ascending limb is presumed to be via diffusion as evidence for a gas transporter for NH_3 in the thick ascending limb is lacking. However, the concept that gasses (NH_3 and CO_2) and water diffuse over the membranes has been questioned over the last years. Instead of diffusion, gasses and water are carried over the membrane by transporters, such as aquaporins and the recently discovered rhesus glycoproteins [25]. The thick ascending limb buffers intracellular produced protons via basolateral bicarbonate transport. This is mediated by the sodium-bicarbonate cotransporter (NBCn1) leading to the formation of H_2CO_3 [26]. H_2CO_3 will be dissociated into H_2O and CO_2, after which CO_2 will be transported over the basolateral membrane into the peritubular lumen. Ammonium in the peritubular space will be transported in the collecting duct via Na^+-K^+-ATPase and Rhesus glycoproteins Rhbg and Rhcg [25,27]. Intracellular ammonia will be secreted over the apical membrane via the Rhcg glycoprotein and becomes available to buffer secreted protons [25]. Formed ammonium in the collecting tubular lumen is trapped and will be excreted. The complex system of ammonia transport through the nephron provides the collecting tubule a chemical and concentration gradient over the apical membrane. By altering these gradients, ammonia secretion over the apical membrane in the collecting tubule can be regulated to buffer the secreted protons.

Proximal acidification

As described before, reabsorption of bicarbonate is mainly achieved by proximal convoluted tubule cells **(Figure 2)**. Secreted H^+ binds to HCO_3^- to form carbonic acid (H_2CO_3) in the tubular lumen. Subsequently, formed H_2CO_3 will become H_2O and CO_2, a reaction catalyzed by the membrane-bound enzyme carbonic anhydrase type 4. Luminal CO_2 and H_2O are transported over the apical membrane via aquaporin 1 (AQP1) in the proximal tubule, after which they hydrate into H_2CO_3. This reaction is catalyzed by intracellular carbonic anhydrase type 2 (CAII). Intracellular H_2CO_3 ionizes to H^+ and HCO_3^-, after which HCO_3^- will be transported over the basolateral membrane via the Na^+-HCO_3^- cotransporter (NBCe-1) [28]. Protons remain in the cytoplasmatic compartment to be secreted again in the tubular lumen. At the end, this process results in the reabsorption of one molecule HCO_3^- and zero net secretion of one molecule of H^+.

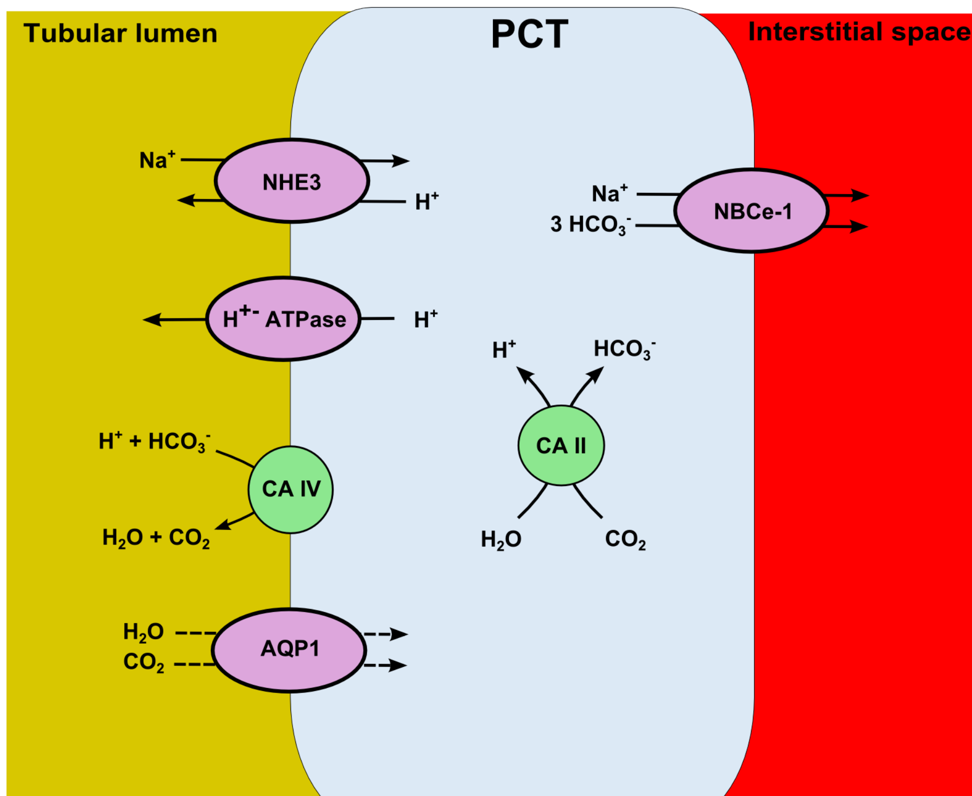

Figure 2 – The process of bicarbonate reabsorption in the proximal tubule cell
Filtered bicarbonate is catalyzed by carbonic anhydrase type 4 into carbon dioxide and hydroxide. Carbon dioxide is transported over the apical membrane via aquaporin 1 (AQP1) in the proximal tubule, after which it hydrates into H_2CO_3. This reaction is catalyzed by intracellular carbonic anhydrase type 2. Intracellular formed bicarbonate will leave the cell via the NBCe-1 transporter localized on the basolateral membrane.
Abbreviations: PCT, proximal convulated tubule cell; CA, carbonic anhydrase; NHE-3, Na^+-H^+ exchanger isoform 3; NBCe-1, Na^+-HCO_3^- cotransporter,

Distal acidification

The α-intercalated and principal cells, located in the collecting tubule, are responsible for the secretion of protons **(Figure 3)**. The principal cell's main function is to reabsorb sodium via the epithelium Na^+ channel (ENaC) located in the apical membrane [29]. This causes an electronegative tubular lumen, favoring the secretion of potassium or protons. Proton secretion is achieved by the vacuolar H^+-ATPase, stored in vacuoles in the cytoplasm of α-intercalated cells. The expression of this pump is largely dependent on the electrical gradient over the luminal membrane. The electronegative luminal potential, driven by ENaC activity, results in expression of

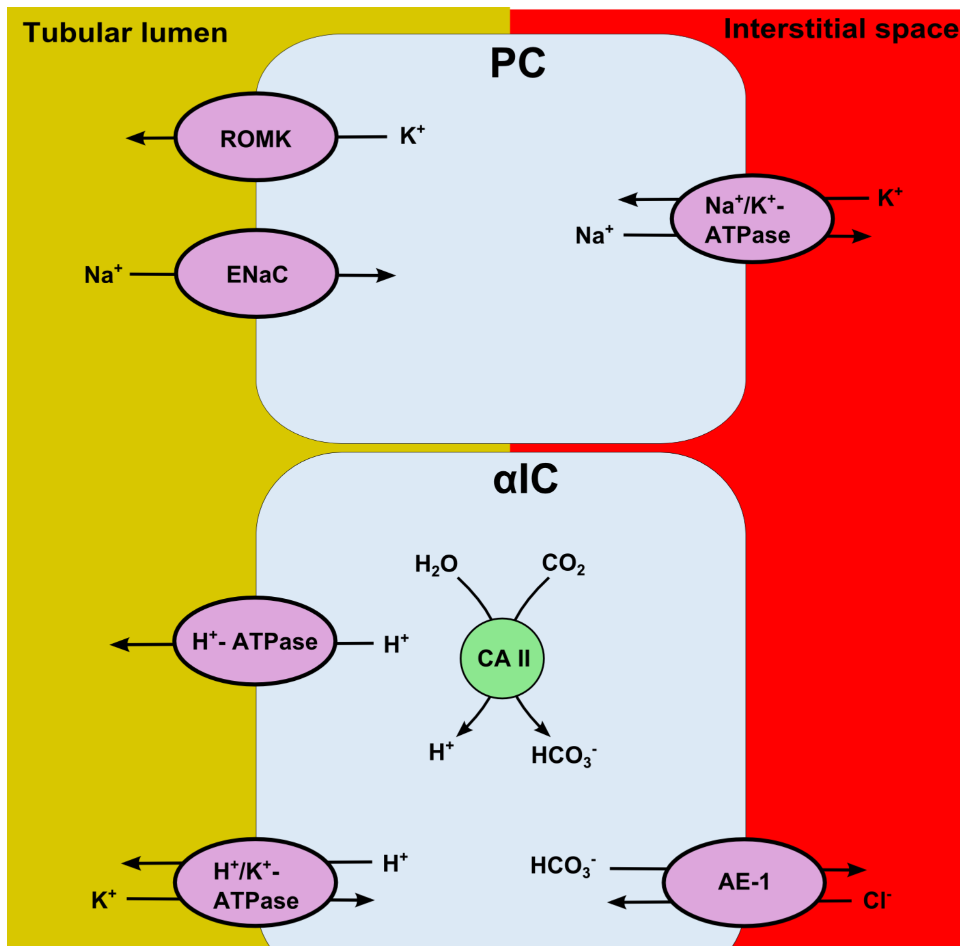

Figure 3 – The process of proton secretion in the collecting duct

The principal cell reabsorbs intraluminal sodium creating an electronegative gradient. The alpha-intercalated cells contain vacuoles which stores H+-ATPases. These proton pumps are built in the apical membrane for proton secretion. The secretion of protons is enhanced by sodium reabsorption and an electrical gradient.

Abbreviations: PC, principal cell; αIC, alpha intercalated cell; CA, carbonic anhydrase; ROMK, renal outer medullary potassium channel; ENaC, epithelium Na+ channel; AE-1, chloride-bicarbonate cotransporter

H^+-ATPase on the apical membrane of the α-intercalated cells and excretion of protons into the lumen [27]. The protons are generated by intracellular activity of the CAII enzyme, which also forms HCO_3^- ions. HCO_3^- will be exchanged with Cl^- over the basolateral membrane via the chloride-bicarbonate cotransporter (AE-1) [27]. Still another ATPase expressed in the apical membrane of the α-intercalated cell, is the H^+/K^+ exchanger. This exchanger contributes to proton

secretion, but is less important than the vacuolar H^+-ATPase and is considered to be more relevant for potassium reabsorption.

Distal renal tubular acidosis

The characteristic features of distal renal tubular acidosis (dRTA) are the presence of systemic acidosis together with the inability to acidify the urine to a pH < 5.3 dRTA is associated with many diseases each with their own pathophysiology. To provide a clear overview of the causes of dRTA, we divided dRTA in to four groups based on their pathophysiologic defect: 1) voltage defect, 2) H^+ secretion defect, 3) H^+ gradient defect and 4) ammonium generation defect **(Table)**.

Voltage defect

As outlined before an electronegative luminal potential in the collecting tubule contributes to proton secretion. The ENaC is responsible for this driving force by reabsorbing Na^+. ENaC's activity is predominantly regulated by aldosterone. Apart from regulation of ENaC activity aldosterone can enhance distal urinary acidification by increasing the activity of H^+-ATPase in the cortical collecting tubule [29,30].

Both genetic and acquired forms of decreased ENaC activity exist. Genetic causes are related to mutations in genes encoding for the alpha, beta or gamma subunit of the channel (respectively *SCNN1A*, *SCNN1B*, *SCNN1G* genes), resulting in autosomal recessive pseudohypoaldosteronism type 1. An autosomal dominant form in which the genetic defect (*NR3C2*) affects the mineralocorticoid receptor is also known [31]. Acquired forms of decreased ENaC activity are more common. They are common due to hypoaldosteronism. The most common cause of hypoaldosteronism is hyporeninemia as can occur in diabetes mellitus, renal insufficiency or use of nonsteroidal anti-inflammatory drugs or calcineurin inhibitors. Furthermore aldosterone is diminished in Addison's disease. Additionally, medication can directly or indirectly decrease ENaC activity (e.g. amiloride, cyclosporine, tacrolimus, lithium, ACE-inhibitors, angiotensin II receptor blocker, aldosterone receptor blockers and heparin) [31].

H^+ secretion defect

Alpha-intercalated cells are responsible for both generation and secretion of protons. The intracellular enzyme CAII catalyzes the reaction leading to the formation of protons and bicarbonate ions. The main proton transporter is the vacuolar H^+-ATPase, built in the apical mem-

Table – Overview of the aetiologie of dRTA

Voltage defect		Proton secretion defect		Proton gradient defect	Ammonium secretion defect
Inherited	***Acquired***	***Inherited***	***Acquired***	***Acquired***	***Acquired***
ENaC subunit mutations	Hypoaldosteronism	Vacuolar H^+ ATPase mutation	Autoimmune disease	Medication	Hyperkalemia
- *SCNN1A*	- Hyporeninemia	- *ATP6V1B1*	- Sjögren	- Amphoterocin B	
- *SCNN1B*	- Addison's disease	- *ATPV6V0A4*	- SLE		
- *SCNN1G*	- Chronic UT obstruction	AE1 transporter mutation	- PBC		
	- Diabetes	- *SLC4A1*	- AIH		
MCR mutations	- Renal insufficiency	CA type 2 mutation	- AIT		
- *NR3C2*		Medullary sponge kidney	Medication		
	Medication		- Topiramate		
	- NSAID		- CA inhibitor		
	- Amiloride				
	- Cyclosporin				
	- Lithium				
	- ACE-inhibitor				
	- Angiotensin inhibitor				
	- (Low molecular weight) heparin				

Abbreviations: ENaC, epithelial sodium channel; MCR, mineralocorticoid receptor; UT, urinary tract; ACE, angiotensin converting enzyme; AE1, anion exchanger 1; CA, carbonic anhydrase; SLE, systemic lupus erythematoses;, PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; AIT, autoimmune thyroiditis

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brane. The bicarbonate ion is transported over the basolateral membrane by the AE1. A defect in one of those subparts of the H^+ secreting machinery can lead to dRTA.

Primary causes for a defect in one of the compartments are due to mutations in genes encoding subunits of the vacuolar H^+-ATPase (*ATP6V1B1* and *ATPV6V0A4*), resulting in impaired transporter function. These mutations lead to autosomal recessive forms of dRTA that can coexist with and without deafness. Also an autosomal dominant form of dRTA is known, caused by a mutation of a gene coding for the AE1 (*SLC4A1*), leading to a decreased number of this transporter in the basolateral membrane. Carbonic anhydrase enzyme type 2 deficiency by genetic mutations leads to both proximal and distal RTA [32]. Medullary sponge kidney is also a primary cause of dRTA, related to the malformation of the distal tubules. The presence of dRTA in these patients depends on the number of nephrons affected [33].

Acquired impaired transporter function of the H^+ secreting machinery is often associated with auto-immune diseases like Sjögren syndrome and systemic lupus erythematosus (SLE). In patients with primary Sjögren syndrome inhibitory autoantibodies against the CAII enzyme have been reported [34]. Also certain medications, such as topiramate and acetazolamide, can inhibit the function of the CAII enzyme [35].

H^+ gradient defect

Proton secretion is dependent on the H^+ gradient over the apical membrane, which is achieved by vacuolar H^+-ATPase. Notwithstanding an appropriately working vacuolar H^+-ATPase, creating of such gradient is not always successful. This is the case in leaky membrane, sometimes seen in patients using amphoterocin B [36,37]. In experimental models, amphotericin B increases the permeability for protons of the apical membrane in the collecting duct, causing back diffusion of the secreted protons [38,39].

Ammonium secretion defects (hyperkalemia)

Ammonium plays a major role in renal urinary acidification. In case of low availability of ammonium in urine, urinary acid excretion is impaired to a certain pH. The most important cause of decreased urinary ammonium is hyperkalemia [40]. Hyperkalemia reduces the expression of ammoniagenic enzymes and acid transport proteins [41]. Additionally, hyperkalemia decreases the secretion of ammonia in the loop of Henle and the collecting duct. This probably is due to

competition between NH_4^+ and potassium. NH_4^+ and potassium use the same binding spot on the transporters in the thick ascending limb (respectively NKCC2 and Na^+-K^+-ATPase) [42]. Hyperkalemia will also drive protons from intracellular to extracellular, leading to a decreased concentration of protons in the distal tubule cells.

Clinical presentation

The most common symptom of dRTA is nephrolithiasis and metabolic acidosis. Fatigue is a frequent complaint, possibly related to the metabolic acidosis-induced hyperventilation. Patients with chronic metabolic acidosis are prone to develop osteoporosis. Metabolic acidosis affects bone by exchanging protons for sodium, potassium, calcium, carbonate and phosphate [43]. The continuous sequestration of protons in bone stimulates both osteoclast development and osteoclast activity. As a consequence bone resorption increases, enhancing release from the bone surface of calcium and mineral buffers like bicarbonate and phosphate [43,44]. Eventually, this mechanism leads to net bone loss and hypercalciuria.

Metabolic acidosis also leads to enhanced proximal tubular reabsorption of citrate resulting in hypocitraturia. Alkaline urine in combination with hypocitraturia and hyperphosphaturia promotes calcium phosphate precipitation leading to nephrocalcinosis and/or kidney stones [45].

Additionally, patients with dRTA often develop abnormalities in the potassium balance. In general, metabolic acidosis will lead to hyperkalemia as a result of the exchange of protons for intracellular potassium. However, patients with dRTA due to a proton secretion defect tend to waste potassium in urine in order to maintain electroneutrality over the apical membrane. Despite potassium wasting, these patients usually have normal levels of serum potassium, because of potassium movement from intracellular to extracellular. Nevertheless, case-reports have been described of patients with dRTA who present to the emergency department with hypokalemic paralysis, including respiratory arrest [1,46].

Incomplete dRTA

Of the RTA syndromes, also an incomplete form of dRTA is known, including patients with nephrocalcinosis or urolithiasis but without metabolic acidosis. Patients with incomplete dRTA cannot acidify their urine, but a higher amount of NH_4^+ excretion compensates for the acid secretion defect. Donnelly *et al*. hypothesized that this increased NH_4^+ excretion originates from

an increased production and secretion of ammonium in the proximal convoluted tubule. Additionally, hypocitraturia in these patients is often present. Diagnosis and treatment is the same as for complete dRTA [47].

Association of dRTA with autoimmune diseases

It is suggested that dRTA is more prevalent in autoimmune diseases. Shearn *et al*. reported in 1965 the first case of distal renal tubular acidosis revealing Sjögren syndrome [48]. Both primary and secondary Sjögren syndrome is associated with dRTA [4,49–51]. Other autoimmune diseases such as SLE [52], primary biliary cirrhosis (PBC) [53], autoimmune hepatitis (AIH) [54] and autoimmune thyroiditis (AIT) [51] are less common associated with dRTA. The prevalence of dRTA in Sjögren syndrome is currently estimated to be 25% [4]. The clinical presentation of dRTA in patients with an autoimmune disease is similar to those patients without a systemic disease.

The pathophysiological mechanism of dRTA in relation to autoimmunity remains unclear. Several reports suggest that autoantibodies against the CAII enzyme [34,55] or the acid-base transporters are involved in the pathogenesis of dRTA in autoimmune disease [56]. Recently, Espinosa *et al*. reported that anti-Ro52 autoantibodies from patients with Sjögren syndrome inhibit Ro52 E3 ligase activity [57]. In-vitro inhibition of the ubiquitination process may increase the transcription of pro-inflammatory genes leading to local inflammation and tissue damage [57]. Interstitial inflammation is often found in renal biopsies.

It is unknown whether treatment with corticosteroids in autoimmune disease has a positive effect on dRTA. We advise to treat dRTA in autoimmune diseases with potassium citrate. Potassium citrate is an effective treatment for both the symptoms and complications of dRTA, by restoring acid-base balance (see below). Studies about prognosis of dRTA in autoimmune diseases are lacking.

Diagnosis

Urinary acidification was assessed by using the oral ammonium chloride loading test (NH_4Cl test). The complete test takes eight hours and does not require blood testing. The test can be unpleasant, because it can induce gastric irritation, nausea, and vomiting. Thus, there was room for the development of a quicker and more patient-friendly urinary acidification test. Walsh *et al.* described in 2007 a urinary acidification test using simultaneous furosemide (40 mg) and

fludrocortisone (1 mg) administration [13]. Simultaneous administration of furosemide and fludrocortisone stimulates the kidney to secrete H^+-ions. Furosemide inhibits the NKCC2 co-transporter, resulting in a higher Na^+ delivery in the collecting tubule. Fludrocortisone binds and activates the mineralocorticoid receptor in the cytoplasm leading to an increased ENaC activity, thereby enhancing sodium reabsorption and potassium secretion. Additionally, fludrocortisone stimulates the expression of vacuolar H^+-ATPase in the apical membrane. Increased sodium reabsorption leads to an electronegative luminal potential, which is the driving force for the secretion of protons by the vacuolar H^+-ATPase in the distal tubule [13].

Walsh *et al*. compared this new test to the NH_4Cl loading test in 10 healthy controls. Every control was capable to acidify their urine to a pH < 5.3. The minimum pH value was 4.92 ± 0.10 after furosemide and fludrocortisone administration.

Both tests had the same result of (impaired) urinary acidification in dRTA patients. All patients failed to acidify their urine to a pH < 5.3. The lowest measured pH was 6.59 ± 0.13 after furosemide/fludrocortisones administration [13]. The furosemide/fludrocortisone test was better tolerated and lasts shorter it may prefer over the NH_4Cl test.

Treatment

The main goal of any treatment for dRTA is to reverse the acidosis, which reduces calciuria and simultaneously increases citrate excretion. This leads to a lower risk of nephrolithiasis and osteoporosis. Currently, potassium citrate (1 to 2 mEq/kg/day) is the treatment of choice for the management of patients with dRTA. With potassium citrate, not only a bicarbonate donor is provided to treat acidosis, but potassium wasting is compensated simultaneously. Potassium citrate treatment in dRTA patients seems to have positive effects on bone mineral density and bone cell function [11]. Additionally, a recent randomized controlled trial showed that potassium citrate increases bone density and reduced fracture risk in healthy elderly without RTA [12].

Conclusions

In this review we discussed the physiology of acid-base homeostasis and translated this mechanism to the RTA syndromes. The pathophysiology is divided in four categories each associated with different etiologies. Physicians should test for dRTA in patients with (recurrent) calcium phosphate stones and/or a chronic metabolic acidosis. The diagnosis of dRTA is made by using a

urinary acidification test, in which the patient is unable to acidify the urine to pH < 5.3. Treatment of dRTA is based on restoring the acid-base balance, which can be achieved with potassium citrate.

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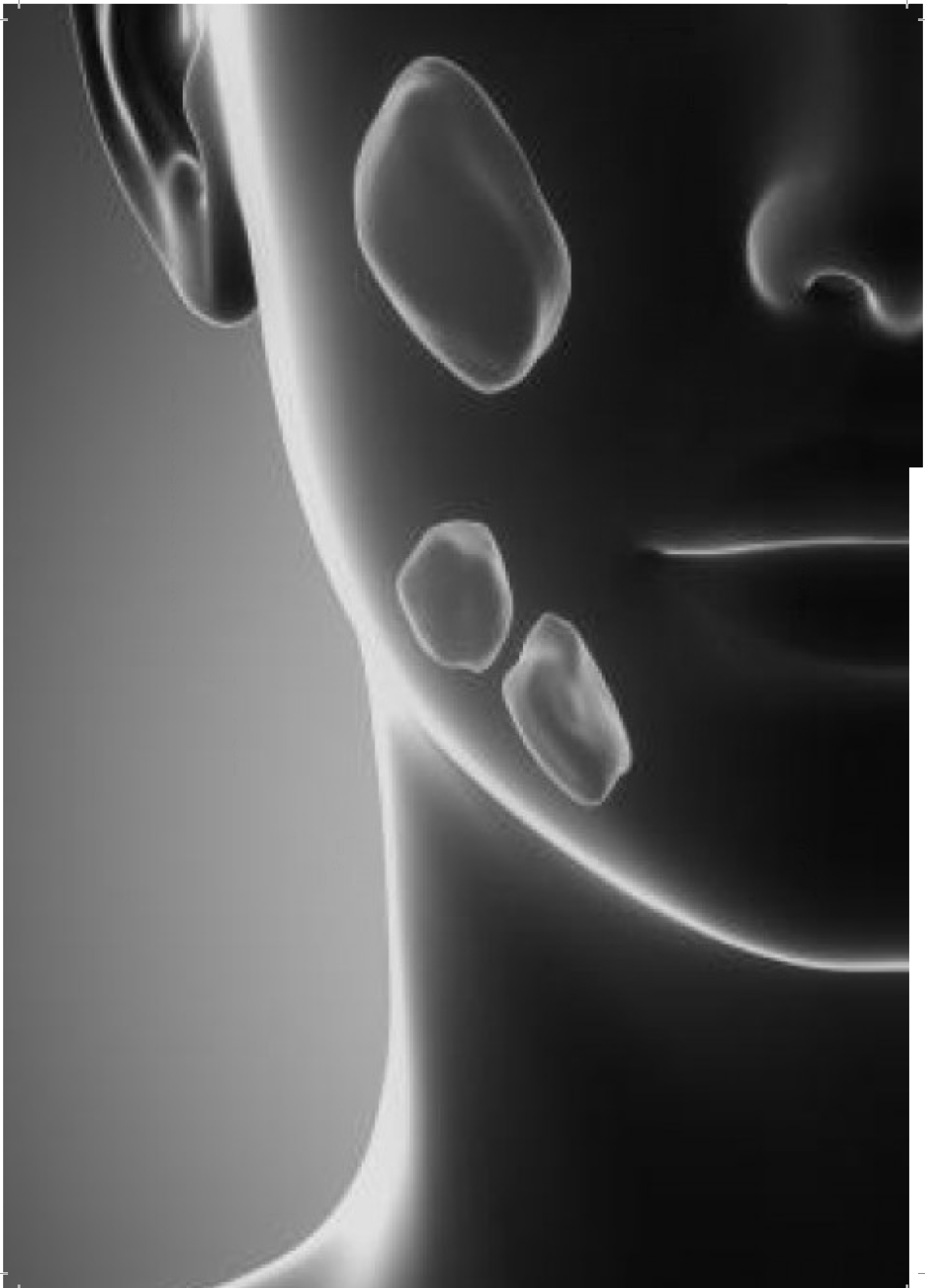

Prevalence of distal renal tubular acidosis in primary Sjögren syndrome

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Abstract

Objectives Our objectives were 1) to analyze the prevalence of distal renal tubular acidosis (dRTA) in primary Sjögren syndrome (pSS) and 2) to compare a novel urinary acidification test with furosemide and fludrocortisone (FF) with the gold standard ammonium chloride (AMCL) to detect dRTA

Methods Urinary acidification was assessed in 57 pSS patients using AMCL and FF. A urinary acidification defect was defined as inability to reach urinary pH < 5.3 after AMCL.

Results The prevalence of complete dRTA (urinary acidification defect with acidosis) was 5% (3 out of 57). All three patients had positive SSA/Ro and SSB/La auto-antibodies and impaired kidney function. The prevalence of incomplete dRTA (urinary acidification defect without acidosis) was 25% (14 out of 57). Compared to patients without dRTA, patients with incomplete dRTA had significantly lower venous pH and serum bicarbonate, and higher urinary pH. SSB/La antibodies were more prevalent in the dRTA groups ($p < 0.05$). Compared to AMCL, the positive and negative predictive values of FF were 46 and 82%, respectively. Vomiting occurred more often during the urinary acidification test with AMCL than with FF (9 vs. 0, $p < 0.05$).

Conclusions Incomplete dRTA is common in pSS and causes mild acidemia and higher urinary pH which may contribute to bone demineralization and kidney stone formation. FF cannot replace AMCL to test urinary acidification in pSS, but may be considered as screening tool, given its reasonable negative predictive value and better tolerability.

Introduction

Distal renal tubular acidosis (dRTA) is a well-known complication of primary Sjögren syndrome (pSS) [1]. dRTA is classified as complete or incomplete dRTA [1]. Complete dRTA is defined as a non-anion gap metabolic acidosis with a urinary pH > 5.3. Patients with incomplete dRTA maintain a serum bicarbonate within the normal range, but are unable to acidify their urine after an acid load [2]. dRTA indicates a failure of the intercalated cells in the kidney collecting duct to secrete hydrogen ions [3,4]. If the secretion of protons is severely impaired, the secretion of other cations, including potassium, is increased to maintain electroneutrality. This explains why complete dRTA is often accompanied by hypokalemia due to renal potassium loss, which may even result in hypokalemic paralysis [5–9]. Other, more long-term complications of dRTA include osteomalacia [10] and kidney stones [11]. Therefore, the detection of dRTA is clinically relevant because treatment with potassium citrate may prevent these complications [12].

In addition to dRTA, other renal manifestations of pSS may include tubulointerstitial nephritis, proximal RTA and nephrogenic diabetes insipidus [13,14]. Proximal RTA is characterized by impaired reabsorption of bicarbonate rather than a failure to secrete protons. Proximal RTA can be differentiated from dRTA by analyzing if other functions of the proximal tubule are perturbed (presence of hypophosphatemia, hypouricemia, glucosuria, tubular proteinuria) or by performing a bicarbonate infusion test [15].

Most of the literature on dRTA in pSS concerns case reports or small case series and therefore the true prevalence of dRTA in pSS remains unclear [7,9]. The prevalence of pSS in patients with new onset dRTA is reported to be 5% [16]. Although Bossini *et al*. analyzed complete and incomplete dRTA more systematically in 60 patients with pSS, the AMCL test was only performed in 12 patients [13]. Recently, Walsh *et al*. proposed an alternative urinary acidification test using the single administration of furosemide and fludrocortisone (FF) [17]. The combination of furosemide and fludrocortisone maximally stimulates urinary acidification because of an increased distal delivery of sodium to the collecting duct by furosemide, and a direct stimulation of hydrogen secretion by fludrocortisone [17]. In this study, FF was shown to be as effective as AMCL to test urinary acidification, and was also quicker and better tolerated [17].

In the present study we determined the prevalence of both complete and incomplete dRTA using the AMCL test. Furthermore, we compared the diagnostic performance of the urinary acidification test with FF to the urinary acidification test with AMCL. To do so, we performed both the AMCL and FF urinary acidification tests in a large cohort of patients with pSS.

Methods

Study cohort

The study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2013-075). pSS was defined according to the Revised American-European classification criteria [18]. Additional inclusion criteria for this study included age >18 years, no other underlying auto-immune disease and estimated glomerular filtration rate >30 ml/min. Patients with pSS were recruited from our outpatient clinic and through the advertisement of this study on the website of the Dutch Sjögren patient society **(Figure 1)**. Anti-nuclear antibodies (ANA), SSA/Ro52, SSA/Ro60, SSB/La auto-antibodies, and rheumatoid factor were measured in all patients using previously reported methods [19]. The results of salivary gland biopsy were retrieved when available. Finally, the EULAR SS Disease Activity Index (ESSDAI) was calculated for all of the patients.

Urinary acidification tests

All subjects underwent both urinary acidification tests using AMCL and FF on separate days with a minimum of one week in between the tests. Patients were allowed to continue their medication except for medication interfering with the urinary acidification tests (mineralocorticoid receptor blockers, loop diuretics, fludrocortisone). Patients were instructed to fast prior to the AMCL test to prevent vomiting; fasting was not necessary prior to the FF test. At baseline, serum and urine were collected followed by the administration of the test medication. AMCL was given at a dose of 1 ml/kg body weight accompanied by water and ingested over a period of 30 minutes to prevent gastric irritation. The FF test included a single oral administration of 40 mg furosemide and 1 mg fludrocortisone, as described previously [17]. In both tests, hourly urine samples were collected for six hours to measure urinary pH. Urinary pH was measured immediately by one of the investigators (T.B.) using an electrode pH meter (Hanna HI 991001, Hanna

Instruments BV, IJsselstein, The Netherlands). During both tests, patients were monitored for nausea and vomiting.

Distal renal tubular acidosis

Complete dRTA was defined as serum bicarbonate < 21 mmol/L, normal anion gap, positive urine anion gap, impaired urinary acidification, and the absence of any other known causes for dRTA (e.g., medication, hypercalciuria) [1]. Incomplete dRTA was defined as an abnormal AMCL test accompanied by a serum bicarbonate in the normal range [1]. In both the AMCL and the FF tests, a urinary acidification defect was defined as the failure to achieve a urinary pH < 5.3 within four hours after intake of the study drugs [17].

Statistics

All results are expressed as means with standard deviations. Sensitivity, specificity, positive and negative predictive values were calculated by comparing the FF test results with the AMCL test results, considering an abnormal response to the AMCL test as definition of disease. Comparisons of continuous variables between the three groups (no dRTA, incomplete dRTA, complete dRTA) were performed using one-way analysis of variance (ANOVA) with the least significant difference post-hoc test. Categorical data (presence or absence of auto-antibodies) were analyzed using the Fisher's Exact test. A p-value < 0.05 was considered significant. All analyses were performed in SPSS (version 21, IBM).

Results

Study cohort and baseline characteristics

The study cohort included 57 patients with pSS **(Figure 1)**. Although 62 patients were invited to participate in the study, 5 patients were excluded, because they were unable to complete both urinary acidification tests, due to repeated vomiting or because of withdrawal of consent. The baseline characteristics of the study cohort are shown in **Table 1**. Of the 30 patients with a retrievable salivary gland biopsy, the mean focus score was 3.1 ± 2.5. In addition to medication for pSS, other commonly used drugs in this cohort were renin-angiotensin inhibitors (9 patients) and non-steroidal anti-inflammatory drugs (7 patients).

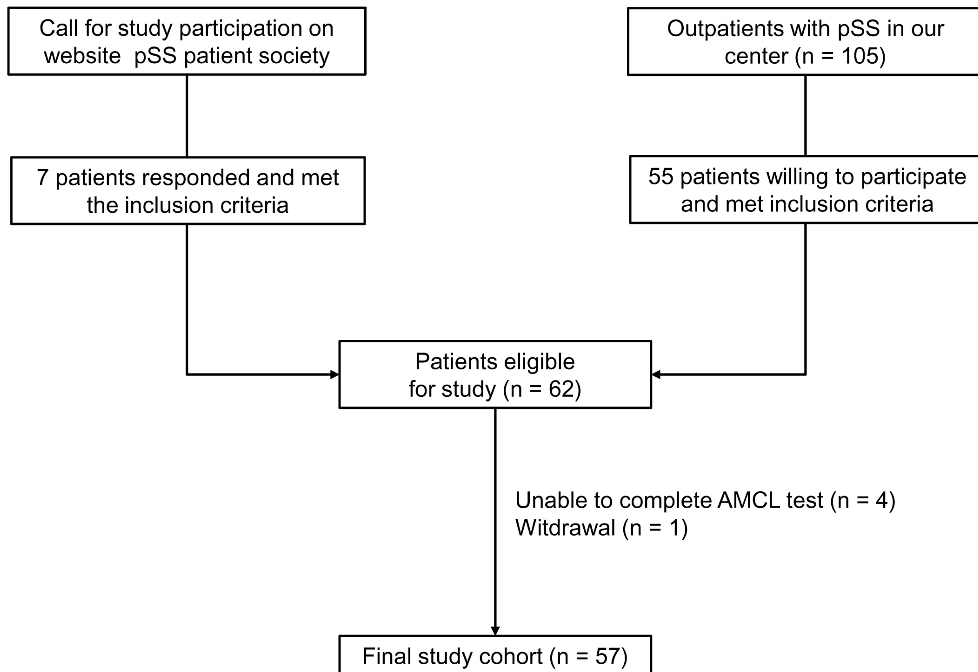

Figure 1 – Study cohort
Abbreviations: AMCL, ammonium chloride; pSS, primary Sjögren syndrome

Prevalence of dRTA

Seventeen out of fifty-seven patients were unable to acidify their urine to a pH < 5.3 after AMCL **(Figure 2A-B)**. None of these patients used non-steroidal anti-inflammatory drugs, which can also cause dRTA [20]. Among these 17 patients three patients **(Table 1)** had a baseline serum bicarbonate < 21 mmol/L (16.9 ± 0.58 mmol/L). They were diagnosed as complete dRTA secondary to pSS, because they also had a normal serum anion gap (10.3 ± 0.5 mEq/L), a positive urine anion gap (+33 ± 9 mEq/L), and no other explanations for dRTA, including medication or hypercalciuria. They also showed no signs of proximal renal tubular acidosis, as indicated by the absence of hypophosphatemia, hypouricemia, and glucosuria. Although all three patients had prior episodes of hypokalemia, they were normokalemic at baseline in this study **(Table 1)**;
two patients were receiving potassium supplementation. The remaining 14 patients were diagnosed as incomplete dRTA, because they had normal baseline serum bicarbonate, but were unable to acidify their urine to pH < 5.3. In summary, the prevalence of complete dRTA was 5%

(3 out of 57) and the prevalence of incomplete dRTA was 25% (14 out of 57).

Table 1 – Characteristics of the study cohort

Category	Variable	Study cohort (N = 57)
Demographics	Age, years	57 ± 11
	Female gender, n (%)	49 (86)
Antibodies	Anti-nuclear antibodies, n (%)	43 (75)
	SSA/Ro52, n (%)	43 (75)
	SSA/Ro60, n (%)	42 (74)
	SSB/La, n (%)	32 (56)
	Rheumatoid factor, n (%)*	21/46 (46)
Other tests	Positive salivary gland biopsy, n (%)*	25/30 (83)
	EULAR SS Disease Activity Index	2.7 ± 2.0
pSS medication	Hydroxychloroquine, n (%)	34 (60)
	Glucocorticoids, n (%)	4 (7)
	Other immunosuppressive drugs, n (%)†	3 (5)
Laboratory values	Serum potassium < 3.5 mmol/l, n (%)	0 (0)
	Serum bicarbonate < 21 mmol/l, n (%)	3 (5)
	Estimated GFR < 60 ml/min, n (%)	9 (16)
	Hypercalciuria, n (%)¶	3 (5)

Abbreviations: EULAR, European League Against Rheumatism; SS, Sjögren's syndrome

** Salivary gland biopsies were performed in 36/57 patients, but a focus score could be retrieved for only 30 patients; Rheumatoid factor was measured in 46/57 patients.*

†Other immunosuppressive therapy consisted of azathioprine, colchicine or methotrexate.

¶ Defined as a urine calcium to creatinine ratio > 0.6

The FF test is less sensitive but better tolerated

In order to compare the results of the AMCL test to the novel FF test, we performed both tests in the same cohort of patients. Twenty-four patients were unable to acidify their urine to a pH < 5.3 with the FF test **(Figure 2C-D)**. A comparison between the AMCL and FF tests showed not only that more patients were unable to acidify their urine to a pH < 5.3 (24 vs. 17 patients), but also that six patients did acidify their urine with FF but not with AMCL **(Table 2)**. Considering the AMCL test as gold standard, the sensitivity and specificity of the FF test were 65% and 68%

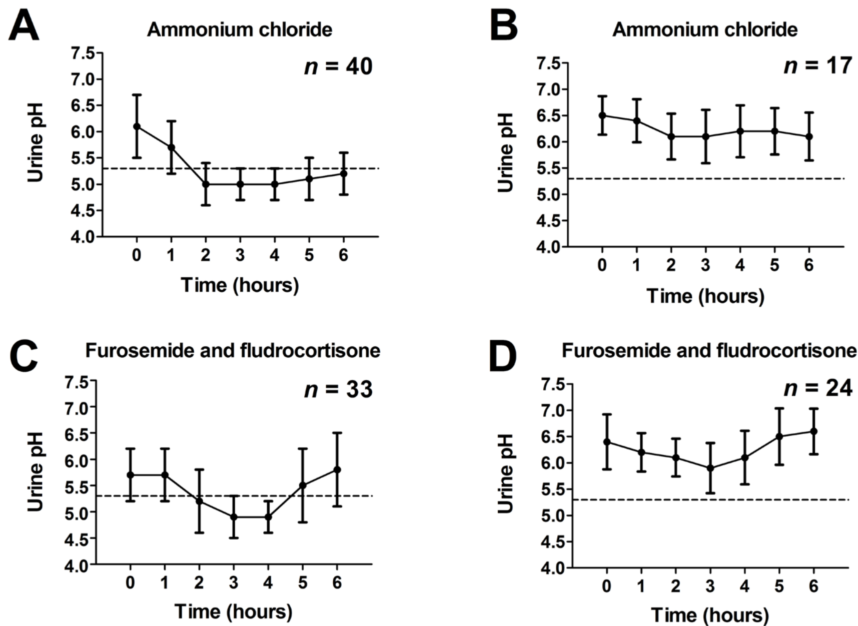

Figure 2 – Results urinary acidification test with ammonium chloride or furosemide and fludrocortisone
*The four panels (**A**-**D**) show the results of the urinary acidification tests with ammonium chloride (**A**-**B**) and furosemide and fludrocortisone (**C**-**D**). The left panels (**A** and **C**) show patients who were able to acidify their urine to pH < 5.3 during the test. The right panels (**B** and **D**) show the patients who were unable to acidify their urine to pH < 5.3 during the test.*

and the positive and negative predictive values were 46% and 82%. Comparison of the side-effects during both tests showed that vomiting was significantly more common in patients undergoing the AMCL test than in patients undergoing the FF test (9 vs. 0, $p < 0.05$). The three patients diagnosed with complete dRTA also failed to reach a urine pH < 5.3 with the FF test. None of the patient characteristics reported in **Table 1** were different in the six patients with a false negative FF test ($p > 0.05$ for all). The six patients used the following drugs: hydroxychloroquine

Table 2 – Comparison of both urinary acidification in patients with primary Sjögren syndrome

	AMCL U_{pH} ≥ 5.3	AMCL U_{pH} < 5.3	Total
FF U_{pH} ≥ 5.3	11	13	24
FF U_{pH} < 5.3	6	27	33
Total	17	40	57

Abbreviations: FF, furosemide and fludrocortisone; AMCL, ammonium chloride

(n = 4), renin-angiotensin inhibitors (n = 1), and non-steroidal anti-inflammatory drugs (n = 1); none of these patients used immunosuppressive drugs.

Correlation of dRTA with disease parameters

We also analyzed whether acid-base related parameters and kidney function differed between patients without dRTA, incomplete dRTA, and complete dRTA **(Figure 3)**. Patients with incomplete dRTA had significantly lower values of serum bicarbonate and venous pH and higher values of urinary pH than patients without dRTA **(Figure 3)**. The three patients with complete dRTA had the lowest venous pH, lowest serum bicarbonate, and lowest urinary pH, although the

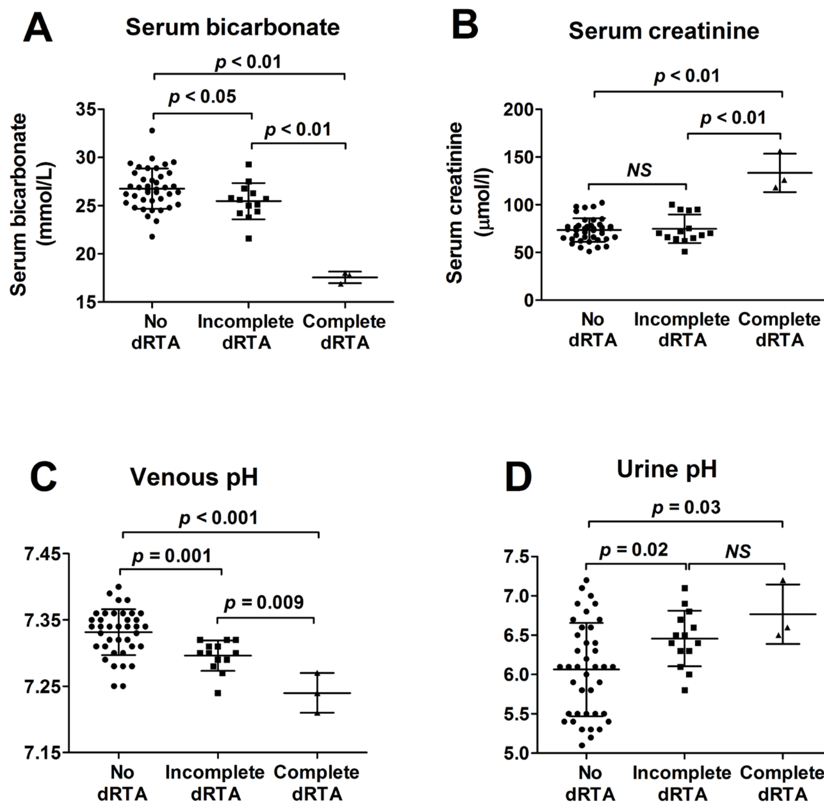

Figure 3 – Comparison of acid-base related parameters and kidney function
*The four panels show the distribution of serum bicarbonate (**A**), serum creatinine (**B**), venous pH (**C**), and urine pH (**D**) under baseline conditions in the three groups. The three groups are classified as 'no dRTA' (n = 40), 'incomplete dRTA' (n = 14), and 'complete dRTA' (n = 3). Abbreviations: dRTA, distal renal tubular acidosis; NS, not significant.*

latter was not different from the patients with incomplete dRTA. The patients with complete dRTA also had a higher serum creatinine than the two other groups. None of these patients used non-steroidal anti-inflammatory drugs. When we analyzed the auto-antibody prevalence in the three groups, SS-B/La auto-antibodies were more prevalent in patients with incomplete dRTA (79%) and complete dRTA (100%) than in patients without dRTA (45%) **(Figure 4)**. These prevalences showed a statistically significant difference only between the patients without dRTA and those with incomplete dRTA, probably due to the low number of patients with complete dRTA.

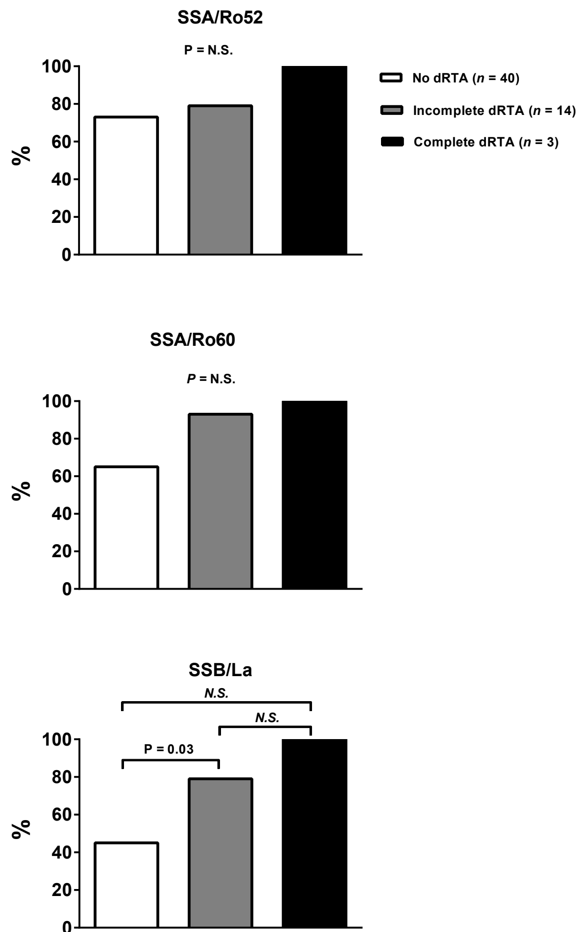

Figure 4 – Comparison of auto-antibodies
The prevalence of a positive auto-antibody test for SSA/Ro52, SSA/Ro60, or SSB/La is shown for the three groups (no dRTA, incomplete dRTA, complete dRTA). Abbreviations: dRTA, distal renal tubular acidosis; N.S., not significant.

Finally, we analysed whether the ESSDAI or disease duration were different in patients with dRTA (complete and incomplete combined) compared to patients without dRTA. The ESSDAI was 3.0 ± 1.9 in dRTA vs. 2.4 ± 1.9 in controls and the disease duration was 13.0 ± 5.0 years in dRTA vs. 11.4 ± 8.0 years in controls ($p > 0.05$ for both).

Discussion

Our objective was to analyze the prevalence of dRTA in pSS and to compare the diagnostic performance of FF with AMCL in assessing urinary acidification. The prevalence of complete dRTA was 5% and that of incomplete dRTA 25%. The prevalence of complete dRTA in this study was the same as in the cohort of 60 patients with pSS reported by Bossini *et al.* [13]. All three patients with complete dRTA also had reduced kidney function. This may be due to tubulointerstitial nephritis which is a common renal manifestation of pSS, although this was not confirmed with a kidney biopsy.

The prevalence of incomplete dRTA in our study was much higher than in the cohort reported by Bossini *et al.* (25% vs. 0%), although they performed the AMCL test in only 12 patients [13]. Although patients with incomplete dRTA by definition do not have metabolic acidosis, the patients in our cohort did have mild acidemia and a higher urinary pH at baseline. This is a novel finding with potential clinical implications, because even mild acidemia may contribute to bone demineralization [12], and higher urinary pH may predispose to kidney stone formation [11]. Indeed, Arampatzis *et al.* diagnosed incomplete dRTA in 1 out of 15 males with recurrent calcium stone formation [21]. Eriksson *et al.* reported ten patients who presented with dRTA and urolithiasis who went on to develop pSS in subsequent years [11].

The higher auto-antibody prevalence in patients with complete and incomplete dRTA may have pathophysiological significance. Although auto-antibodies causing dRTA have not been identified, several reports suggest that auto-antibodies against carbonic anhydrase [22] or acid-base transporters [23] are involved in the pathogenesis of dRTA in pSS. If the presence of auto-antibodies causing dRTA is confirmed, screening for these antibodies would facilitate early identification and treatment of dRTA.

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tion test with furosemide and fludrocortisone (FF) could replace AMCL [17]. We confirmed the better tolerability of FF, but, unfortunately, the overall diagnostic performance of FF was poor. One exception was the reasonable negative predictive value. Therefore, we believe FF could be considered as a first screening test, and AMCL could be reserved for those patients who fail to acidify their urine to pH < 5.3 with FF. One caveat with this approach is the possibility of a false negative test result. For example, if the FF-test would have been used as initial screening test for dRTA in this cohort, dRTA would have been missed in 6 out of 17 patients (35%). Why these 6 patients had a false negative test result remains unclear, but is probably due to the different mechanisms by which urinary acidification is tested (directly giving an acid load vs. indirectly stimulating H^+ secretion).

Because it remains unclear if incomplete dRTA always results in complications, it may be acceptable to miss these false negatives initially. Conversely, early identification of patients with a urinary acidification defect could provide a rationale for treatment with potassium citrate, which was recently shown to improve bone mineral density even in healthy older adults [12].

Why did the urinary acidification test with FF in the present study perform worse than in the initial report by Walsh *et al*.? One important difference is that half of the patients in the study by Walsh *et al*. had complete dRTA, which is more likely to result in a positive FF test. In addition, FF was only tested in patients with previously confirmed dRTA and not in patients in whom dRTA may be absent, incomplete, or complete. Indeed, in response to Walsh's report, Viljoen *et al*. reported the results of performing both urinary acidification tests in 10 patients with recurrent nephrolithiasis and/or nephrocalcinosis [24]. They also identified 3 patients who were able to acidify their urine to a pH < 5.3 with AMCL but not with FF. In agreement with our recommendation, Viljoen *et al*. proposed that the urinary acidification test with FF should be used as initial screening test to be followed up by AMCL if urine pH remains ≥ 5.3 [24]. It is unclear whether the FF test should be repeated after a certain period of time in case of a normal test result. There are no data to indicate what the disease-free period is after a negative test result. Therefore, at present, we recommend to leave it to the discretion of the treating physician to repeat the FF test after a few years.

The strength of our study is the large cohort of patients with pSS in whom urinary acidification was tested functionally, which allowed us to establish the true prevalence of incomplete dRTA. A limitation of our study was that we do not know whether the presence of incomplete dRTA leads to poorer outcomes during follow-up, because our study was cross-sectional. A prospective cohort study aiming to determine the clinical significance of incomplete dRTA is currently ongoing.

In conclusion, incomplete dRTA is common in pSS and causes mild acidemia which may potentially contribute to organ damage. FF cannot replace AMCL to test urinary acidification in pSS, but may be considered as screening test, given its reasonable negative predictive value and better tolerability.

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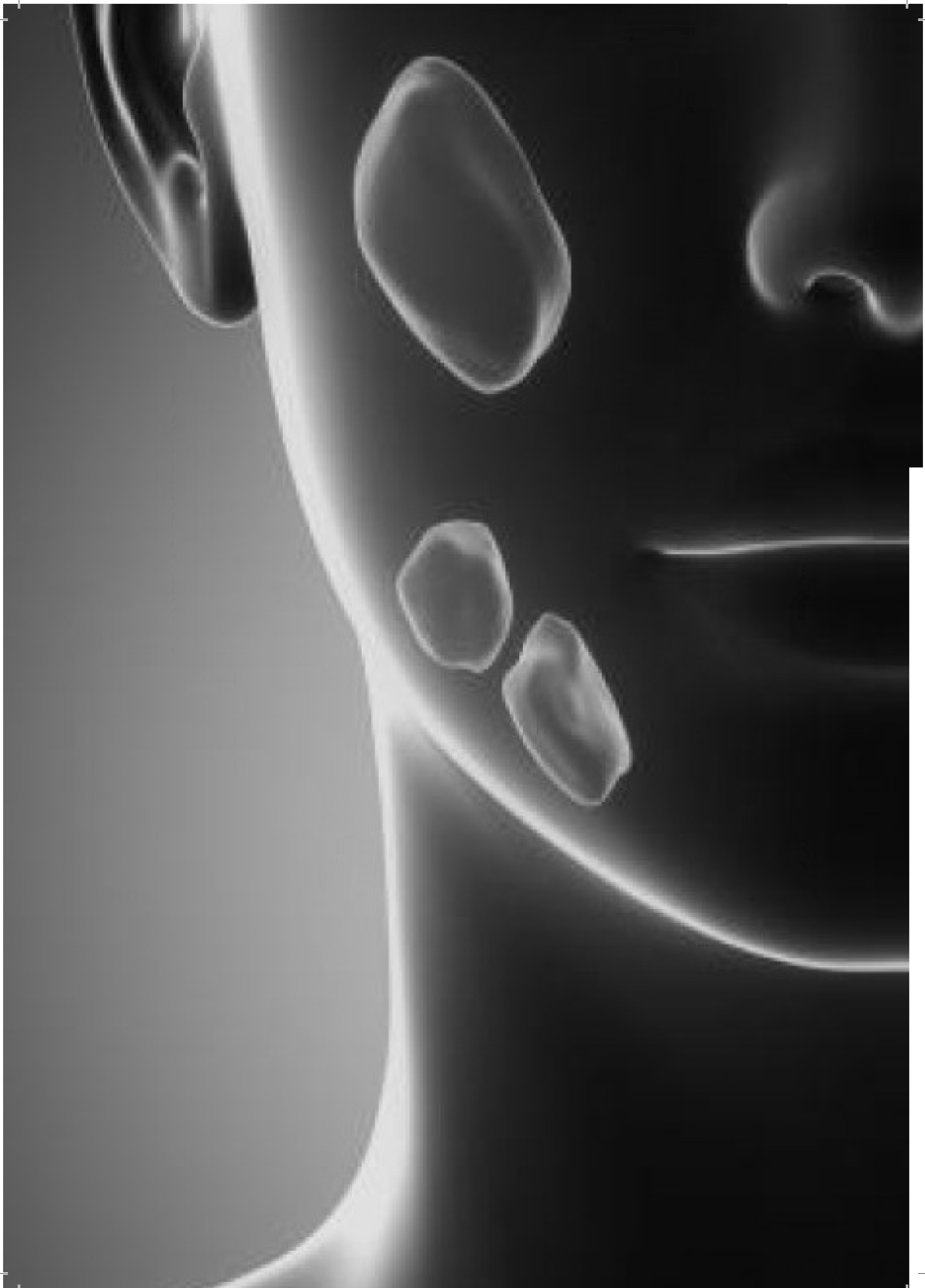

Bone mineral density in Sjögren syndrome patients with and without distal renal tubular acidosis

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Abstract

Introduction Primary Sjögren's syndrome (pSS) can be complicated by distal renal tubular acidosis (dRTA), which may contribute to low bone mineral density (BMD).

Aim Our objective was to evaluate BMD in pSS patients with and without dRTA as compared with healthy controls.

Methods BMD of lumbar spine (LS) and femoral neck (FN) was measured in 54 pSS patients and 162 healthy age- and sex-matched controls by dual-energy X-ray absorptiometry (DXA). dRTA was defined as inability to reach urinary pH < 5.3 after an ammonium chloride (NH_4Cl) test.

Results LS- and FN-BMD were significantly higher in pSS patients compared with controls (1.18 ± 0.21 g/cm^2 for patients vs. 1.10 ± 0.18 g/cm^2 for controls, P = 0.008 and 0.9 ± 0.16 g/cm^2 for patients vs. 0.85 ± 0.13 g/cm^2 for controls, P = 0.009 respectively). After adjustment for BMI and smoking, the LS- and FN-BMD remained significantly higher. Patients with dRTA (N=15) did not have a significantly different LS- and FN-BMD compared with those without dRTA (N=39) after adjustment for BMI, age and gender. Thirty-seven (69%) pSS patients were using hydroxychloroquine (HCQ).

Conclusions Unexpectedly, pSS patients had a significantly higher LS- and FN-BMD compared with healthy controls. Patients with dRTA had similar BMD compared with patients without dRTA. We postulate that an explanation for the higher BMD in pSS patients may be the frequent use of HCQ.

Introduction

Sjögren syndrome (SS) is a prevalent chronic autoimmune disease characterized by impairment of exocrine glands and systemic manifestations, affecting between 1% and 3% of the general population [1]. SS can be present alone (primary Sjögren syndrome (pSS)) or accompanied by other autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) and is then called secondary Sjögren syndrome (sSS) [2,3]. The classical symptoms of SS include dryness of mouth (xerostomia), dryness of eyes (xerophtalmia) and less commonly dryness of pharynx, larynx and/or vagina [4]. Extraglandular manifestations can be divided into general symptoms (e.g. fatigue, arthralgia and myalgia) and in systemic manifestations [5]. Common systemic manifestations include renal tubular acidosis [6], non-erosive symmetrical arthritis [7], interstitial lung disease [8], peripheral polyneuropathy [9], autoimmune thyroiditis [10] and B-cell lymphoma [11].

Distal renal tubular acidosis (dRTA) is one of the less well recognized complications. Recently, we reported a considerably high prevalence of dRTA in pSS [6]. dRTA is characterized by the inability of patients to lower urinary pH < 5.3 due to a defect in the proton secreting machinery of the alpha-intercalated cells in the collecting ducts of the kidney [12]. Patients with dRTA have a non-anion gap metabolic acidosis with urinary pH ≥ 5.3.

The association between chronic metabolic acidosis and alteration in bone cell function has been demonstrated both *in vitro* and *in vivo* [13,14]. During metabolic acidosis there appears to be an exchange of protons for calcium ions in bone mineral to buffer the excess of protons [13]. The metabolic effects of dRTA in pSS remain conflicting. Several case-series have shown a low bone mineral density (BMD) in patients with dRTA in pSS [15,16]. Some recent studies report an increased prevalence of low BMD in patients with dRTA [17,18], while other studies did not report a significant difference in patients with dRTA [19,20]. Epidemiologic data on BMD in SS are lacking. We hypothesized that BMD is significantly decreased in patients with pSS and especially in those with dRTA. Therefore, our aim was to evaluate BMD in pSS patients with and without dRTA as compared with healthy controls.

Methods

Study cohort

Patients were selected from the outpatient clinic of the department of internal medicine (division of clinical immunology) of Erasmus MC in Rotterdam, The Netherlands. pSS was defined according to the Revised American-European classification criteria [21]. The results of salivary gland biopsy were retrieved when available. Additional inclusion criteria for this study included age >18 years and an estimated glomerular filtration rate >30 ml/min. The exclusion criteria were: other underlying auto-immune diseases, known risk factors for osteoporosis (vitamin D level < 20 nmol/L, untreated hyperthyroidism, hyperparathyroidism, use of corticosteroids (prednisone equivalent of > 7.5 mg for > 3 months in the last year, use of bisphosphonates, multiple myeloma, mastocytosis). All participants were asked about menopausal status (if applicable), current smoking and history of fractures and use of medication **(Table 1)**. Data from BMD in the healthy control group were obtained from the ERF (Erasmus Rucphen Family) study database [22]. The matching criteria for the controls were age and sex. For every pSS patient, three controls were selected. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2013-075). Informed consent was obtained from every participant.

Bone mineral density

In all subjects we measured BMD of the lumbar spine (L2-L4) and femoral neck using a dual-energy X-ray absorptiometry (DXA) scanner (Prodigy Pro Full P8, enCORE™ Software Platform, GE Medical Systems Lunar, Belgium). Scans were performed according to the manufacturer's guidelines and analyzed according to ISCD rules [23]. The healthy control group was scanned with a different DXA device from the same type (GE Lunar Prodigy device, GE Healthcare, USA) [24]. As described by Enneman *et al*. a cross-calibration was performed using a spine phantom which showed that the measurements of the new scanner (the one we used for the patients in the current study) were slightly higher by a factor 1.0101 [24]. Therefore, we divided our results by this factor for comparison with the data from the ERF study. BMD was expressed in grams per square centimeters.

Biochemical parameters

In all patients we measured vitamin D status. Anti-nuclear antibodies (ANA), SSA/Ro52, SSA/Ro60, SSB/La auto-antibodies, and rheumatoid factor (RF) were also measured in all patients using

Table 1 – Characteristics of the study cohort

	Total (N = 54)	No dRTA (N = 39)	dRTA (N = 15)	Control group (N = 162)
Demographics				
Age, years ± SD	57.3 ± 10.6	60.1 ± 9.4	50.2 ± 10.5	57.3 ± 10.6
Female gender, n (%)	50 (93)	36 (92)	14 (93)	150 (93)
Body mass index, kg/m^2 ± SD	26.8 ± 6.2	26.9 ± 6.2	26.6 ± 6.6	27.3 ± 5.3
Current smokers, n (%)	2 (4)	0 (0)	2 (13)	60 (37)
Postmenopausal, n (%)	36/50 (72)	5/36 (14)	5/14 (36)	119 (69)
Age at menopause, years ± SD	47.4 ± 6.1	47.0 ± 6.5	48.4 ± 4.9	47.9 ± 6.1
Previous fractures, n (%)§	19 (35)	11 (28)	8 (53)	n.a
Disease duration, years ± SD	12.1 ± 7.2	12.2 ± 8.0	11.5 ± 5.6	-
Biochemical				
Serum 25-OH-Vitamin D, nmol/L ± SD	70.4 ± 21,8	69.7 ± 22.1	72.5 ± 21.7	
Serum intact PTH, pmol/L ± SD	4.3 ± 1.5	4.5 ± 1.5	3.7 ± 1.5	
Serum calcium, mmol/L ± SD	2.39 ± 0.08	2.39 ± 0.07	2.38 ± 0.16	
Serum phosphate, mmol/L ± SD	1.09 ± 0.15	1.10 ± 0.14	1.06 ± 0.16	
Serum creatinine, µmol/L ± SD	76.8 ± 18.6	72.9 ± 11.8	86.7 ± 27.9	
Serum PINP, µg/L ± SD	38.6 ± 18.5	41 ± 19.2	32.5 ± 15.4	
Serum BAP, µg/L ± SD	14.2 ± 4.1	14.7 ± 4.3	12.8 ± 3.3	
Serum NTX, nM BCE ± SD	17.2 ± 4.4	17.3 ± 4.6	16.8 ± 3.8	
Immunology				
Anti-nuclear antibodies, n (%)	41 (76)	28 (72)	13 (87)	
Rheumatoid factor, n (%)	32/42 (76)	21/29 (72)	11/13 (85)	
SSA/Ro52, n (%)	42 (78)	29 (74)	13 (87)	
SSA/Ro60, n (%)	40 (74)	26 (67)	14 (93)	
SSB/La, n (%)	31 (57)	18 (46)	13 (87)	
Positive salivary gland biopsy, n (%)¶	23/27 (85)	14/18 (78)	9/9 (100)	
Medications				
Hydroxychloroquine, n (%)	35 (69)	23 (59)	12 (80)	
Vitamin D supplements, n (%)	11 (20)	9 (23)	2 (13)	
Glucocorticoids, n (%)	3 (6)	2 (5)	1 (7)	
Other immunosuppressive drugs, n (%)†	4 (7)	2 (5)	2 (13)	

Salivary gland biopsies were performed in 34/54 patients, but a focus score could be retrieved for only 27 patients; Rheumatoid factor was measured in 42/54 patients. † Other immunosuppressive therapy consisted of azathioprine, colchicine or methotrexate. § Data about previous fractures could not accurately be retrieved. Data are presented as mean ± standard deviation (SD) and no. (%)

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previously reported methods [25]. Serum was collected before 10:00 AM and analyzed the same day. Patients were not instructed to be fasting. The following bone turnover markers (BTMs) were measured in patients: serum N-terminal propeptide of type I procollagen (PINP) and serum bone-specific alkaline phosphatase (BAP, both as measures of bone formation) and serum N-terminal crosslinking telopeptide of type I collagen (NTX, as measure for bone resorption). There were no data available on BTMs in the healthy control group.

Distal renal tubular acidosis

dRTA was defined as an abnormal NH_4CL test and the absence of any other known causes for dRTA (e.g., medication, hypercalciuria) [12]. The NH_4CL test is defined as abnormal if patients fail to achieve a urinary pH < 5.3 within four hours after intake of ammoniumchloride (1 ml/kg body weight) [26].

Statistics

All results are expressed as means with standard deviations. Comparisons of the normally distributed continuous variables between two groups were performed using the student T-test. Since BTMs were not normally distributed we compared BTMs between the two groups using the Mann-Whitney U test. Linear regression analysis was used to estimate the effect of having pSS on BMD before and after adjustment for body mass index (BMI) and smoking. Linear regression analysis was used to estimate the effect of having dRTA on BMD before and after adjustment for BMI, age and gender. A P-value < 0.05 was considered significant. All analyses were performed in SPSS (version 21, IBM).

Results

Study cohort and baseline characteristics

The study cohort included 54 patients with pSS and the control group consisted of 162 subjects. Initially, 62 patients participated in the study. Eight patients were excluded, including four patients who were unable to complete the NH_4Cl test due to repeated vomiting, one patient in whom the DXA-scan was not reliable due to scoliosis and three patients were using bisphosphonates. No patients were excluded because of long-term use of corticosteroids and only three

patients were using low dose corticosteroids for a medical condition other than pSS. The baseline characteristics of the study cohort are shown in **Table 1**. Similar to previous studies on pSS, our cohort has a female:male ratio of approximately 10:1 [27]. Thirty-seven (69%) patients with pSS were using HCQ. In addition to medication for pSS, other commonly used drugs in this cohort were vitamin D with calcium supplements (N=11). None of these patients reported a fracture in their medical history.

BMD of pSS patients compared with healthy controls

BMD of fifty-four pSS patients was compared with the age- and sex matched control group of 162 subjects. The LS- and FN-BMD were significantly higher in the pSS patients compared with the healthy control group (1.18 ± 0.21 g/cm^2 for pSS patients vs. 1.10 ± 0.18 g/cm^2 for the control group, P = 0.008 and 0.9 ± 0.16 g/cm^2 for pSS patients vs. 0.85 ± 0.13 g/cm^2 for the control group, P = 0.009 respectively) **(Figure)**. After adjustment for BMI and smoking, the LS- and FN-BMD remained significantly higher (β = 0.10 ± 0.030 g/cm^2, P < 0.001 and β = 0.077 ± 0.022 g/cm^2, P < 0.001) **(Table 2)**.

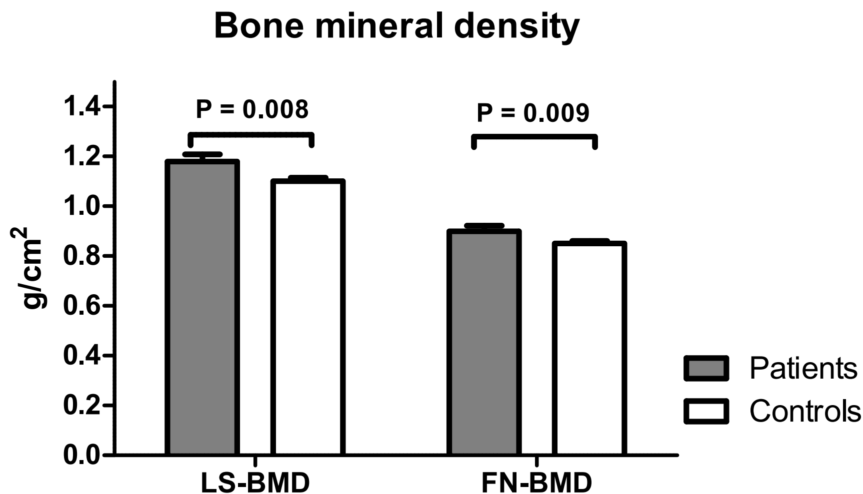

Figure – Comparison of BMD between patients and controls

The graph shows the bone mineral density of the lumbar spine and the femoral neck of patients with primary Sjögren syndrome (N =54) compared with healthy age- and sex matched controls (N = 162).

Abbreviations: SD, standard deviation; BMD, bone mineral density; LS-BMD, bone mineral density of the lumbar spine; FN-BMD, bone mineral density of the femoral neck.

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Table 2 – Multiple regression analysis of factors related to LS- and FN-BMD between patients (N = 54) vs. controls (N = 162)

Variable	LS-BMD			FN-BMD		
	B	Std. Error	Beta	B	Std. Error	Beta
(Constant)	0.797	0.063		0.587	0.047	
pSS	0.103	0.030	0.237**	0.077	0.022	0.235**
BMI	0.010	0.002	0.298**	0.009	0.002	0.350**
Smoking	0.061	0.029	0.147*	0.045	0.021	0.143*

*Abbreviations: Std. error, standard error of the mean; pSS, primary Sjögren syndrome; BMI, body mass index; LS-BMD, bone mineral density of the lumbar spine; FN-BMD, bone mineral density of the femoral neck. * P 0.01 < 0.05, ** P < 0.01*

The effect of distal renal tubular acidosis on BMD

Fifteen pSS patients had an urinary acidification defect as measured by the NH_4CL test. Between both groups the levels of biochemical parameters and the use of medication were similar **(Table 1)**. Patients with dRTA were significantly younger compared with those without dRTA **(Table 1)**. Both the LS- and FN-BMD were significantly higher in patients with an urinary acidification defect compared with those without an urinary acidification defect (LS: 1.29 ± 0.16 g/cm^2 vs. 1.14 ± 0.21 g/cm^2, P = 0.018 and FN: 1.0 ± 0.19 g/cm^2 vs. 0.87 ± 0.14 g/cm^2, P = 0.007). After adjustment for BMI, age and gender, both the LS- and FN-BMD were not significantly higher

Table 3 – Multiple regression analysis of factors related to LS- and FN-BMD between patients with dRTA (N = 15) vs. patients without dRTA (N = 39)

Variable	LS-BMD			FN-BMD		
	B	Std. error	Beta	B	Std. Error	Beta
(Constant)	1.292	0.217		1.175	0.166	
dRTA	0.121	0.064	0.264	0.070	0.049	0.184
BMI	0.007	0.004	0.220	0.003	0.003	0.113
Gender	-0.177	0.100	-0.226	-0.008	0.076	-0.012
Age	-0.003	0.003	-0.157	-0.006	0.002	-0.405*

Abbreviations: Std. error, standard error of the mean; dRTA, distal renal tubular acidosis; BMI, body mass index; LS-BMD, bone mineral density of the lumbar spine; FN-BMD, bone mineral density of the femoral neck.
** P < 0.01*

anymore (LS: $\beta = 0.12 \pm 0{,}064$ g/cm^2, $P = 0.065$ and FN: $\beta = 0.07 \pm 0{,}049$ g/cm^2, $P = 0.16$) **(Table 3)**.

Bone turnover markers in pSS patients

In patients with dRTA serum PINP was not significantly higher compared with patients without dRTA (P = 0.093). The other marker for bone formation, BAP, was also not significantly different between both groups (P = 0.11). The bone resorption marker NTX, was not significantly different between patients with and without dRTA (P = 0.92).

Discussion

In the present study we found that, contrary to expected, pSS patients have significantly higher BMD than healthy age- and sex-matched controls. We searched the available literature but did not find another study reporting BMD measurements in pSS patients as compared with a healthy control group. Studies concerning BMD in autoimmune diseases are mainly performed in lupus patients. In agreement with Arampatzis *et al*. and Pongchaiyakul *et al*. we found that patients with an urinary acidification defect did not have a significantly different LS- and FN-BMD compared with those patients without an urinary acidification defect [19,20].

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Bushinsky *et al*. reported a decreased bone mineralization in an acidotic environment in both in-vitro and in-vivo studies [13,14]. This makes us wonder what the reason is that we did not find a lower BMD in patients with dRTA.

We hypothesize that the observed BMD in pSS patients may be related to the use of hydroxychloroquine (HCQ) which the majority (69%) of patients in our study was using. In case of systemic manifestations, therapy with non-steroidal anti-inflammatory drugs or HCQ is advised. HCQ has proven to be effective against fatigue, arthralgia and myalgia [28,29]. Lakshminarayanan *et al*. and Mok *et al*. reported that in lupus the use of HCQ was associated with increased BMD of the hip [30,31]. In both studies, disease activity and use of corticosteroids were not significantly different between both groups. Additionally, Xiu *et al*. recently reported a reduced osteoclastogenesis by TRAF3 degradation due to the effects of chloroquine in mice, which may suggest that HCQ has direct effects on bone metabolism [32]. Based on these clinical and biochemical studies we hypothesize that HCQ may have beneficial effects on BMD.

In our cohort, it is unknown how long these patients were treated with HCQ. We also did not have information about past use of HCQ in patients, who are not using it currently. Therefore, analyzing a possible association between HCQ use and BMD would not be reliable in our cohort. To demonstrate whether the use of HCQ has beneficial effects on human bone cells, *in vitro* studies should be performed.

We analyzed whether patients with dRTA also had different BTM measurements compared with patients without an urinary acidification defect. Since patients with dRTA had similar LS- and FN-BMD compared with those without dRTA, we expected that the BTMs measurements would not be significantly different between both groups. Indeed, all three BTMs (PINP, NTX and BAP) were not significantly different between patients with and without an urinary acidification defect. Unfortunately, we could not compare BTM measurements between pSS patients and the healthy control group since data about BTM measurements in the healthy controls is lacking.

The strength of this study is that we have reported new data about the BMD values in a large cohort of pSS patients. In addition, we analyzed the effects of dRTA, a common complication of pSS, on BMD in pSS patients. A limitation of this study is that we used a different DXA scanner compared to Zillikens *et al*. although the type of machine was the same and calibration was performed with a spine phantom, making this an unlikely explanation for our findings [22].

In conclusion, we found that both the LS- and FN-BMD were higher in patients with pSS than in age and sex-matched healthy controls. In addition, LS- and FN-BMD in patients with an urinary acidification defect is comparable with patients without an urinary acidification defect. An explanation for the high BMD in pSS patients may be the frequent use of HCQ, but future studies will have to confirm whether indeed use of HCQ is associated with higher BMD.

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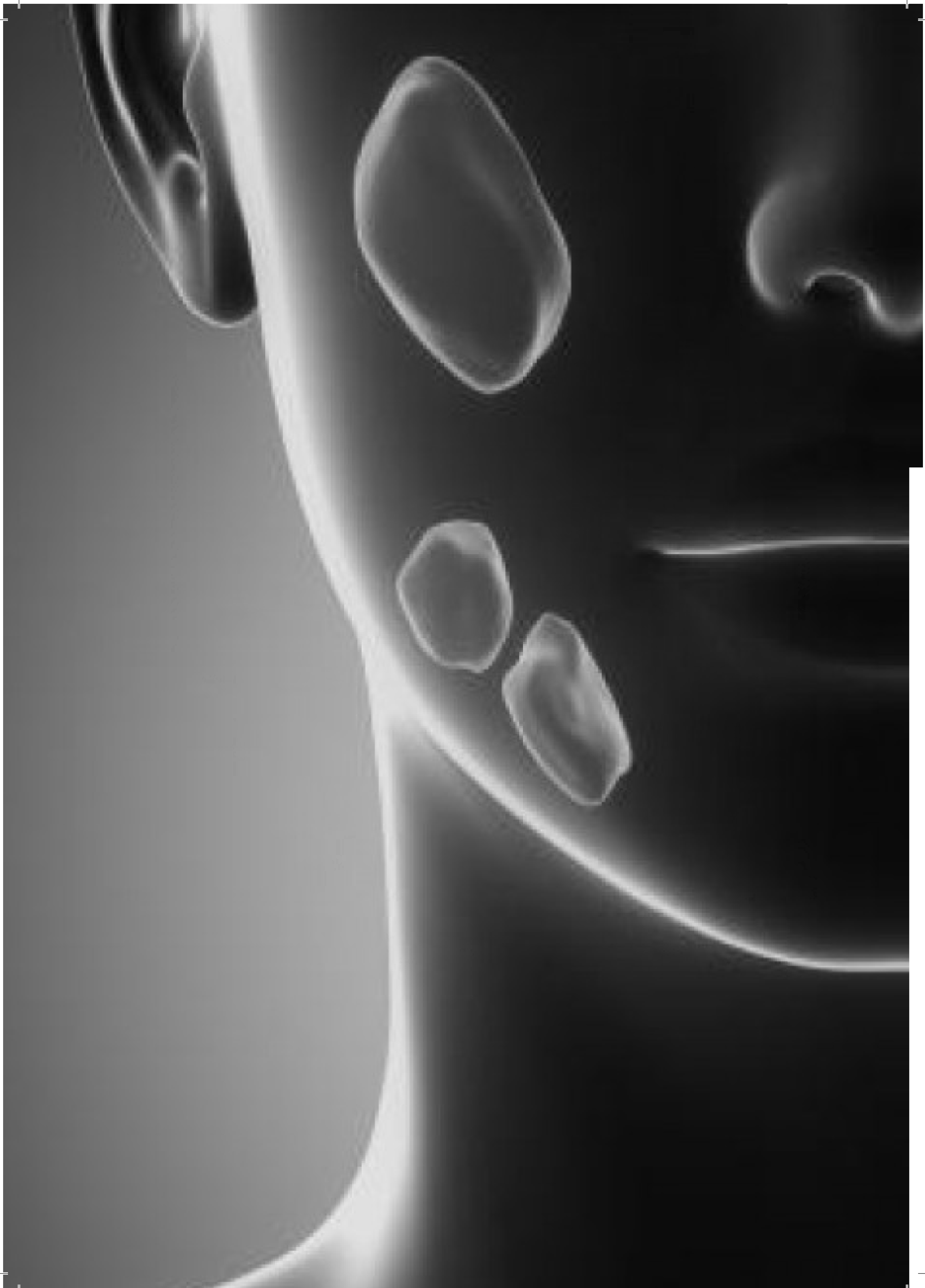

Hydroxychloroquine decreases human MSC-derived osteoblast differentiation and mineralization *in vitro*

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Abstract

Introduction We recently showed that patients with primary Sjögren Syndrome (pSS) have significantly higher bone mineral density (BMD) compared to healthy controls. The majority of those patients (69%) was using hydroxychloroquine (HCQ), which may have favorable effects on BMD.

Aims To study the direct effects of HCQ on human MSC-derived osteoblast activity

Methods Osteoblasts were cultured from human mesenchymal stromal cells (hMSCs). Cultures were treated with different HCQ doses (control, 1 and 5 µg/ml). Alkaline phosphatase activity and calcium measurements were performed to evaluate osteoblast differentiation and activity, respectively. Detailed microarray analysis was performed in 5 µg/ml HCQ-treated cells and controls followed by qPCR validation. Additional cultures were performed using the cholesterol synthesis inhibitor simvastatin (SIM) to evaluate a potential mechanism of action.

Results HCQ inhibits both MSC-derived osteoblast differentiation and mineralization *in vitro*. Microarray analysis and additional PCR validation revealed a highly significant upregulation of the cholesterol biosynthesis, lysosomal and extracellular matrix pathways in the 5 µg/ml HCQ-treated cells compared to controls. Besides, we demonstrated that 1 µM SIM also decreases MSC-derived osteoblast differentiation and mineralization compared to controls.

Conclusion HCQ suppresses MSC-derived osteoblast differentiation and mineralization *in vitro*. It appears that the positive effect of HCQ on BMD cannot be explained by a stimulating effect on the MSC-derived osteoblast. The discrepancy between high BMD and decreased MSC-derived osteoblast function due to HCQ treatment might be caused by systemic factors that stimulate bone formation and/or local factors that reduce bone resorption which is lacking in cell cultures.

Introduction

Hydroxychloroquine (HCQ) is an antimalarial agent now often used in systemic autoimmune diseases such as primary Sjögrens Syndrome (pSS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) due to its anti-inflammatory properties [1–3]. The pharmacokinetics of HCQ has been described extensively but the exact mechanism of action remains unclear [4].

In addition to its anti-inflammatory effects, the literature concerning the pharmacodynamics of HCQ is extensive. In vivo studies showed that HCQ has beneficial effects on the lipid profile of patients with RA and pSS by lowering serum levels of low density lipoprotein (LDL) cholesterol, triglycerides and total cholesterol as well as increasing high density lipoprotein (HDL) cholesterol [5, 6]. Additionally, HCQ has been associated with beneficial cardiovascular and anticancer effects but it is not used for these conditions since there are better alternatives available [7, 8]. In vitro studies have shown that HCQ is capable of inhibiting Toll-like receptors (TLR) [7] and [9], which are involved in the pathogenesis of SLE [9–11]. Although Raicevic *et al*. reported that osteoblasts do not express TLR 7 and 9, other studies did show TLR 9 expression in osteoblasts [12–14]. HCQ has also been identified as an autophagy inhibitor by blocking the degradation of autophagosomes and promoting apoptosis in endometriosis, cervical cancer cells and myeloid leukemia [15–17]. In addition to the effects on autophagosomes, HCQ also acts on lysosomes. Some studies reported an increased lysosomal pH by HCQ treatment, which is associated with decreased lysosomal function [18, 19], while other studies did not observe a significant difference in lysosomal pH [10, 11]. Furthermore, HCQ has been associated with increased lysosomal membrane permeabilization (LMP), a process occurring prior to mitochondrial membrane permeabilization (MMP) leading to apoptosis [20].

We recently reported that patients with pSS, of which the majority was using HCQ, had a higher bone mineral density (BMD) compared to healthy controls [21]. Additionally, we found two studies showing a positive association between BMD and HCQ use in SLE patients, which was corrected for patient characteristics and disease activity [22, 23], while one study reported a negative effect of HCQ on BMD [24]. We recently showed that HCQ leads to decreased osteoclast differentiation and activity due to HCQ treatment [25]. Based on our previous studies, we hypothesized

that HCQ stimulates the activity of the bone forming cells, the osteoblasts, which has not been studied before.

Methods

Cell cultures

Human mesenchymal stromal cells (hMSCs; Lonza, Basel, Switzerland) were differentiated into osteoblasts as described before [26]. Briefly, hMSCs were differentiated into mineralizing osteoblasts within 2 to 3 weeks, using dexamethasone and β-glycerophosphate. The media were refreshed twice a week and cells were treated without (control) and with HCQ (1 or 5 µg/ml). Alkaline phosphatase (ALP) activity was measured at day 7 of culture. Osteoblast mineralization was analyzed by measuring the amount of precipitated calcium corrected for total protein at day 18 as extensively described before [26]. Images were taken during culture to evaluate cell morphology. For microarray analysis, osteoblast cultures with and without 5 µg/ml HCQ were stopped at day 5.

Mineralization staining assays

Calcium depositions were visualized with the Alizarin red staining assay as described before [26]. Briefly, cells were fixed with 70% (vol/vol) ethanol and, after washing, stained for 10–20 min with alizarin Red S solution. Phosphate depositions were visualized with the von Kossa staining assay as described before [26]. Cells were washed with water and the wells were stained for 30 minutes with 5% silver nitrate (in bright daylight), incubated for one minute in 5% sodium carbonate in 25% formalin and finally for two minutes in 5% sodium thiosulphate.

Activation of simvastatin

hMSCs were differentiated to osteoblasts as described before. In addition, cells were treated with a dose range from 100 nM to 100 µM simvastatin (SIM; Sigma Aldrich, The Netherlands) with and without 5 µg/ml HCQ to evaluate whether the effects of HCQ on both osteoblast differentiation and mineralization could be antagonized by SIM. Simvastatin was activated before use as previously described [27]. Briefly, 5 mg simvastatin was dissolved in 125 µl of 100% ethanol, with subsequent addition of 187.5 µl of 0.1 N NaOH. The solution was heated to 50°C for 2

hours in a water bath and then activated by neutralizing to pH 7.0 using 0.1 N HCl. The resulting solution was brought to a final concentration of 4 mg/ml using distilled water and aliquots were stored at 4°C until use.

Immunocytochemistry assays

hMSCs were cultured for 5 days and stained for cytoskeletal actin. Briefly, cells were washed with phosphate buffer solution (PBS) and fixed with 10% formalin. PBS + Triton X100 was added for ten minutes, followed by blocking aspecific binding sites, using PBS + Tween 0.05% + BSA 1% for 30 minutes. Cells were then incubated with a rhodamine-conjugated phalloidin antibody for 1 hour at room temperature and washed with PBS + Tween 0.05% followed by DAPI staining. Staining of the cytoskeleton was visualized under a fluorescent microscope using a 535 nm filter. Additionally, a DAPI filter (365 nm) was used to visualize the nuclei and evaluate any apoptotic events (e.g. nuclear fragmentation, chromatin condensation).

For visualization and quantification of focal adhesions, cells were labeled for 1 h with rabbit monoclonal anti-vinculin antibody at 1:200 dilution at RT, followed by secondary Alexa Fluor 488 goat anti-rabbit IgG at 1:400 dilution for a total of 1 h [28].

Illumina gene chip–based gene expression

Total RNA of hMSCs was isolated as described before [26]. Illumina Human HT-12 v4 BeadChip (Illumina, Inc, San Diego, USA) human whole-genome expression arrays were used. RNA integrity of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA of 3 biologic replicates for each condition (control, 1 and 5 µg/ml HCQ) was analyzed. The Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) was used for RNA amplification of each sample according to manufacturer's instructions. In short, T7 oligo(dT) primer was used to generate single-stranded cDNA, followed by a second strand synthesis to generate double-stranded cDNA. In vitro transcription was done to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was column purified and checked for quality by RNA 6000 Nano assay. A total of 750 ng of cRNA was hybridized for each array using the standard Illumina protocol, with streptavidin-Cy3 (GE Healthcare, Piscataway, NJ, USA) being used for detection. Slides were scanned on an iScan and analyzed using GenomeStudio (both from Illumina, Inc.).

Microarray analysis

Background was subtracted from the raw data using GenomeStudioV2010.1 (Gene Expression Module 1.6.0, Illumina), and data were processed using the Bioconductor R3.3 lumipackage (www.bioconductor.org) [29]. The data were transformed by variance stabilization and quantile normalization. Probes that were detected at least three times in the experiments (Illumina detection p-value < 0.01) were considered to be expressed and were further analyzed. Differentially expressed probes were identified using Bioconductor Package Limma (www.bioconductor.org), with adjusted p-values adjusted to reduce the false discovery rate (FDR; $p < 0.01$) [30]. Gene ontology (GO) analysis, selected Illumina IDs were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 hosted by the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (Bethesda, MD, USA) and at GeneMANIA (http://www.genemania.org/). Merging of overlapping GO annotations was performed by using the Reduce and Visualize Gene Ontology (REVIGO) tool (www.revigo.irb.hr) .

Quantitative real-time PCR analyses

The methods used for RNA extraction and cDNA synthesis and real-time (RT) PCR have been described previously [26]. Real-time qPCR was performed by using the ABI Prism 7900 sequence detection system (Applied Biosystems), and the results were analyzed using SDS version 2.3 software (Applied Biosystems). Data are presented as relative mRNA levels calculated and corrected for gene expression of the housekeeping gene *GAPDH* by the formula: $2^{-\Delta(\text{Ct of gene of interest - Ct of housekeeping gene})}$. All primers used are summarized in **Table 1**.

Statistics

All results are expressed as means with standard error of the mean (SEM). Comparisons of the continuous variables between three groups (control, 1 and 5 µg/ml HCQ) and two groups (control and 5 µg/ml HCQ) were performed using the one-way analysis of variance (ANOVA) and students T-test, respectively. For ANOVA analysis, the least significant difference post-hoc test was used. A P-value < 0.05 was considered significant. All analyses were performed in SPSS (version 21, IBM).

Table 1 – Primer sequences of the analyzed genes

Gene	Forward primer	Reverse primer
GAPDH	CCGCATCTTCTTTTGCGTCG	CCCAATACGACCAAATCCGTTG
TNC	CACAGCCACGACAGAGGC	AAAGGCATTCTCCGATGCCA
ALP	TAAAGCAGGTCTTGGGGTGC	GGGTCTTTCTCTTTCTCTGGCA
ACAT2	GAGCTTTGCCTAGCTTGCAG	TGAAGGAACCTATGATGGTCCG
DHCR7	GAGGTGTGCGCAGGACTTTA	CTTCTTGAACCGGCCCCTTA
CTSK	TGCCCACACTTTGCTGCCGA	GCAGCAGAACCTTGAGCCCCC
CTNS	AACGCGGTGCATTCCTGA	GCGTCTCCAAAGCAATCTGA
GPNMB	TAAACCTTGAGTGCCTGCGT	TGAAATCGTTTGGCGGCATC
HMGCR	TCTAGTGAGATCTGGAGGATCCAA	GGATGGGAGGCCACAAAGAG

Abbreviations: GO, gene ontology; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TNC, Tenascin C; ALP, Alkaline Phosphatase; ACAT2, Acetyl-CoA Acetyltransferase 2; DHCR7, 7-Dehydrocholesterol Reductase; CTSK, Cathepsin K; CTNS, Cystinosin, Lysosomal Cystine Transporter; GPNMB, Glycoprotein Nmb; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

Results

HCQ inhibits osteoblast differentiation and activity

Osteoblast differentiation, as measured by ALP activity at day 7, was significantly decreased dose-dependently between HCQ doses of 1 and 5 µg/ml vs. controls (1.54 ± 0.11 mU/µg for HCQ dose 1 µg/ml and 0.8 ± 0.044 mU/µg for HCQ dose 5 µg/ml vs. 2.7 ± 0.15 mU/µg for the controls, $P < 0.001$ for both and $P < 0.001$ for the dose-dependent trend) **(Figure 1A)**. Mineralization at day 18 was significantly decreased between 5 µg/ml HCQ and controls (0.40 ± 0.015 nmol/µg vs. 9.75 ± 1.76 nmol/µg, $P = 0.011$ and $P < 0.001$ for the dose dependent trend). In fact, using the highest HCQ dose, mineralization was virtually absent at 18 days of culture **(Figure 1B)**. Additionally, using alizarin red and von Kossa stainings, mineralization in the HCQ-treated cells was absent compared to the controls **(Figure 1C)**. During culture, evaluation of the cells showed an altered morphology in the 5 µg/ml HCQ-treated cells compared to the controls at day 14 **(Figure 1D)**. We performed vinculin stainings at day 5 of culture to analyze for differences in cell-surface attachment between HCQ-treated cells and controls. HCQ-treated cells showed significantly less staining compared to the controls indicating less cell-surface attachment due to HCQ **(Figure 1E)**. Furthermore, there is no evidence for a difference in apoptotic events between the conditions **(data not shown)** or cytoskeletal malformations (actin) between controls and HCQ-treated cells based on rhodamine-phalloidin staining **(Figure 1E)**.

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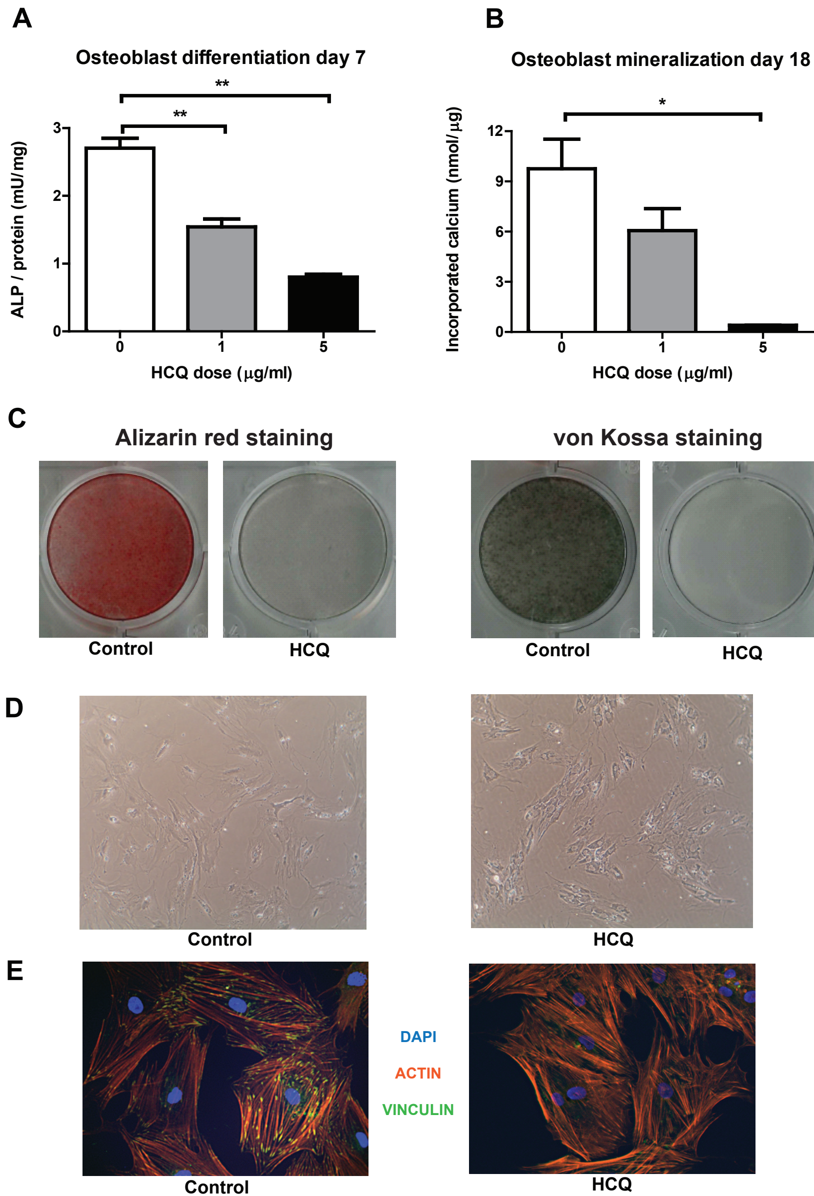

Figure 1 – Effect of HCQ on MSC-derived osteoblast differentiation and mineralization

All experiments are performed twice with N = 4 for every condition. ***A*** *ALP measurement at day 7.* ***B*** *Mineralization at day 18* ***C*** *Alizarin red staining and von Kossa staining in controls vs. HCQ-treated cells* ***D*** *Morphology of MSC- derived osteoblasts at day 14 of culture in controls vs. 5 µg/ml HCQ-treated cells.* ***E*** *DAPI/Actin/Vinculin staining in controls vs. 5 µg/ml HCQ-treated cells. Data are presented as mean ± SEM. * = P < 0.05, ** = P < 0.01. Abbreviations: HCQ, hydroxychloroquine; ALP, alkaline phosphatase*

Microarray analysis of HCQ-treated hMSCs yields 4 regulated processes in MSC-derived osteoblasts

In order to gain insight into processes regulated by HCQ during osteoblast differentiation, we performed microarray gene expression analysis using Illumina Human HT-12 v4 expression arrays. hMSCs were cultured and treated without or with HCQ (1 or 5 µg/ml) for 5 days as described above. Next, whole-genome analysis of mRNAs was assessed following induction of osteogenic differentiation. When evaluating 2-fold up- and downregulated genes in HCQ-treated cells vs. controls, a clear dose response between 1 µg/ml and 5 µg/ml HCQ treatment was observed **(Figure 2A-B)**. In addition, none of the genes was stronger regulated by 1 µg/ml HCQ compared to 5 µg/ml HCQ. Therefore, we excluded the 1 µg/ml HCQ-treated cells from further analysis. A total of 119 gene probes corresponding to 72 genes were differentially expressed ($q < 0.05$ and 2-fold change) at day 5 compared to controls. GO analysis of these gene probes resulted in a significant overrepresentation of 14 functional categories. Evaluation of the regulated genes within the categories showed a large overlap between the GO terms and using REVIGO, we narrowed them down based on the largest number of genes to four main processes, namely 1) lipid metabolic process (GO:000629), 2) developmental process (GO:0032502), 3) lysosome (GO:0005764) and 4) extracellular matrix (GO:0031012) **(Table 2)**.

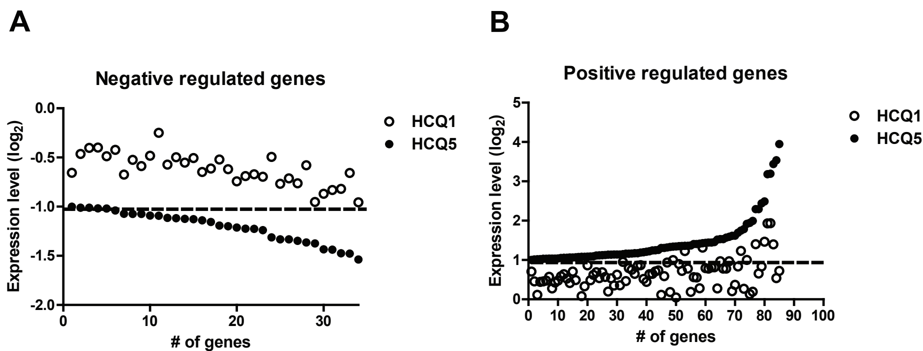

Figure 2 – Dose response curve of gene expression profiles between 1 and 5 µg/ml HCQ compared to controls

All experiments are performed with N = 4 for every condition. The dotted line indicates the threshold of 2-fold up- or downregulation. ***A*** *Dose response for all genes that are negative regulated by HCQ.* ***B*** *Dose response for all genes that are positive regulated by HCQ. Abbreviations: HCQ, hydroxychloroquine*

Table 2 – GO term enrichment analysis of 5 µg/ml HCQ treatment vs. control at day 5 of MSC-derived osteogenesis

GO	Name	Fold enrichment	Number of genes	P-value
Biological process				
GO:0006629	Lipid metabolic process	4.1	20	0.0002
GO:0032502	Developmental process	2.0	37	0.014
Cellular component				
GO:0005764	Lysosome	9.9	11	0.0002
GO:0031012	Extracellular matrix	5.5	10	0.012

Abbreviations: GO, gene ontology

PCR validation of HCQ-regulated genes underlying selected GO terms from microarray analysis

From every GO term we selected two genes of interest for PCR validation **(Table 2)**. For the GO term 'lipid metabolism' process we selected acetyl-CoA acetyltransferase 2 (*ACAT2*) and 7-dehydrocholesterol reductase (*DHCR7*), which encode the first and last enzyme involved in the cholesterol biosynthesis pathway [32]. For the GO term 'extracellular matrix' we selected tenascin C (*TNC*) and alkaline phosphatase (*ALP*) since these genes were highly regulated by HCQ and are known to be involved in osteoblast differentiation. Genes belonging to the GO term 'lysosome' include cathepsin K (*CTSK*) (a cysteine proteinase) and cystinosin, lysosomal cystine transporter (*CTNS*) (a small lysosomal membrane protein). All selected genes were also regulated in the GO term 'developmental process' and therefore we only selected glycoprotein Nmb (*GPNMB*) from this GO term, since this was the strongest regulated gene upon HCQ treatment in our experiment. We validated these seven genes using real-time PCR. Although expression of two genes (*ALP* and *TNC*) did not reach significance between controls and HCQ treatment, all genes showed the same direction of regulation compared to our results from the microarray analysis **(Figure 3A-G)**.

Simvastatin decreases osteoblast differentiation and mineralization alone and in combination with 5 µg/ml HCQ

Since HCQ upregulates the cholesterol synthesis pathway, we hypothesized that SIM (a cholesterol synthesis inhibitor) would antagonize the inhibitory effects of HCQ on osteoblast dif-

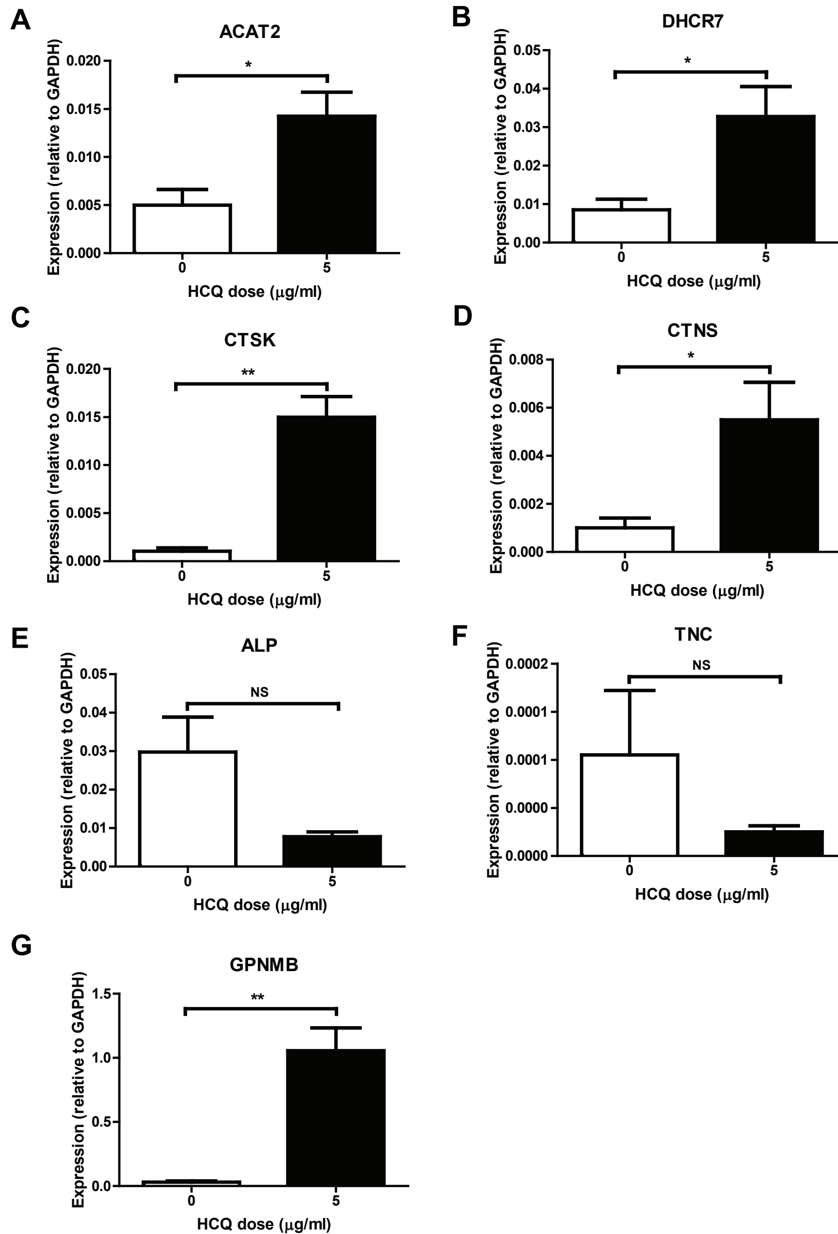

Figure 3 – Validation of multiple genes regulated using real-time qPCR

*All experiments are performed with N = 4 for every condition. Total RNA was isolated from by 5µg/ml HCQ-treated hMSCs at day 5 followed by qPCR for **A** ACAT2, **B** DHCR7, **C** CTSK, **D** CTNS, **E** ALP, **F** TNC and **G** GPNMB. Gene expression was corrected for the housekeeping gene GAPDH. Data are presented as mean ± SEM. * = P < 0.05, ** = P < 0.01. Abbreviations: HCQ, hydroxychloroquine*

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ferentiation and mineralization. Therefore, we treated MSCs with SIM in multiple doses in the presence or absence of 5 μg/ml HCQ to evaluate the effects of SIM alone and in combination with HCQ on MSC-derived osteoblasts. We found that SIM doses of 100 nM and 10 nM were ineffective, while SIM doses above 1 μM increased cell death in the early phase of the culture probably due to its cellular toxicity **(data not shown)**.

We showed that 1 μM SIM significantly decreased osteoblast differentiation, as measured by ALP activity, compared to untreated controls (0.67 ± 0.038 mU/μg for 1 μM SIM vs. 1.9 ± 0.33 mU/μg for the controls, $P < 0.001$) **(Figure 4A)**. The effect of 1 μM SIM was similar to the effect of HCQ only as well as to the combination of these two drugs. Additionally, both 0.2 and 1 μM SIM significantly decreased osteoblast mineralization compared to the controls (1.49 ± 0.072 nmol/μg for 0.2 μM SIM and 1.63 ± 0.018 nmol/μg for 1 μM SIM vs. 2.67 ± 0.32 nmol/μg for the controls, $P < 0.001$ for both) **(Figure 4B)**. However, the observed decreased mineralization by both doses of SIM was less severe compared to the HCQ treatment. The combination of HCQ with either SIM doses significantly decreased the mineralization compared to either SIM dose alone and is similar to the cells treated with HCQ only (1.63 ± 0.18 nmol/μg for 1 μM SIM vs. 0.78 ± 0.59 nmol/μg for HCQ and 0.77 ± 0.047 nmol/μg for 1 μM SIM + HCQ, $P < 0.05$ for both). We also analyzed gene expression for *HMGCR* (the enzyme inhibited by SIM) in HCQ and/or SIM-treated cells compared to control. Although gene expression in SIM-treated cells was higher, the effect was not significant. HCQ significantly increased *HMGCR* gene expression compared to controls ($P < 0.05$) **(Figure 4C)**. In addition, the combination with SIM and HCQ resulted in a significantly increased expression compared to either drug alone and to controls ($P < 0.05$ and $P < 0.001$, respectively). Furthermore, we analyzed gene expression of *ALP* and *DHCR7* in HCQ- and/or SIM-treated cells compared to control. Expression of *ALP* was significantly increased by 0.2 μM SIM compared to control ($P < 0.001$) **(Figure 4D)**. The combination of SIM and HCQ was similar to HCQ alone, but significantly lower compared to control ($P < 0.001$). Expression of *DHCR7* was significantly increased by both HCQ and 1 μM SIM compared to control ($P < 0.01$ and $P < 0.05$, respectively) **(Figure 4E)**. The combination of SIM and HCQ showed a synergistic effect leading to an increased *DHCR7* expression compared to control ($P < 0.001$) **(Figure 4E)**.

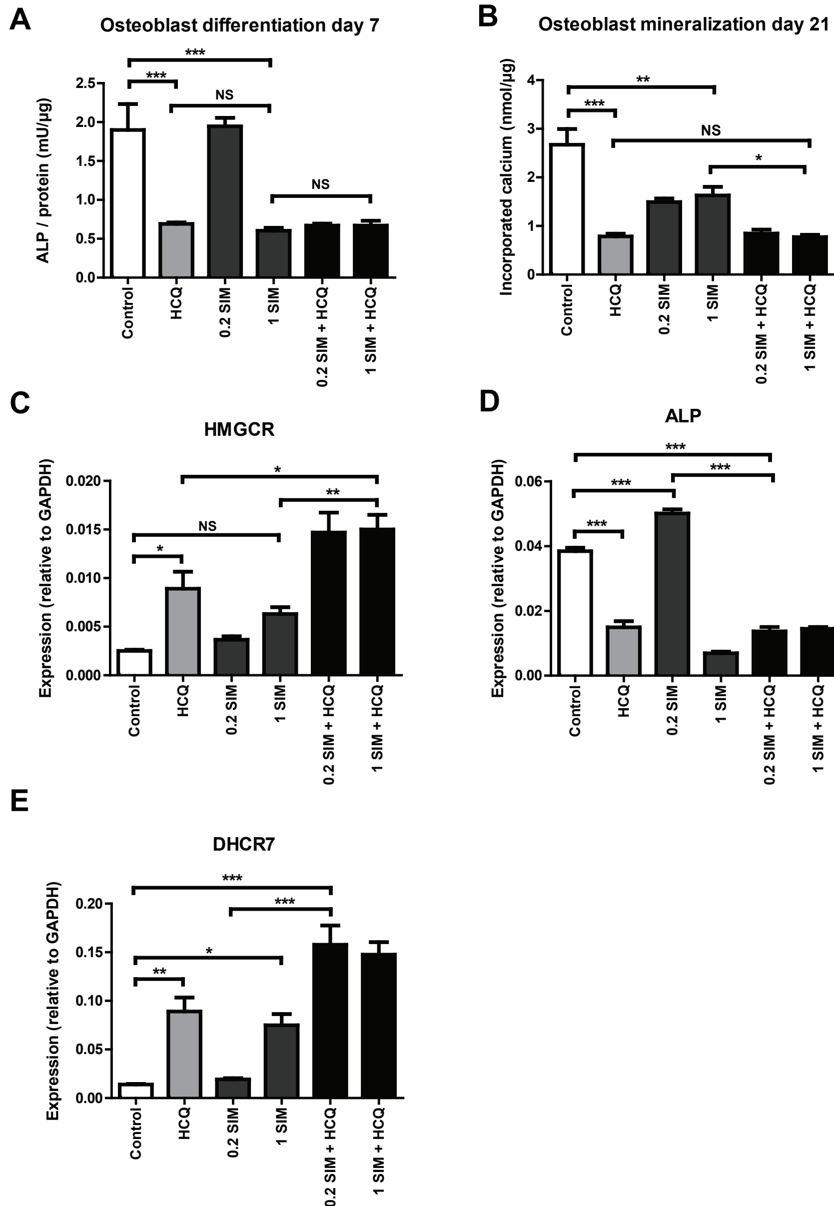

Figure 4 – Effect of 5 µg/ml HCQ and SIM on MSC-derived osteoblast differentiation and mineralization
All experiments are performed twice with N = 4 for every condition. SIM doses are 0.2 µM and 1 µM. ***A*** *MSC-derived osteoblast differentiation, as measured by ALP, at day 7 in HCQ- and/or SIM-treated cells compared to control.* ***B*** *MSC-derived osteoblast mineralization, as measured by calcium incorporation, at day 21 in HCQ and/or SIM-treated cells compared to control. qPCR analysis of* ***C*** *HMGCR,* ***D*** *ALP and* ***E*** *DHCR7 in HCQ- and/or SIM-treated cells compared to control. Data are presented as mean ± SEM. * = P < 0.05, ** = P < 0.01, *** = P < 0.001.*
Abbreviations: HCQ, hydroxychloroquine; SIM, simvastatin; ALP, alkaline phosphatase

Discussion

In the present study we demonstrated that HCQ suppresses both MSC-derived osteoblast differentiation and mineralization *in vitro*. Although some of the pharmacodynamics of HCQ may apply to specific biological processes in MSC-derived osteoblasts, we did not come across studies reporting the direct effects of HCQ on MSC-derived osteoblast differentiation or activity. Furthermore, we demonstrated results of the microarray analysis comparing 5 μg/ml HCQ-treated hMSCs to controls. Upregulation of genes belonging to the cholesterol biosynthesis pathway, lysosomal pathway and extracellular matrix were the most significantly influenced processes by 5 μg/ml HCQ treatment. Since SIM is a cholesterol synthesis inhibitor and beneficial for osteoblast differentiation and mineralization, we evaluated whether SIM could antagonize the negative effects of HCQ and enhance MSC-derived osteoblast function simultaneously. Contrary to expected, SIM significantly decreased both MSC-derived osteoblast differentiation and mineralization and the combination of SIM and HCQ yielded similar outcomes compared to HCQ treatment alone.

Since patients with pSS, of which the majority is using HCQ, have a higher BMD compared to healthy controls, we hypothesized that HCQ is beneficial for either MSC-derived osteoblast differentiation or mineralization [21]. However, our *in vitro* work showed that both MSC-derived osteoblast differentiation (as measured by ALP activity) and mineralization (as measured by calcium incorporation and shown by mineralization stainings) are significantly decreased by 5 μg/ml HCQ treatment compared to controls.

We performed microarray analysis on both control and 5 μg/ml HCQ-treated cells to assess potential mechanisms causing decreased MSC-derived osteoblast differentiation and mineralization. We showed that the upregulation of genes involved in the cholesterol metabolism pathway was the most significantly regulated process by 5 μg/ml HCQ compared to control samples. From this pathway, 10 out of 24 enzymes were significantly upregulated. Indeed, we confirmed the upregulation of this pathway by validating two of the involved genes (*ACAT2* and *DHCR7*) using RT-PCR. Based on this finding, we speculate that either 1) HCQ has a direct positive regulatory effect on cholesterol synthesis or 2) HCQ causes an intracellular cholesterol depletion leading indirectly to increased cholesterol synthesis or increased cholesterol uptake. The latter

is in agreement with the observed depletion of LDL cholesterol *in vivo* in patients that receive HCQ [5, 6].

The role of cholesterol in MSC-derived osteoblast differentiation has mainly been studied by the use of statins (e.g. SIM). SIM inhibits 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and thereby blocks the synthesis of mevalonate and its downstream products leading to decreased levels of cholesterol [32]. SIM activates Ras signaling by inhibiting the synthesis of cholesterol leading to overexpression of *BMP-2* through the PI3K/Akt/MAPK pathway. BMP-2 upregulates the expression of *RUNX2*, and phosphorylated RUNX2 stimulates a series of bone-specific gene transcriptions and promotes the differentiation of osteoblasts [33-35]. Indeed, both *in vitro* and *in vivo* studies have reported the beneficial effects of statins on osteoblast differentiation and mineralization [36, 37]. Additionally, *in vivo* studies showed that statins are a potential treatment for osteoporosis [38, 39]. Based on these studies, we expected to find improved MSC-derived osteoblast differentiation and/or mineralization and we speculated that SIM may antagonize the negative effects of HCQ. However, we found that both MSC-derived osteoblast differentiation and mineralization were significantly decreased in SIM-treated cells compared to controls. Furthermore, MSC-derived osteoblast mineralization was significantly decreased by the combination of SIM and HCQ compared to cells treated with SIM only. A potential explanation might be that HCQ leads, due to an unknown mechanism, to an intracellular cholesterol depletion resulting in upregulation of cholesterol synthesizing enzymes as described earlier. Treatment with SIM would then block this compensatory mechanism of the cell which may lead to decreased MSC-derived osteoblast development and activity. Indeed, gene expression of *HMGCR* is significantly increased in HCQ and SIM-treated cells and it seems that both drugs have synergistic effects supporting our hypothesis. It remains unclear, however, why SIM did not have beneficial effects on MSC-derived osteoblasts in our experiments. Another possible explanation might be the use of hMSCs, since many studies showing beneficial effects of SIM used different type of cell-lines [40]. A third explanation might be that 1µM SIM has still toxic effects leading to impaired MSC-derived osteoblast activity without leading to apoptosis. Despite using a dose response experiment for SIM and following the methods as described in other papers, we could not confirm previously reported beneficial effects of SIM.

We showed a highly significant upregulation of the endosomal/lysosomal system by HCQ com

pared to the controls in our microarray analysis. Surprisingly, the most upregulated gene was *CTSK*, a lysosomal protease, which is predominantly known to be involved in bone resorption by osteoclasts [41, 42]. The role of CTSK in osteoblasts is less well understood and the majority of these studies are performed in mice. Mandelin *et al*. reported that osteoblast-like cells indeed produce *CTSK* mRNA and release processed cathepsin K into culture media *in vitro* [43]. A study performed in a *CTSK* knockout mouse showed a significantly increased number of osteoblasts in the fracture callus with associated increased callus mineral density and strength compared to wild-type mice [44]. Since we demonstrated a significantly decreased MSC-derived osteoblast differentiation and mineralization and a significant upregulation of *CTSK* expression in HCQ-treated MSC-derived osteoblasts, a direct relation between *CTSK* upregulation and the observed phenotype is too premature at this stage.
According to literature, HCQ has been associated with increased LMP leading to apoptosis [20]. LMP is caused by loss of cholesterol in the lysosomal membrane leading to the release of cathepsins and protons from the lysosomal lumen into the cytosol where they participate in apoptosis signaling [45]. This may lead to the observed upregulation of *CTSK* gene expression in order to compensate for the loss. Additionally, cholesterol is identified as a stabilizer of the lysosomal membrane and may therefore counter LMP.

Finally, the decreased mineralization may be caused by HCQ-induced alteration in the extracellular matrix (ECM) gene expression profile as this was one of the regulated GO terms following HCQ treatment. Eijken *et al*. reported that activin signaling in human osteoblasts changes the expression of a specific range of ECM proteins prior to the onset of mineralization, leading to a matrix composition with reduced or no mineralizing capacity [28]. In agreement with this, we found similar ECM gene expression alterations due to HCQ treatment compared to controls in our microarray experiment (downregulation of *ALPL* and *CLEC3B*; upregulation of *POSTN*, *MMP7* and *MMP15*). In addition, we showed that staining for yet another ECM protein, vinculin, was significantly decreased in HCQ-treated cells compared to controls. Therefore, we speculate that HCQ leads to reduced cell-surface attachment and altered ECM composition leading to decreased matrix mineralization.
Based on these findings, our final hypothesis is that HCQ ‘attacks’ the lysosomal membrane by

removing cholesterol leading to decreased osteoblast differentiation and mineralization. As a compensatory mechanism, both the cholesterol synthesis pathway and the lysosomal pathway are upregulated in an attempt to restore osteoblast function. In addition, HCQ may also affect ECM composition leading to decreased cell attachment, differentiation and matrix mineralization. The discrepancy between high BMD and decreased MSC-derived osteoblast function due to HCQ treatment might be caused by systemic factors that stimulate bone formation and/or systemic or local factors that reduces bone resorption which is lacking in cell cultures. In fact, we have shown that HCQ strongly suppresses bone resorption *in vitro* and *in vivo* and in women with an high bone turnover state, this may lead to a net increase in bone mass [25].

The strength of this study is that we performed an unbiased evaluation of potential mechanisms of action for the observed decreased MSC-derived osteoblast differentiation and mineralization using microarrays. Additionally, genetic data from the microarray was translated into functional experiments, but the precise mechanism remains elusive.

In conclusion, we demonstrated that HCQ suppresses MSC-derived osteoblast differentiation and mineralization *in vitro*. Furthermore, we reported results of our microarray analysis showing significant upregulation of the cholesterol biosynthesis and lysosomal pathway. Surprisingly, treatment with SIM and HCQ also resulted in decreased MSC-derived osteoblast differentiation and mineralization. A potential mechanism could be HCQ-induced LMP leading to decreased MSC-derived osteoblast development and activity.

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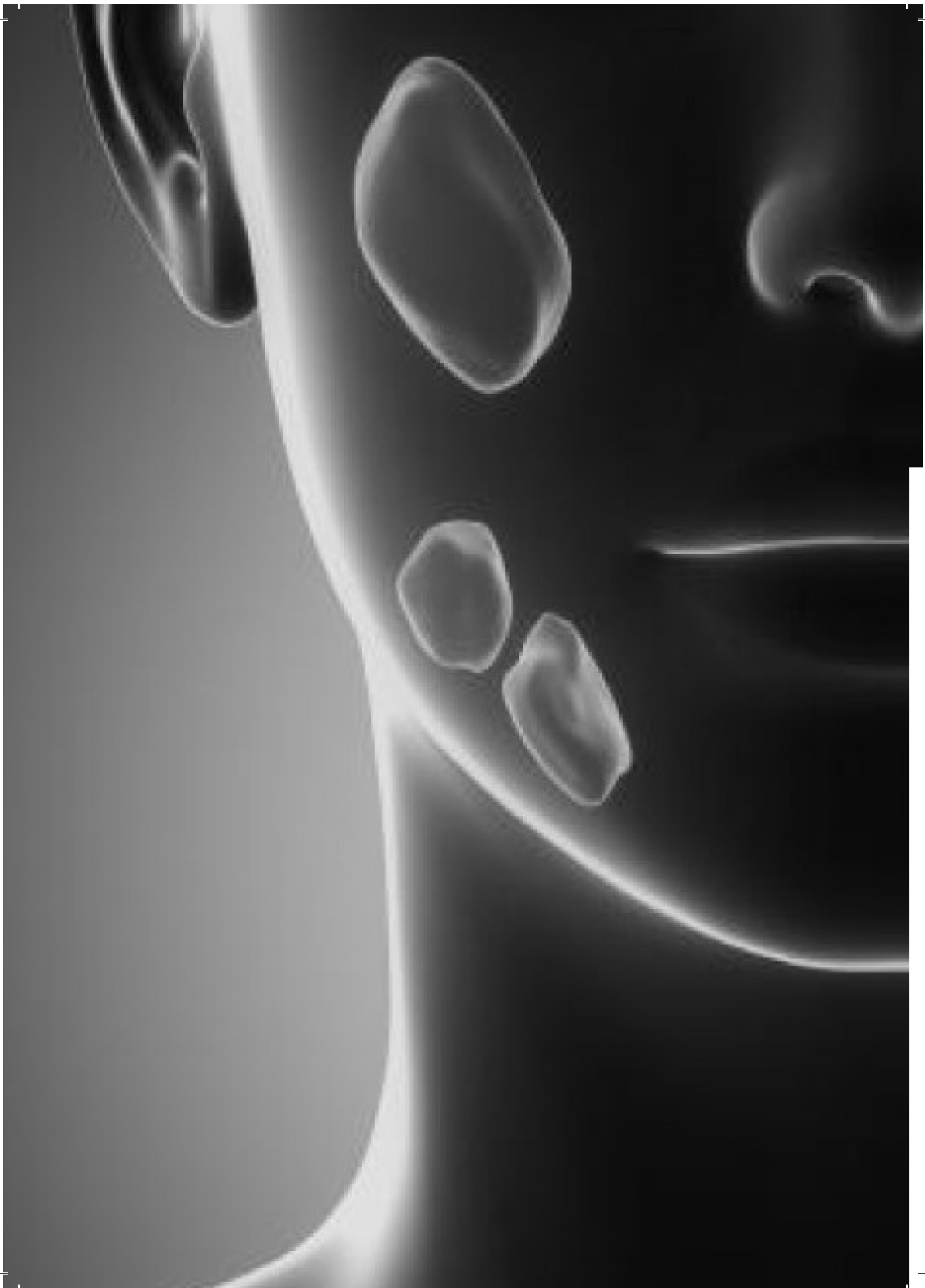

Hydroxychloroquine affects bone resorption both *in vitro* and *in vivo*

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Abstract

We recently showed that patients with primary Sjögren syndrome (pSS) have significantly higher bone mineral density (BMD) compared to healthy controls. The majority of those patients (69%) was using hydroxychloroquine (HCQ), which may have favorable effects on BMD. The aim of the study was to evaluate whether HCQ modulates osteoclast function. Osteoclasts were cultured from PBMC-sorted monocytes for 14 days and treated with different HCQ doses (control, 1 and 5 μg/ml). TRAP staining and resorption assays were performed to evaluate osteoclast differentiation and activity, respectively. Staining with an acidification marker (acridine orange) was performed to evaluate intracellular pH at multiple timepoints. Additionally, a fluorescent cholesterol uptake assay was performed to evaluate cholesterol trafficking. Serum bone resorption marker β-CTx was evaluated in rheumatoid arthritis patients. HCQ inhibits the formation of multinuclear osteoclasts and leads to decreased bone resorption. Continuous HCQ treatment significantly decreases intracellular pH and significantly enhanced cholesterol uptake in mature osteoclasts along with increased expression of the lowdensity lipoprotein receptor. Serum β-CTx was significantly decreased after six months of HCQ treatment. In agreement with our clinical data, we demonstrate that HCQ suppresses bone resorption *in vitro* and decreases the resorption marker β-CTx *in vivo*. We also showed that HCQ decreases the intracellular pH in mature osteoclasts and stimulates cholesterol uptake, suggesting that HCQ induces osteoclastic lysosomal membrane permeabilization (LMP) leading to decreased resorption without changes in apoptosis. We hypothesize that skeletal health of patients with increased risk of osteoporosis and fractures may benefit from HCQ by preventing BMD loss.

Introduction

Hydroxychloroquine (HCQ) has primarily been registered for prevention and treatment of malaria [1]. In recent decades, HCQ was found to be effective in inflammatory diseases as well. Nowadays, HCQ is used to treat many autoimmune diseases such as systemic lupus erythematosus (SLE), primary Sjögren syndrome (pSS) and rheumatoid arthritis (RA) [2–4]. However, the exact molecular target(s) of HCQ remains unclear. Over the recent decades, the effect of HCQ on inflammatory processes has been studied. Important actions of HCQ on the inflammatory response are: 1) inhibition of the local inflammatory response (e.g. inhibition of lysosomal enzyme release by polymorphonuclear leucocytes); 2) reduced chronic inflammatory response (e.g. reduction in lymphocyte proliferation, reduced MHC-antigen presentation and pro-inflammatory cytokine production) and 3) cellular effects (e.g. reduced function of intracellular organelles) [5–9]. In addition to the anti-inflammatory effects, HCQ is also associated with anti-thrombotic and anti-atherosclerotic actions and anti-diabetic effects leading to cardioprotective outcomes [10,11]. Furthermore, HCQ has beneficial effects on the lipid profile by decreasing low density lipoprotein (LDL) and triglycerides (TG) as well as increasing high-density lipoprotein (HDL) in patients with pSS and RA [12,13].

We recently reported that patients with pSS have a significantly higher bone mineral density (BMD) in the lumbar spine (LS) and femoral neck (FN) compared to age/sex matched healthy controls [14]. Of this predominantly female cohort, 72% was postmenopausal, a period known to manifest with elevated bone turnover due to increased osteoclast (bone resorbing cells) activity [15]. Most of these patients were using HCQ (69%), which could be a potential explanation for our findings since a positive association between HCQ and BMD has been reported before in SLE [16,17]. However, we could not accurately analyze the effect of HCQ on BMD between HCQ-users (N = 23) and non HCQ-users (N = 16) due to lack of information concerning dose and duration of HCQ treatment. The underlying mechanism of this is unknown. *In vitro* studies on the effect of HCQ on bone remodeling are very limited. Lee *et al*. showed that HCQ did not affect human osteoclastogenesis [18]. Furthermore, Xiu *et al*. showed reduced osteoclastogenesis by preventing TRAF3 degradation following chloroquine (drug from the same family as HCQ) treatment in mice [19]. A study from 1978 showed by using acridine orange staining that HCQ is

capable of increasing the lysosomal pH in macrophages [20]. In addition, it was shown that HCQ is able to increase lysosomal pH in mice osteoclasts that is associated with decreased signaling and nuclear translocation of the key osteoclast marker NFATc1, leading to an impairment of osteoclastogenesis [21]. Recent reports suggest, however, that HCQ does not affect lysosomal pH [22,23]. To our knowledge, studies concerning the direct effect of HCQ on bone resorption *in vitro* have not been performed yet.

We hypothesized that HCQ affects osteoclastogenesis and/or bone resorption leading to decreased bone turnover resulting in net less bone loss. This would be a potential explanation for our BMD findings in pSS patients. The aim of the current study is therefore to evaluate the effects of HCQ on human osteoclasts *in vitro* and bone resorption *in vivo* as well as to investigate underlying mechanisms.

Methods

Cell cultures

Human peripheral blood mononuclear cell (PBMCs)-sorted monocytes, using a CD14 antibody-conjugated magnetic bead system (Miltenyi Biotec, Germany) were cultured towards osteoclasts as described before [24]. The media were refreshed twice a week as described previously and cells were treated without and with 5 µg/ml HCQ. After 14 days of culture, cells were fixed in 10% formalin and stained for tartrate-resistant acid phosphatase (TRAP). Cells were counted and categorized according to the number of nuclei (1, 2, 3-5 and ≥6 nuclei).

Bone resorption assay

Osteoclasts were cultured on an osteoassay surface plate (Corning, USA) for 14 days and treated as described above. In this way, the capability of mineral resorption by acid secretion can be assessed. Briefly, cells were washed with water and the wells were stained for 30 minutes with 5% silver nitrate (in bright daylight), incubated for one minute in 5% sodium carbonate in 25% formalin and finally for two minutes in 5% sodium thiosulphate. Pictures were obtained and the area of the resorption pits was quantified using ImageJ (version 1.47).

Acidification assay

Cell cultures were performed as described above. In addition, some plates received only a single HCQ treatment three days prior to the assay to assess whether the effect of HCQ is achieved after a single dose or repetitive treatment is required. At different time points, 5 µg/ml acridine orange (Sigma Aldrich, The Netherlands) was added for 15 minutes as described before [25]. Cells were then washed with phosphate buffer solution and fixed with 10% formalin following DAPI staining. Plates were kept in the dark and analyzed, using a Zeiss Axiovert 200 MOT fluorescent microscope (Zeiss, the Netherlands). Depending on the intracellular pH, the acridine orange staining changes color which can be analyzed under a fluorescent microscope using 485 nm (neutral pH) and 535 nm (acidic pH) filters. Using the DAPI filter (365 nm), any staining in the nuclei for the other two wavelengths was excluded, leaving the cytoplasm for analysis. Images were made for each experiment using both wavelengths. Quantification and comparison of the intensities (expressed as 485:535 ratio) of each plate individually was performed using ImageJ (version 1.47).

Phalloidin staining protocol

Cell cultures were performed as described above. Briefly, cells were washed with phosphate buffer solution (PBS) and fixed with 10% formalin. PBS + Triton-X100 was added for 10 minutes, followed by PBS + 0.05% Tween and 1% BSA for 30 minutes. Cells were then incubated with rhodamine-conjugated phalloidin antibodies for 1 hour and washed with PBS + 0.05% Tween followed by DAPI staining. Staining of the cytoskeleton was visualized under a Zeiss Axiovert 200 MOT fluorescent microscope using the 535 nm filter (Zeiss). A 365 nm filter was used to evaluate any apoptotic events (e.g. nuclear fragmentation, chromatin condensation).

Cholesterol uptake assay

Cell cultures were performed as described above. Twenty-four hours before cholesterol uptake evaluation, medium was replaced by serum free medium with the addition of fluorescent cholesterol (1:50) from the Cholesterol Uptake Cell-Based Assay Kit (Cayman Chemical, Michigan, USA). Thirty-two hours after incubation, medium was removed and cells were washed with phosphate buffer solution. Fluorescent intensities (excitation 485 nm and emission 535 nm)

were measured using a plate reader and expressed as relative units (RU). Images were made by using a fluorescent microscope at the best optical settings for each experiment with excitation and emission at 485 nm and 535 nm, respectively.

Quantitative real-time PCR analyses

The methods used for RNA extraction and cDNA synthesis and RT-PCR have been described previously [26]. Real-time qPCR was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems), and the results were analyzed using SDS version 2.3 software (Applied Biosystems). Data are presented as relative mRNA levels calculated by the formula: $2^{-\Delta(\text{Ct of gene of interest - Ct of housekeeping gene})}$. All primer sequences used are summarized in **Table 1**.

Table 1 – Primer sequences of the analyzed genes

Gene	Forward primer	Reverse primer
GAPDH	CCGCATCTTCTTTTGCGTCG	CCCAATACGACCAAATCCGTTG
TM7SF4	AAGCAGCCGCTGGGAGAAGT	TTTTCAGGACTGGAAGCCAGAAATGAA
CTSK	TGCCCACACTTTGCTGCCGA	GCAGCAGAACCTTGAGCCCCC
LDLR	CTACCCCTCGAGACAGATGGTC	GCGAGGTCTCAGGAAGGGTT
BAX	CTGAGCAGATCATGAAGACAGG	CTGCTCGATCCTGGATGAAA
BCL2	AGTACCTGAACCGGCACCT	ACAGTTCCACAAAGGCATCC
CASP3	TGGAATTGATGCGTGATGTT	TGGCTCAGAAGCACACAAAC

Abbreviations: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TM7SF4, Transmembrane 7 Superfamily Member 4; CTSK, Cathepsin K; LDLR, Low Density Lipoprotein Receptor; BAX, BCL2 Associated X Protein; BCL2, B-Cell CLL/Lymphoma 2; CASP3, caspase 3

Patient cohort

Serum from patients was obtained from the tREACH study, which included only RA patients with intermediate disease activity [27]. From those subjects, baseline serum and serum after six months of treatment with HCQ was used to measure beta C-terminal telopeptide (β-CTx) as marker for bone resorption. As control group we used serum from patients from the same database (same time points) who were treated with methotrexate (MTX) since joint inflammation can lead to local bone destruction associated with increased serum β-CTx levels. A reduction in disease activity with any kind of treatment will then lead to a decrease of serum β-CTx. We re-

trieved serum levels of C-reactive protein (CRP) from the patient medical record. Patients were not instructed to be fasting.

Additionally, we recruited patients from the outpatient clinic of the departments of Internal Medicine (division of clinical immunology) and Rheumatology of Erasmus Medical Center, Sint Franciscus Gasthuis and Maasstad Hospital in Rotterdam, The Netherlands. From those patients, we measured serum β-CTx at baseline and after three months of HCQ treatment to evaluate a potential early change in serum β-CTx. In addition, CRP levels were also measured. All subjects had to meet the following inclusion criteria: age > 18 years with either inflammatory arthritis, pSS, sarcoidosis, RA, SLE or osteoarthritis and starting with HCQ. The exclusion criteria were: use of immunosuppressive drugs except for corticosteroids equivalent of < 7.5 mg in the last year, severe renal insufficiency (glomerular filtration rate < 30ml/min), known risk factors for osteoporosis (vitamin D level < 20 nmol/L, untreated hyperthyroidism, hyperparathyroidism, use of bisphosphonates, multiple myeloma, mastocytosis). All participants were asked about menopausal status (if applicable) and use of medication. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2015-046). Informed consent was obtained from every participant.

Statistics

All results are expressed as means with standard error of the mean (SEM). Comparisons of the continuous variables were performed using two-way analysis of variance (two-way ANOVA) with the least significant difference post-hoc test. Comparisons between baseline and after treatment (three and six months) and comparisons between both treatment regimens were performed using the paired-T-test and the students-T-test, respectively. Linear regression analysis was used to estimate the effect of treatment on serum β-CTx levels before and after adjustment for CRP and age. A P-value < 0.05 was considered significant. All analyses were performed in SPSS (version 21, IBM).

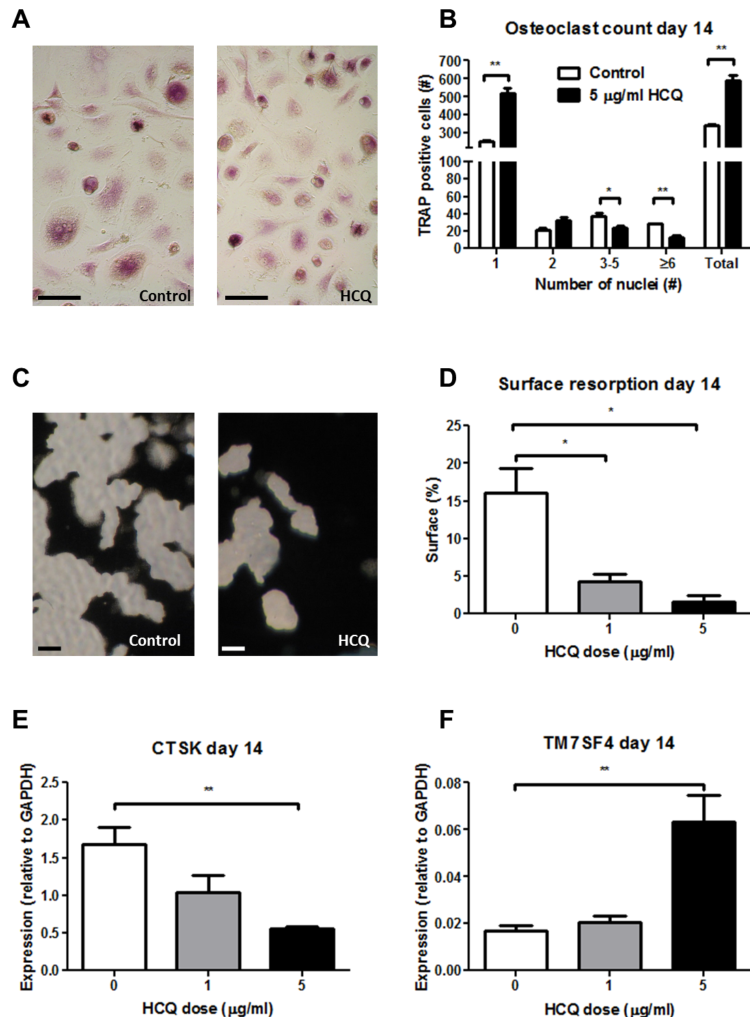

Figure 1 – Effect of HCQ on osteoclastogenesis and osteoclast activity

*All experiments are performed twice with N = 4 for every condition (control vs. HCQ). A TRAP stained osteoclasts. Scale bar = 50 mm. B TRAP staining reveals no difference in mono- and multinuclear osteoclast numbers between HCQ treatment and controls. C VonKossa staining of controls vs. 5 µg/ml HCQ treated osteoclasts D Surface resorption is significantly decreased by HCQ treatment (both doses) compared to control with a significant dose dependent effect. E Expression of CTSK is significantly decreased in 5 µg/ml HCQ compared to control with a significant dose dependent effect. F Expression of the fusion marker TM7SF4 is increased in 5 µg/ml HCQ compared to control. * = P < 0.05, ** = P < 0.01*

Abbreviations: HCQ, hydroxychloroquine; TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K; TM7SF4, Transmembrane 7 Superfamily Member 4

Results

Effect of HCQ on human osteoclasts in vitro

HCQ inhibits osteoclast activity, but not differentiation

The osteoclasts were stained for TRAP at day 14 and sorted by number of nuclei (1, 2, 3-5 and ≥6 nuclei) **(Figure 1A)**. Quantification of the TRAP staining showed a significant increased number of mononuclear osteoclasts in the HCQ group compared to the controls ($P < 0.01$). Additionally, multinuclear osteoclasts were significantly less observed in the HCQ group compared to the controls ($P < 0.05$) **(Figure 1B)**. Surface resorption (as measured by VonKossa staining) showed a significant dose dependent decreasing trend with increasing HCQ dose ($P = 0.005$) **(Figure 1C)**. The difference in amount of surface resorption was significant between HCQ dose 1 and 5 μg/ml vs. controls (4.3 ± 1.0% for HCQ dose 1 μg/ml and 1.6 ± 0.8% for HCQ dose 5 μg/ml vs. 16.2 ± 3.2% for the controls, $P = 0.037$ and $P = 0.011$, respectively), but not between 1 and 5 μg/ml **(Figure 1D)**. The expression of cathepsin K (*CTSK*) mRNA at day 14 displayed a significant dose dependent decreasing trend with increasing HCQ dose ($P = 0.003$), however, a significant effect was only observed between the 5 μg/ml HCQ treated cells compared to the controls ($P = 0.006$) **(Figure 1E)**. Gene expression of the osteoclast fusion marker *TM7SF4* at day 14 was significantly increased in the cells treated with 5 (but not 1) μg/ml HCQ compared to the controls ($P = 0.002$) **(Figure 1F)**.

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HCQ affects the intracellular pH of osteoclasts

We performed a staining on the osteoclasts with an acidification marker (acridine orange) to visualize the intracellular acidification (**Figure 2A**). In total, seven time points were evaluated with either continuous or single HCQ treatment. We found that the 485 nm : 535 nm ratio in the 5 μg/ml HCQ group was significantly lower at day 12 and day 14 following continuous HCQ treatment compared to the controls (day 12: 0.87 ± 0.03 for 5 μg/ml HCQ vs. 1.06 ± 0.05 for the control, $P = 0.034$ and day 14: 0.59 ± 0.03 for 5 μg/ml HCQ vs. 0.92 ± 0.03 for the controls, $P = 0.037$) **(Figure 2B-C)**. In contrast, the intracellular pH (485:535 ratio) in both immature osteoclasts (day 7) and mature osteoclasts (day 13), which received a single HCQ treatment, was not significantly different between the controls and HCQ treatment groups **(Figure 2D)**.

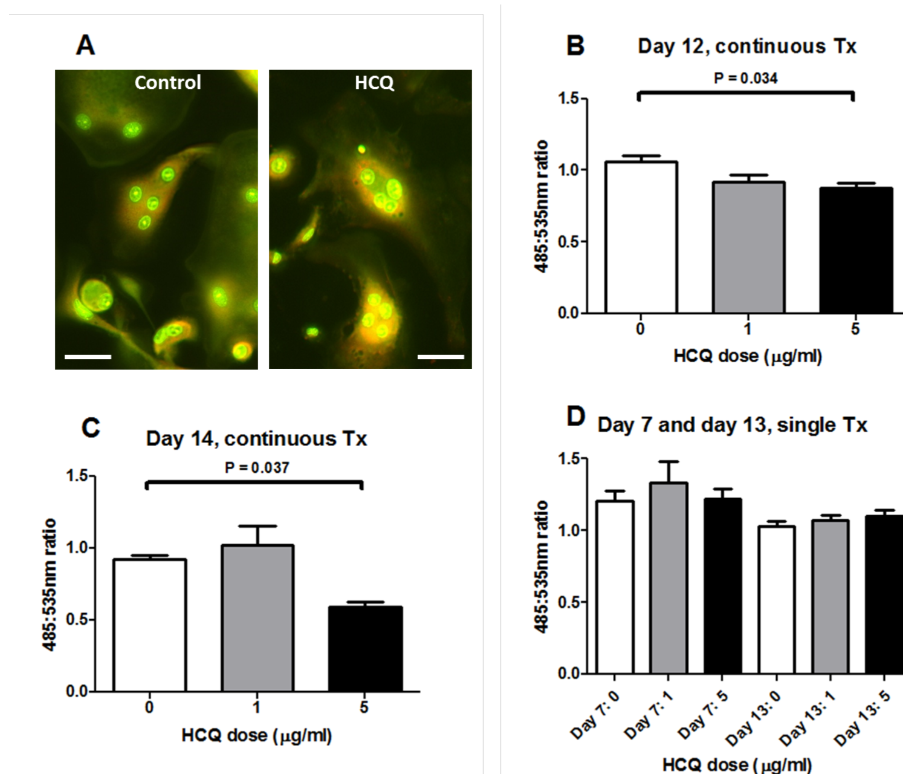

Figure 2 – Effect of HCQ on intracellular pH

*All experiments are performed twice with N = 4 for every condition (control vs. HCQ). (**A**) Osteoclasts stained with the acidification marker acridine orange (green is neutral pH and red is acidic pH). Scale bar = 50 mm. (**B**) Intracellular pH at day 12 is decreased after continuous treatment with HCQ compared to control. (**C**) Intracellular pH at day 14 is decreased after continuous treatment with HCQ compared to control. (**D**) In immature osteoclasts and after a single dose of HCQ, the intracellular pH is similar to the control. Abbreviations: HCQ, hydroxychloroquine; Tx, treatment*

HCQ stimulates cholesterol uptake by osteoclasts

Since osteoclasts lack the capacity to synthesize cholesterol endogenously, we speculated that HCQ-induced lysosomal membrane permeabilization (LMP) leads to increased LDL cholesterol significantly increased in the cells treated with 5 µg/ml HCQ compared to the controls (day 7: 31673 ± 1922 RU for 5 µg/ml HCQ vs. 14583 ± 3217 RU for the control, P = 0.0015, day 11: 18297 ± 229.7 RU for 5 µg/ml HCQ vs. 4902 ± 259.6 RU for the controls, P = 0.0003 and day 21: 6474 ± 747.5 RU for 5 µg/ml HCQ vs. 3720 ± 651.0 RU for the controls, P = 0.023) **(Figure**

3A). Additionally, at day 11 also 1 µg/ml HCQ caused a significant increase in cholesterol uptake compared to the controls **(Figure 3A)**. Although the same trend was observed as the other days, at day 14 the cholesterol uptake was not significantly different. We also evaluated the mRNA expression of the LDL receptor (*LDLR*) at day 7 and 14 showing an significantly increased expression at day 14 in cells treated with 5 µg/ml HCQ compared to the controls (0.22 ± 0.05 for 5 µg/ml HCQ vs. 0.093 ± 0.013 for the controls, P = 0.03) **(Figure 3B)**.

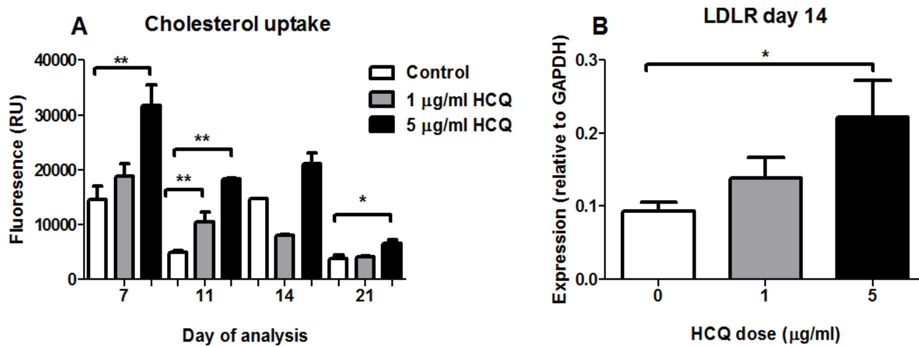

Figure 3 – Effect of HCQ on cholesterol uptake

*All experiments are performed twice with N = 4 for every condition (control vs. HCQ). (**A**) Cholesterol uptake is significantly increased by osteoclasts treated with HCQ compared to the controls at multiple time points. (**B**) Expression of LDLR is significantly increased in HCQ treated osteoclasts compared to the controls. Abbreviations: HCQ, hydroxychloroquine; LDLR, low-density lipoprotein receptor*

HCQ does not affect actin ring formation and/or apoptosis in osteoclasts

We performed phalloidin staining at day 17 to analyze a potential effect of HCQ on the formation of actin rings. We did not find any differences in appearance and/or number of actin rings between the HCQ-treated osteoclasts compared to the controls **(Figure 4A)**. Furthermore, we performed additional DAPI staining, which provided us with the opportunity to evaluate possible apoptotic events in the nuclei of osteoclasts at multiple time points. Based on the DAPI staining there was no evidence for a difference in apoptotic events between the controls and HCQ treated cells **(Figure 4B)**. In addition, we analyzed the gene expression of the apoptotic markers *BAX/BCL2* and *CASP3*. In agreement with the morphologic aspect of the cells, both apoptotic markers were not significantly different in the HCQ groups compared to the controls at day 14 **(Figure 4C-D)**.

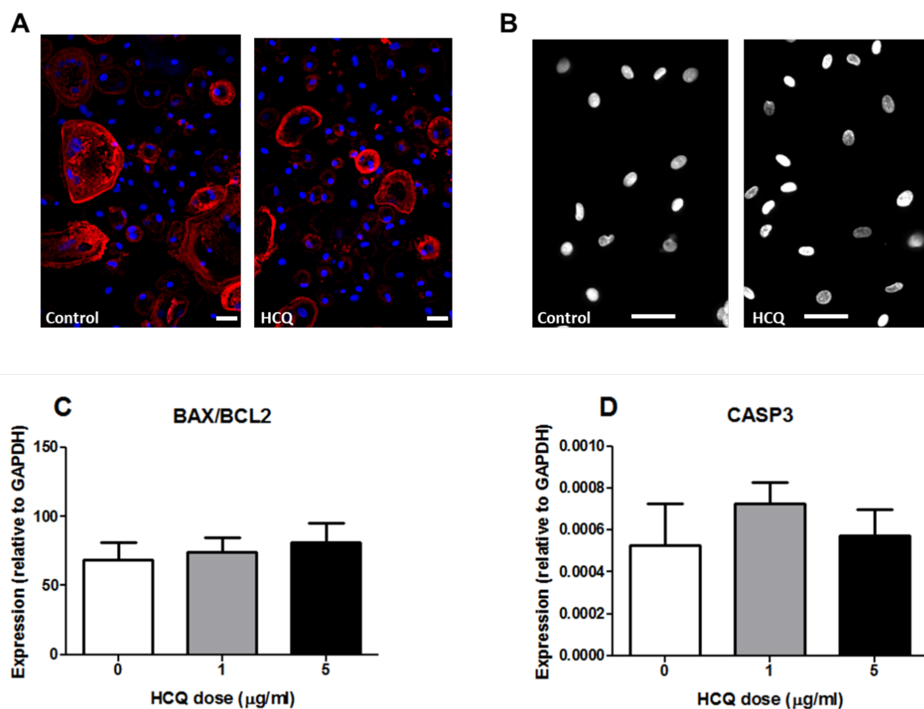

Figure 4 – Effect of HCQ on actin ring formation and apoptotic markers
*All experiments are performed twice with N = 4 for every condition (control vs. HCQ). Scale bar = 50 mm. (**A**) Phalloidin and DAPI double staining of osteoclasts on bone surface. Scale bar = 50 mm. (**B**) DAPI staining of osteoclasts (**C**) Expression of the BAX/BCL2 ratio was not significantly affected by HCQ compared to controls at day 14 (**D**) Expression of the CASP3 was not significantly affected by HCQ compared to controls at day 14*

Effect of HCQ on bone turnover in vivo

Study cohort and baseline characteristics

We obtained serum from 63 RA patients (tREACH database) at baseline and after six months of treatment. Of those, 33 subjects received HCQ and thirty subjects were treated with MTX. We recruited another 17 patients from the outpatient clinics of all three hospitals. Of those, 7 patients were excluded due to the use of immunosuppressive drugs (N = 6) or use of bisphosphonates (N = 1). In total, 10 patients whom used HCQ for three months were included. Baseline characteristics from all subjects are shown in **Table 2**.

Table 2 – Characteristics of the study cohort

	3 months treatment	6 months treatment	
	HCQ (N = 10)	**HCQ (N = 33)**	**MTX (N = 30)**
Demographics			
Age, years ± SEM	59.1 ± 3.7	49.3 ± 2.7	55.9 ± 2.5
Female gender, n (%)	9 (90)	18 (55)	15 (50)
Postmenopausal, n (%)	6/9 (67)	6/18 (33)	9/15 (60)
Biochemical			
Serum CRP, mg/L ± SEM ¶	-4.08 ± 2.6	-11.7 ± 3.6*	-14.0 ± 4.4*
Serum ß-CTx, µg/L ± SEM ¶	-0.037 ± 0.02	-0.063 ± 0.2*	-0.038 ± 0.3

Data are presented as mean ± standard error of the mean (SEM) and no. (%)
¶ Data are presented as the difference between the two time points (after treatment - baseline)
** P < 0.05*

HCQ decreases bone resorption in vivo

β-CTx was measured in all subjects at baseline and treatment (HCQ or MTX). β-CTx was not significantly different between both treatment regimens after six months (P = 0.16). β-CTx was significantly decreased after six months of HCQ treatment compared to baseline (0.363 ± 0.167 µg/L at baseline vs. 0.300 ± 0.166 µg/L after six months, P = 0.01) **(Table 2)**. Although we observed a decreasing trend of serum β-CTx, no significant difference after six months of MTX treatment (P = 0.24) was measured **(Table 2)**. Additionally, in those 10 patients who received HCQ for three months, serum β-CTx was not significantly decreased (P = 0.14) **(Table 2)**. Since both HCQ and MTX reduce inflammation, the observed decrease in serum β-CTx may be explained by a decrease of inflammation-induced bone resorption. Therefore, we adjusted serum β-CTx for the decrease in serum CRP after six months compared to baseline and age. Serum β-CTx was not significantly associated with a decrease of CRP in the HCQ group, suggesting that HCQ has a direct inhibitory effect on bone resorption (β = 0.001 ± 0,001 µg/L, P = 0.44) **(Table 3)**. In contrast, serum β-CTx was significantly associated with a decrease in serum CRP in the MTX group (β = 0.004 ± 0,001 µg/L, P = 0.003) **(Table 3)**.

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Table 3 – Linear regression analysis of factors related to serum β-CTx between HCQ treatment (N = 33) and MTX treatment (N = 30)

Serum β-CTx (μg/L)	HCQ			MTX		
	B	Std. Error	Beta	B	Std. Error	Beta
(Constant)	0.127	0.070		0.032	0.012	
CRP (mg/L) ¶	0.001	0.001	0.126	0.004	0.001	0.530*
Age	-0.004	0.001	-0.435*	0.000	0.002	-0.024

Abbreviations: Std. error, standard error of the mean; β-CTx, beta C-terminal telopeptide; HCQ, hydroxychloroquine; MTX, methotrexate; CRP, C-reactive protein
¶ Data are presented as the difference between the two time points (after treatment - baseline)
** $P < 0.05$*

Discussion

In the present study we demonstrated that HCQ suppresses bone resorption *in vitro* and decreases the resorption marker β-CTx *in vivo*. We searched the available literature but were not able to find another study reporting an association between HCQ and human osteoclast activity. We report here that HCQ treatment leads to a decreased number of multinuclear osteoclasts and diminished resorptive activity. Furthermore, we demonstrated a significantly decreased intracellular pH and increased cholesterol uptake in osteoclasts upon HCQ treatment compared to controls, which has been reported before in cells undergoing LMP. LMP may lead to decreased bone resorption due to decreased delivery of the required protons and lysosomal enzymes. Supporting our *in vitro* data, we showed a significant decrease in serum β-CTx after six months of HCQ treatment compared to baseline, which was not the case for MTX treatment.

Our *in vitro* work showed that osteoclast activity is inhibited by increasing doses of HCQ as measured by bone resorption assay and by reduced gene expression of the resorption marker *CTSK*. We also showed that HCQ leads to a decreased number of multinuclear osteoclasts, which indicates a disturbance in cell fusion leading to decreased resorption. We indeed found that gene expression of the fusion marker *TM7SF4* was significantly increased by HCQ compared to the controls. We speculate that the upregulation of *TM7SF4* is a compensatory mechanism in an attempt to increase the number of multinuclear osteoclasts. Despite reduced bone resorption, phalloidin staining did not show a difference in either the formation or morphology

of the actin ring between the HCQ treated cells and the controls.

Another potential mechanism for the decreased osteoclast activity could be an increase in lysosomal pH, which is a known but disputed effect of HCQ in mice osteoclasts, leading to decreased secretion of acid [21]. Therefore, we stained the osteoclasts with an acidification marker at multiple time points and with different treatment regimens. Contrary to expectation, we found that mature osteoclasts with continuous HCQ treatment have a lower intracellular pH compared to the controls, which was not occurring in immature osteoclasts and in mature osteoclasts receiving a single dose of HCQ.

A potential explanation for this is the association of HCQ with increased LMP [30]. LMP is caused by loss of cholesterol in the lysosomal membrane leading to the release of cathepsins and protons from the lysosomal lumen into the cytosol where they participate in apoptosis signalling and lead to a decreased intracellular pH, respectively [31,32]. We hypothesize that the acid required for resorption canot leave the cell due to LMP leading to decreased bone resorption. Additionally, cholesterol is identified as a stabilizer of the lysosomal membrane and therefore counters LMP [33,34]. Since osteoclasts lack the capacity to synthesize cholesterol endogenously, we speculated that HCQ-induced LMP leads to increased LDL cholesterol uptake in order to prevent HCQ-induced LMP [29,35]. Indeed, HCQ has beneficial effects on cholesterol metabolism *in vivo* [13,36]. We reported a significant increase of *LDLR* expression in the HCQ treated cells compared to the controls. In addition, we evaluated the cholesterol uptake by osteoclasts at multiple time points, which showed a significantly increased uptake due to HCQ treatment supporting our hypothesis. Nevertheless, we could not find evidence of increased apoptosis due to LMP in the HCQ treated cells compared to the controls.

It may be possible that the osteoclast is capable of defending itself from apoptosis (e.g. by increasing cholesterol uptake). Additionally, BAX-dependent mitochondrial membrane permeabilization (MMP), downstream of LMP, is an obligatory step of LMP-triggered apoptosis [30]. MMP may be a rate limiting step in HCQ-induced apoptosis. Indeed, we found no significant difference in *BAX/BCL2* and *CASP3* expression due to HCQ treatment.

Summarizing our *in vitro* work, we propose a mechanism of action for HCQ in osteoclasts leading to increased LMP (not leading to apoptosis), which would be in agreement with the observed lower intracellular pH (due to LMP) and increased cholesterol uptake as a mechanism to prevent HCQ-induced LMP **(Figure 5)**.

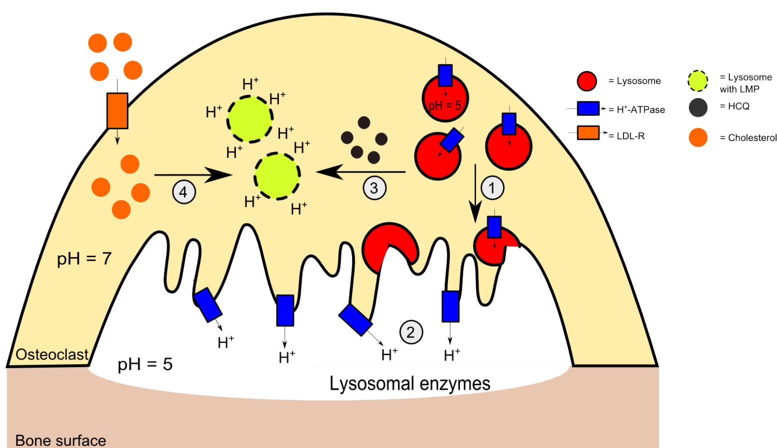

Figure 5 – Proposed model of the mechanism of action for HCQ in the osteoclast
1) *Lysosomes containing H⁺-ATPases on the surface, cathepsins and acid move intracellularly to the ruffled border.* **2**) *Lysosomes fuse with the ruffled border and build in the H⁺-ATPases in order to secrete protons. In addition, the lysosomal enzymes (e.g. cathepsins) are released in the resorption pit.* **3**) *HCQ treatment may lead to an increased rate of LMP leading to leakage of protons (decreased pH) and enzymes into the intracellular space. Additionally, a decreased number of lysosomes will move to the ruffled border leading to decreased bone resorption.* **4**) *Since cholesterol is a stabilizer of the lysosomal membrane, mRNA expression of the LDLR and cholesterol uptake are increased to counter HCCQ-induced LMP.*
Abbreviations: HCQ, hydroxychloroquine; LMP, lysosomal membrane permeabilization; LDLR, low-density lipoprotein receptor

We also analyzed the effect of HCQ on bone resorption *in vivo* by measuring the serum resorption marker β-CTx in patients with RA. Although the subjects in the HCQ group were relatively younger and more often premenopausal compared to the subjects in the MTX group, there was no significant difference in age and postmenopausal rate between both groups. In this cohort, we did find a modest, but very significant, lower serum level of β-CTx after 6 months of HCQ treatment compared to baseline. In addition, the effect remained significant after correction for the decrease in serum CRP. In contrast, MTX did not lead to a significant reduction of serum β-CTx. Based on these findings, we conclude that HCQ has a direct effect on bone resorption since the decrease in serum β-CTx was not explained by a decrease in inflammation-induced bone resorption. Additionally, our findings *in vivo* are in agreement with our *in vitro* data, suggesting that HCQ has a direct inhibitory effect on bone resorption by inhibiting osteoclast function.

The strength of this study is that we demonstrated both *in vivo* and *in vitro* effects of HCQ on bone resorption. In addition, we evaluated a potential mechanism of action for HCQ on osteoclast function. A limitation of this study is that β-CTx measurements were taken at random moments during the day without fasting, which may have influenced the test results. Rather, we speculate that measurement of β-CTx in patients who fast and from whom blood is collected in the morning, may result in a stronger effect than what has been demonstrated currently.

In conclusion, we demonstrate that HCQ suppresses bone mineral resorption both *in vitro* and decreases the bone resorption marker β-CTx *in vivo*. We also showed that HCQ decreases the intracellular pH in mature osteoclasts and stimulates cholesterol uptake. We postulate that HCQ induces osteoclastic LMP leading to decreased bone resorption. Based on these findings, we hypothesize that skeletal health of patients with increased risk of osteoporosis and fractures, including postmenopausal women and patients with inflammatory diseases such as pSS and RA, may benefit from HCQ by preventing BMD loss.

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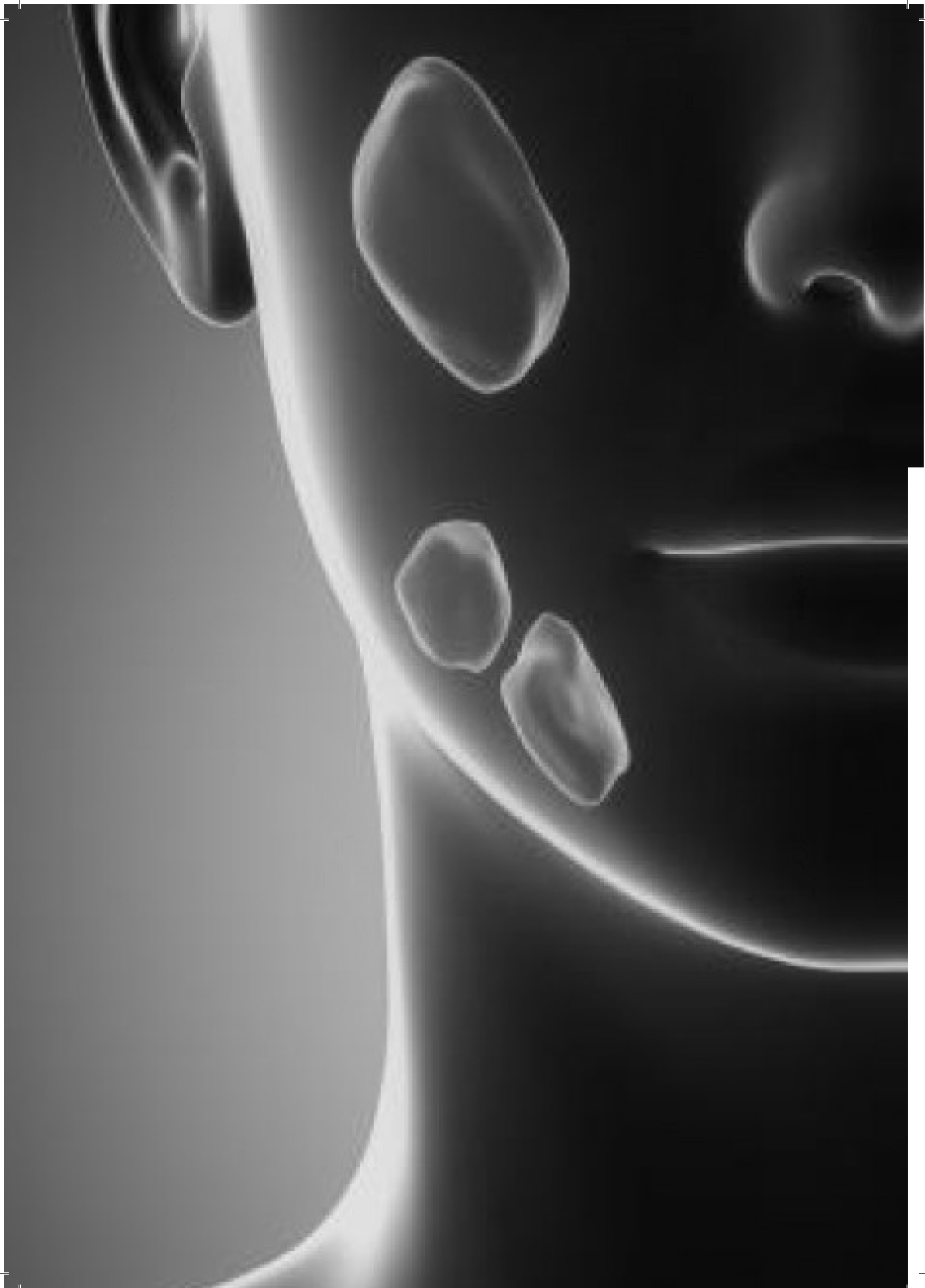

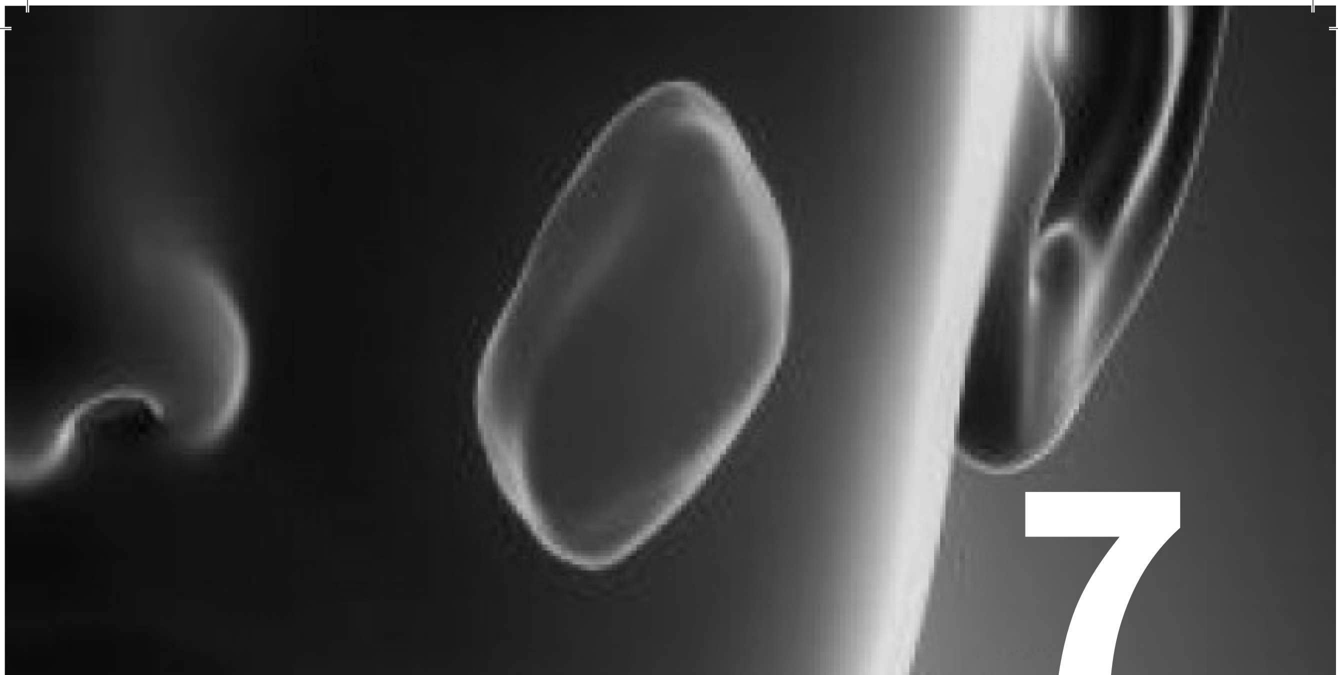

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General discussion and clinical implications

General discussion and clinical implications

Evaluation of metabolic disturbances – renal involvement

We showed that the prevalence of dRTA in pSS is high as measured by the AMCL urinary acidification test. It is unknown whether dRTA is also more prevalent in other autoimmune diseases such as RA and SLE. Determining the prevalence of dRTA and its complications is important because there is an effective treatment with potassium citrate for both the symptoms and complications of dRTA, by restoring acid-base balance with potassium citrate [1,2]. Unfortunately, there is no curative therapy for dRTA available. The symptoms of dRTA in pSS patients are comparable to patients without pSS and include fatigue, muscle weakness and hypercalciuria (leading to nephrolithiasis and decreased BMD), which are caused by the metabolic acidosis [3–5]. First, since fatigue is a major problem for patients with pSS and is associated with a low quality of life, we speculate that dRTA may contribute (partially) to the fatigue complaints of pSS patients [6,7]. Treatment of dRTA would then hopefully lead to an improved quality of life of pSS patients, which can be measured by questionnaires concerning fatigue and/or depression over time. Secondly, we did not find a case of hypercalcemia and none of the patients had documented nephrocalcinosis / nephrolithiasis in their medical history or had complaints of ongoing nephrolithiasis. Unfortunately, we did not measure urinary calcium excretion. Still, we observed that patients with complete dRTA had a decreased glomerular filtration rate (GFR) compared to those without dRTA or with incomplete dRTA. A potential explanation for decreased GFR would be tubulointerstitial nephritis, which is a common renal manifestation of pSS, although this was not confirmed by a kidney biopsy. Therefore, treatment with corticosteroids may lead to improvement of renal function and possibly resolve dRTA with it. In addition, since patients with even a mild acidemia and higher urinary pH may predispose to kidney stone formation, it may be interesting to perform an ultrasound examination of the kidneys in patients with dRTA. Currently, dRTA is diagnosed using the AMCL urinary acidification test, although studies report a more patient-friendly alternative using FF [8]. After comparison of both tests, we recommend to use AMCL to test urinary acidification in pSS, but the use of FF may be considered as screening test, given its reasonable negative predictive value and better tolerability. Furthermore, it is unclear whether the FF test should be repeated after a certain period of time in case of a normal test result. There are no data to indicate what the disease-free period is after

a negative test result. Therefore, at present, we recommend to leave it to the discretion of the treating physician to repeat the FF test after a few years.

Evaluation of metabolic disturbances – bone involvement

Some recent studies report an increased prevalence of low BMD in patients with dRTA [9,10], while other studies did not report a significant difference in patients with dRTA [11,12]. Although metabolic acidosis leads to bone loss, we showed that patients with a urinary acidification defect (complete and incomplete dRTA combined) did not have a significantly different BMD in the lumbar spine (LS) and femoral neck (FN) compared to patients without a urinary acidification defect [13]. A potential explanation may be that patients with incomplete dRTA may have an intermittent metabolic acidosis instead of chronic academia, which does not lead to increased bone loss. Furthermore, only three patients had complete dRTA in our cohort making it unreliable to draw any conclusions concerning the effect of complete dRTA on BMD. Therefore, the same study should be repeated with a larger cohort of patients with complete dRTA. Contrary to expectation, we found that pSS patients have significantly higher BMD than healthy age- and sex-matched controls. A possible explanation for the high BMD could be that patients receive lifestyle advice and/or medication from their doctor to improve their condition that also is beneficial for bone health. Also, it has been shown before that seasonal fluctuation of vitamin D is associated with differences in bone metabolism (e.g., during summer time more sun exposure leading to higher vitamin D, which may result in to a higher BMD) [14,15]. The BMD data of the controls was obtained throughout the whole year without peaks in a certain season and our patients were tested during summer time. However, we used three age- and sex matched controls for every pSS patient to reduce the likelihood of comparing BMD data obtained in different seasons. In addition, it is unlikely that such small fluctuations will lead to a significantly higher BMD. Another explanation would be the use of medications by the pSS patients. In our cohort, the most prevalent drug used was HCQ (69%). According to literature, the use of HCQ in SLE patients was associated with increased BMD of the hip compared to the non-users [16,17]. In both studies, disease activity and use of corticosteroids were not significantly different between both groups, which may suggest a disease-independent effect of HCQ on bone. Indeed, we demonstrated that HCQ treatment leads to a significantly lower serum level of the resorp-

tion marker β-CTx compared to baseline independent from the reduction of inflammation. Based on these findings, we suggest that HCQ has a direct inhibitory effect on bone resorption since the decrease in serum β-CTx was not explained by a decrease in inflammation-induced bone resorption. This is in agreement with the observed higher BMD in pSS patients.

Hydroxychloroquine decreases bone turnover

HCQ has primarily been registered as an antimalarial drug which was found to be effective in inflammatory diseases as well. Nowadays, HCQ is used to treat many autoimmune diseases such as primary Sjögren syndrome (pSS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [18–20]. It has been reported that HCQ is capable of TLR inhibition, especially TLR 7 and 9, which are involved in the pathogenesis of both pSS and SLE [21–23]. These TLRs are localized intracellular on the surface of lysosomes and their activation depends on lysosomal acidification. Multiple studies have reported that HCQ is associated with an increased lysosomal pH leading to TLR inhibition [24,25]. Recent reports suggest, however, that HCQ does not affect lysosomal pH but rather have a direct inhibitory effect on the TLRs [26,27]. Additionally, HCQ has beneficial effects on the lipid profile by decreasing low density lipoprotein (LDL) and triglycerides (TG) as well as increasing high-density lipoprotein (HDL) in both patients with pSS and RA [28,29]. The effect of HCQ on bone is unknown, whereas studies concerning the effects of HCQ on bone are limited. In vitro studies on the effect of HCQ on bone remodeling are very limited. It was shown that HCQ is able to increase lysosomal pH in osteoclasts that is associated with decreased signaling and nuclear translocation of the key osteoclast marker NFATc1, leading to an impairment of osteoclastogenesis [24,25]. Furthermore, HCQ has been identified to be an autophagy inhibitor by blocking the degradation of autophagosomes and promoting apoptosis, which is essential during osteoblast mineralization and bone homeostasis [30–32]. Also, both cell types express TLR 2, 4 and 9 [33]. While the activation of TLRs in committed osteoclast precursors, mature osteoclasts and osteoblasts results in increased osteoclastogenesis (and is probably the mechanism by which pathogen-induced bone loss occurs, activation of TLRs in early osteoclast precursors exerts an anti-osteoclastogenic effect). This may suggest that inhibition of TLR 9 by HCQ (or other TLR-inhibitors) will affect bone remodeling depending in what stage of differentiation HCQ is introduced. We studied the effect of HCQ on both osteo-

clasts (bone resorbing cells) and osteoblasts (bone forming cells). Since we reported a favorable effect of HCQ on bone in vivo, we hypothesized that HCQ will have either a beneficial effect on the osteoblast and/or an inhibitory effect on the osteoclasts. Contrary to expectation, we demonstrated a significantly decreased osteoblast differentiation and mineralization due to HCQ treatment compared to controls. However, we indeed demonstrated that HCQ significantly decreased bone resorption by osteoclasts. In the osteoblasts, we performed microarray analysis to elucidate the mechanism of HCQ, which showed a highly significant upregulation of both the cholesterol biosynthesis pathway and the lysosomal system. We also showed that SIM treatment led to decreased osteoblast differentiation and mineralization. The beneficial effects of SIM in osteoblast has been studied extensively before [45]. We searched the literature for studies reporting similar observations, however, we could not find these. A potential explanation might be that we used a different type of cell-line compared to previous studies reporting beneficial effects of SIM on osteoblasts [46, 47]. Despite using a dose-response experiment for SIM and following the methods as described in other papers, we could not discriminate between the effects of HCQ and SIM. In the osteoclasts, we showed that continuous HCQ treatment leads to a lower intracellular pH and increased cholesterol uptake compared to the controls. We analysed the difference of pH in the resorption pit using acridine orange stained osteoclasts cultured on mouse bone instead of plastic; however, it was difficult and inaccurate to assess the resorption pits under the fluorescence microscope. We searched literature and we did find a different method to analyse the pH in the resorption pit, but this method involved custom made acid-sensing probes [34]. Although this experiment may validate our hypothesis, we were not able to get access to these probes. Therefore, we were unable to demonstrate an increased pH in the resorption pit due to HCQ treatment. We did not evaluate the effects of SIM on osteoclasts since osteoclasts lack the capacity to synthesize cholesterol endogenously [35,36]. Based on our results, we speculate that the potential mechanism for decreased osteoclast and osteoblast function would be HCQ-induced lysosomal membrane permeabilization (LMP). LMP is caused by loss of cholesterol in the lysosomal membrane leading to the release of cathepsins and protons from the lysosomal lumen into the cytosol and lead to a decreased intracellular pH [37,38]. Additionally, cholesterol is identified as a stabilizer of the lysosomal membrane

and therefore counters LMP [39,40]. In the osteoclasts we demonstrated a decreased intracellular pH compared to controls. In both the osteoclast and the osteoblast we showed that the cholesterol metabolism is significantly influenced by HCQ (cholesterol uptake, microarray). Furthermore, we reported that HCQ affects the gene expression of lysosomal membrane proteins and lysosomal enzymes in both cells. We hypothesize that the upregulation of both pathways are a compensatory mechanism to protect the cell from LMP-induced apoptosis. LMP has been studied before with immunostaining for cathepsin B and a lysosomal membrane protein in mouse embryonic fibroblasts [41]. Since the function of osteoclasts depends on lysosome secretion, we speculate that these cells contain more lysosomes compared to fibroblasts. Therefore, interpretation of the previously described immunostaining may not be accurate in osteoclasts. Additionally, since LMP leads to apoptosis, we expected more apoptotic cells due to HCQ treatment compared to the controls. We observed an altered phenotype in the HCQ-treated osteoblasts; however, both DAPI and phalloidin staining appeared to be normal. We suggest that the rate of LMP may not be sufficient enough to detect by staining methods. Furthermore, it may be possible that the compensatory mechanisms are keeping the cells alive although the function is decreased. In conclusion, we demonstrated that HCQ decreases bone turnover (by inhibiting both osteoclast and osteoblast activity), which may be caused by HCQ-induced LMP. Since the cholesterol pathway seems to be affected the most in both cells, we proposed a combination therapy of HCQ and SIM, in which SIM could antagonize the effects of HCQ. SIM would only attenuate the negative effect of HCQ on osteoblasts and have no effect on osteoclasts since these cells are not capable of synthesizing cholesterol. However, treatment with SIM alone and/or combined with HCQ also decreased osteoblast differentiation and mineralization. Based on this finding, our proposed combination therapy with HCQ and SIM seems to be not valid. Although both cells are inhibited by HCQ, we hypothesize that skeletal health of patients with increased risk of osteoporosis and fractures, including postmenopausal women and patients with inflammatory diseases such as pSS and RA, may benefit from HCQ by preventing BMD loss.

Future research

Potassium citrate reduces fatigue in pSS patients?

The most prevalent general symptom in pSS is fatigue, occurring in up to 70-80% of all patients [11]. Fatigue in pSS has been well studied using the multidimensional fatigue inventory (MFI) on which pSS patients score twice as bad at all dimensions when compared to healthy controls [22,23]. Fatigue has also been reported as symptom of (incomplete) dRTA. In chapter 3, we reported a high prevalence of dRTA in pSS patients. Therefore, we speculate that treatment of (incomplete) dRTA with potassium citrate may reduce fatigue complaints in pSS patients. The outcome parameters for this observational study should include the MFI scale as tool to evaluate the effects of treatment on fatigue. Furthermore, periodic blood and urine measurements for pH evaluation and to prevent hyperkalemia should be performed.

A new therapy for osteoporosis?

In chapter 4, we reported a higher BMD in pSS patients compared to controls. In chapter 5 and 6 we demonstrated that HCQ decreases bone turnover by inhibiting both osteoblasts and osteoclasts. We finished our work by suggesting that HCQ would be beneficial for patients with increased risk of osteoporosis and fractures. We searched the available literature but did not find another study reporting HCQ as potential new therapy. Follow-up research on this topic would be a clinical trial comparing HCQ to the current gold standard (bisphosphonates). The study population should consist subjects who are catabolic, such as patients with a chronic inflammatory disease or postmenopausal women, leading to decreased BMD. DEXA scanning to measure T-scores and serum bone markers (β-CTx and PINP) could be used as main outcome parameters. We hypothesize that HCQ at least equals the current gold standard in this population. HCQ may then be a more favorable treatment since it is known to have a relative few side effects and can be used throughout life. The latter is not the case for bisphosphonates.

HCQ-induced dRTA?

In this thesis, we studied the mechanism of action for HCQ on bone cells. Overall, we found that HCQ is associated with upregulation of the cholesterol synthesis pathway and the lysosomal pathway in both cell lines. We showed that HCQ is associated with 1) decreased

intracellular pH, 2) increased uptake of exogenous cholesterol and 3) upregulation of cholesterol synthesizing enzymes and lysosomal enzymes gene expression, which may be explained by HCQ-induced LMP. In the osteoclast, we hypothesized that HCQ-induced LMP leads to a decreased availability of lysosomes at the ruffled border as potential explanation for the observed decreased bone resorption. Lysosome trafficking and fusion with the ruffled border are an essential step in both the development of the ruffled border and consequently bone resorption [42]. The lysosomes have H^+-ATPases on their surface, which are built in the ruffled border membrane in order to secrete protons in the resorption pit. A similar process occurs in the α-intercalated cells (α-ICs) located in the collecting duct of the kidney. The α-ICs main function is to secrete protons into the tubular lumen via H^+-ATPases, which are stored on the surface of lysosomes located in the cytoplasm. Upon stimulation (e.g. metabolic acidosis, hyperaldosteronism), these lysosomes move to the apical membrane in order to build in their H^+-ATPases for proton secretion. In chapter 3 we reported a high prevalence of dRTA in pSS patients. We hypothesize that HCQ-induced LMP also occurs in the α-ICs leading to decreased availability of H^+-ATPase on the apical membrane causing dRTA. Recently, LMP and lysosomal dysfunction have been reported to be induced by proteinuria and also to be involved in the pathogenesis of diabetic nephropathy [43,44]. However, no studies have reported the effects of HCQ on renal tubular cells. dRTA has also been reported in patients with the extremely rare carbonic anhydrase type 2 (CA2) deficiency syndrome [45,46]. CA2 is present in many tissues (e.g. kidney, bone) and catalyzes the reaction leading to the formation of protons and bicarbonate ions. Patients with CA2 deficiency develop dRTA and osteopetrosis (a condition in which BMD is significantly elevated). We showed that HCQ affects intracellular pH and is associated with a higher BMD. Although CA2 deficiency is a rare and devastating disease, we postulate that there are similarities between the effects of HCQ treatment en CA2 deficiency syndrome. Based on our findings on bone cells, it would be interesting to study the effect of HCQ on renal tubular cells with special aim to cells present in the collecting duct. A first step would be to evaluate the effect on proton secretion to the medium, but also pH changes intracellular. Consequently, a staining for the presence of H^+-ATPase on the apical membrane would potentially show decreased expression of H^+-ATPase due to HCQ.

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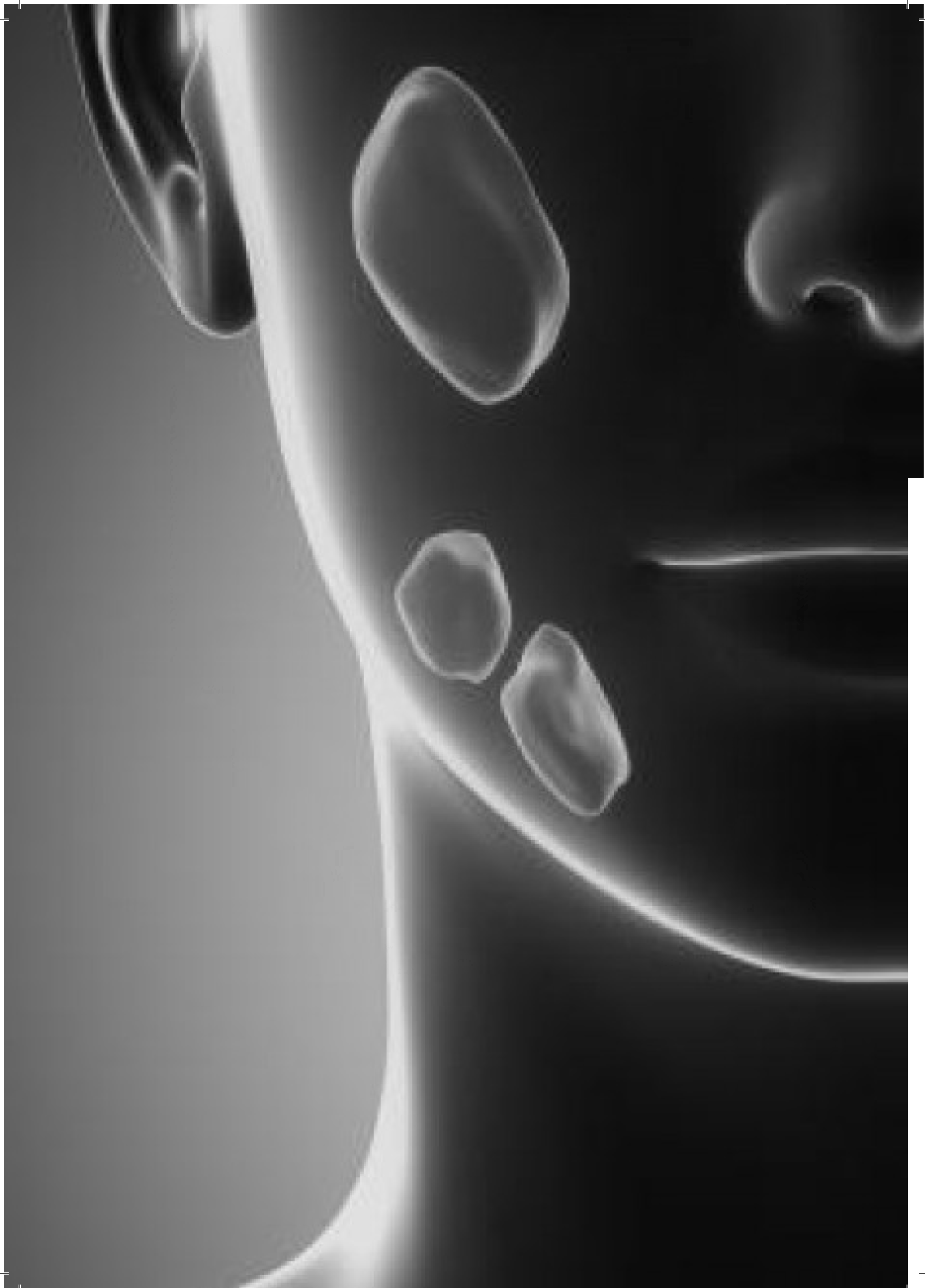

Summary
Samenvatting

Summary

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, which is characterized by lymphocytic infiltration of the secretory glands. The pathogenesis of pSS is currently not well understood, but increased activation of B cells followed by immune complex formation and autoantibody production are thought to play important roles [1]. pSS is diagnosed using the American-European consensus group (AECG) classification criteria for pSS including subjective symptoms and objective tests such as histopathology and serology [2]. The symptoms of pSS include sicca syndrome (dry eyes and/or oral cavity) and systemic manifestations, such as pulmonary, articular, renal and neurological involvement [3]. Systemic involvement is not always easy to recognize by the physician in a complex disease as pSS, since symptoms can be non-specific. Renal involvement, with distal renal tubular acidosis (dRTA), is common in pSS [4,5]. The symptoms of dRTA (e.g. fatigue and/or muscle weakness) are non-specific, and are also occurring in pSS without systemic involvement. Untreated dRTA leads to metabolic acidosis, which is associated with hypercalciuria. During acute metabolic acidosis, protons are exchanged for calcium ions from bone. In case of chronic metabolic acidosis, a cell mediated process, increased activity of osteoclasts, may lead to decreased bone mineral density (BMD) [6,7].

pSS patients are often treated with the immunomodulatory agent hydroxychloroquine (HCQ). The most important effects on the immune system is reducing inflammatory pathways including Toll-like receptor (TLR) activation. In addition to its anti-inflammatory effects, HCQ is also associated with anti-thrombotic and anti-atherosclerotic actions, anti-diabetic effects and beneficial effects on the lipid profile leading to cardioprotective outcomes [8–10]. Furthermore, HCQ has also been reported to positively affect BMD in systemic lupus erythematosus patients [11,12]. In vitro studies on the effect of HCQ on bone remodeling are very limited. Lee et al. showed that HCQ did not affect human osteoclastogenesis [13]. Xiu *et al.* showed reduced osteoclastogenesis by preventing TRAF3 degradation following chloroquine (drug from the same family as HCQ) treatment in mice [14]. In addition, it was shown that HCQ is able to increase lysosomal pH in mice osteoclasts that is associated with decreased signaling and nuclear translocation of the key osteoclast marker NFATc1, leading to an impairment of osteoclastogenesis [15].

In this thesis, we addressed three clinical important issues:

1) the prevalence and consequences of distal renal tubular acidosis in pSS patients;

2) the effect of pSS on bone metabolism;

3) the effect of HCQ on bone metabolism.

Part 1; includes the clinical studies of this thesis and is dedicated to the evaluation of dRTA and its consequences in pSS and the effect of pSS on bone. **Chapters 1** and **2** provide an overview of pSS and dRTA, respectively, concerning the pathogenesis, diagnosis and treatment. **Chapter 3** is the first original article in which we determined the prevalence of dRTA in pSS. We found a positive urinary acidification test with ammonium chloride (AMCL) in 30% of 57 pSS patients. The prevalence of complete dRTA (urinary acidification defect with acidosis) was 5% (3 out of 57). All three patients also had an impaired kidney function. The prevalence of incomplete dRTA (urinary acidification defect without acidosis) was 25% (14 out of 57). Furthermore, we compared the ammonium chloride test with a more patient-friendly test using the combination of furosemide and fludrocortisone (FF). Compared to AMCL, the positive and negative predictive values of FF were 46 and 82%, respectively. Therefore, we suggest that FF cannot replace AMCL to test urinary acidification in pSS, but may be considered as screening tool, given its reasonable negative predictive value and better tolerability. In **chapter 4** we focused on the association between dRTA and BMD, since multiple studies report a decrease of BMD in dRTA patients, which is disputed by other studies. Therefore, we compared the BMD of the lumbar spine (LS) and the femoral neck (FN) between pSS patients with and without dRTA as measured by Dual Energy X-ray Absorptiometry (DXA). We demonstrated that patients with a urinary acidification defect (complete and incomplete dRTA combined) did not have a significantly different LS- and FN- BMD compared to patients without a urinary acidification defect. Since epidemiologic data on BMD in pSS are lacking, we also compared BMD of the complete pSS cohort (with and without dRTA combined) to an age- and sex matched healthy control group. Unexpectedly, pSS patients had a significantly higher LS- and FN-BMD compared to healthy controls, which remained significant higher after adjustment for body mass index and smoking. We speculated that the use of HCQ (in our cohort 69%) is a potential explanation for the observed higher BMD. Unfortunately, we could

not analyze the association between HCQ use and BMD since information (patient history of referring hospitals) about the duration and dose of HCQ treatment in our cohort was lacking.

Part 2; includes the laboratory studies of this thesis in which we demonstrate the effects of HCQ on human bone cells both in vivo and in vitro. In **chapter 5** we studied the effects of HCQ on human osteoblasts in vitro. We demonstrated a significantly decreased differentiation and mineralization of the HCQ-treated osteoblasts compared to controls. Furthermore, we studied the potential mechanism of action for HCQ in osteoblasts using microarray analysis and additional PCR validation. We reported a highly significant upregulation of the cholesterol biosynthesis and lysosomal pathways in the 5 μg/ml HCQ treated cells compared to the controls. Based on this finding, we speculate that either 1) HCQ has a direct positive regulatory effect on cholesterol synthesis, or 2) HCQ causes an intracellular cholesterol depletion leading indirectly to increased cholesterol synthesis and/or increased cholesterol uptake. Since simvastatin (SIM, a cholesterol synthesis inhibitor) has reported to be beneficial for osteoblast differentiation and mineralization, we evaluated whether SIM could antagonize the effects of HCQ. Contrary to expectation, we showed that SIM significantly decreased both osteoblast differentiation and mineralization and that the combination of SIM and HCQ was similar to HCQ treatment alone. We speculate that the potential mechanism would be HCQ-induced LMP leading to decreased osteoblast development and activity. As a compensatory mechanism, both the cholesterol synthesis pathway and the lysosomal pathway are upregulated to restore osteoblast function. It appears that the positive effect of HCQ on BMD cannot be explained by a stimulating effect on the osteoblast. **Chapter 6** focuses on the effects of HCQ on human osteoclasts both in vivo and in vitro. In 63 rheumatoid arthritis (RA) patients who received either HCQ or methotrexate (MTX) treatment for six months, we found a significant lower level of serum β-CTx (the bone resorption marker) compared to baseline in the HCQ group, but not in the MTX group. Since both HCQ and MTX reduce inflammation, the observed decrease in serum β-CTx may be explained by a decrease of inflammation-induced bone resorption. Serum β-CTx was not significantly associated with a decrease of C-reactive protein (CRP) in the HCQ group, suggesting that HCQ has a direct inhibitory effect on bone resorption. In contrast, CRP reduction in the MTX group was

associated with β-CTx.

We also demonstrated that HCQ significantly decreases bone resorption by human osteoclasts in vitro. Furthermore, we showed a significantly decreased intracellular pH and increased cholesterol uptake in osteoclasts upon HCQ treatment compared to controls, which has been reported before in cells with lysosomal membrane permeability (LMP). LMP may lead to decreased bone resorption due to decreased delivery of the required protons and lysosomal enzymes. We postulate that HCQ-induced LMP leads to decreased bone resorption. Based on these findings, we hypothesize that bone health of patients with increased risk of osteoporosis and fractures, including postmenopausal women and patients with inflammatory diseases such as pSS and RA, may benefit from HCQ by preventing BMD loss.

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Samenvatting

Primair Sjögren syndroom (pSS) is een systemische autoimmuunziekte gekarakteriseerd door ontstekingsreacties in de vocht producerende klieren. De pathogenese (oorzaak van de ziekte) is nog onbekend, maar onderzoek wijst uit dat verhoogde activitiet van B-cellen gevolgd door de vorming van immuuncomplexen een belangrijke rol hierin speelt. De diagnose pSS wordt gesteld door middel van de Amerikaanse-Europese classificatie criteria waarin zowel de klachten (droge ogen en droge mond) als objectieve testen (aanwezigheid van autoantistoffen en histopatholgisch onderzoek) staan. Naast de droge ogen en mond (sicca syndroom) kunnen in patiënten met pSS meerdere orgaansystemen zoals de nieren, gewrichten, zenuwstelsel en longen, tot klachten leiden. Gezien deze klachten meestal aspecifiek zijn, is dit moeilijk te herkennen. Nierfunctiestoornissen komt vaak voor in pSS en uit zich meestal door distale renale tubulaire acidose (dRTA), wat een bekende complicatie is van pSS. De klachten die passen bij dRTA (b.v. moeheid en spierzwakte) zijn aspecifiek en komen ook voor bij patiënten met pSS die geen dRTA hebben. Onbehandelde dRTA kan leiden tot een metabole acidose wat geassocieerd wordt met hypercalciurie (veel kalk in de urine). Ten tijde van acute metabole acidose worden protonen uitgewisseld voor calciumionen uit bot. Chronische metabole acidose leidt tot een cel-gemedieerd proces (verhoogde activiteit van osteoclasten, botresorberende cellen) en kan resulteren in een verlaagde botmineraaldichtheid (botontkalking, BMD). Patiënten met pSS worden vaak behandeld met het immuunmodulerende medicijn hydroxychloroquine (HCQ). De belangrijkste acties op de ontstekingsreactie zijn: 1) remming van de lokale onstekings respons; 2) verminderde chronische ontsteking en 3) cellulaire effecten. Naast deze effecten wordt HCQ ook geassocieerd met verminderde thrombotische en atherosclerotische processen, anti-diabetische effecten en een gunstig effect op het vetspectrum wat resulteert in een beschermend effect voor hart- en vaatziekten. Verder is beschreven dat HCQ ook een gunstig effect heeft op de BMD van patiënten met systemische lupus erythematodes (SLE). Er zijn tot nu toe weinig in vitro studies (studies met cellen) gedaan naar het effect van HCQ op botcellen. De studie van Lee et al. toonde dat HCQ geen effect had op de ontwikkeling van menselijke osteoclasten. Xiu *et al.* toont aan dat chloroquine behandeling van muizen leidt tot een verminderde ontwikkeling van osteoclasten door remming van TRAF3 afbraak. Daarnaast is aangetoond dat HCQ in staat is om de lysosomale pH te verhogen in muis osteo-

clasten wat geassocieerd wordt met verlaagde activiteit en nucleaire translocatie van de belangrijke osteoclast marker NFATc1 met als gevolg een verminderde osteoclast ontwikkeling.

In dit proefschrift hebben we drie onderwerpen onderzocht:

1) de prevalentie en complicaties van dRTA in pSS patiënten;

2) het effect van pSS op botmetabolisme;

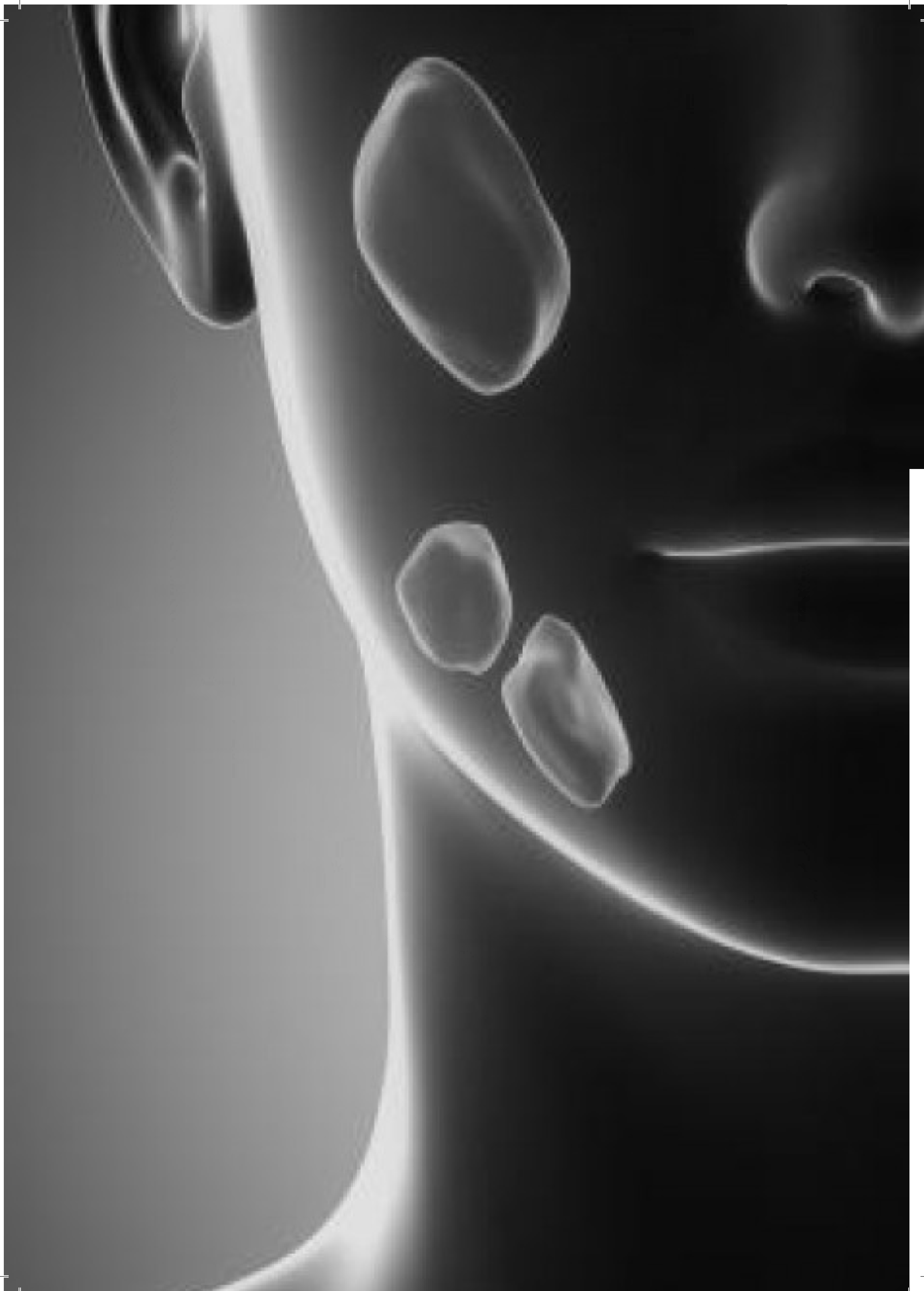
3) het effect van HCQ op botmetabolisme.

Deel 1 van dit proefschrift bevat de klinische studies en betreft het onderzoek naar dRTA en de complicaties hiervan bij pSS patiënten en de effecten op bot. **Hoofdstuk 1** en **2** geven een overzicht van respectievelijk pSS en dRTA waarin de pathogenese, diagnose en behandeling worden beschreven. **Hoofdstuk 3** is het eerste originele artikel waarin we de prevalentie van dRTA in pSS hebben onderzocht. Uit ons onderzoek blijkt dat 30% van de pSS patiënten (17 van 57) een positieve zuurbelastingstest met ammoniakchloride (AMCL) had. De prevalentie van complete dRTA (positieve zuurbelastingstest en metabole acidose) was 5% (3 van de 57 patiënten). Deze drie patiënten hadden ook nierfunctiestoornissen. De prevalentie van incomplete dRTA (positieve zuurbelastingstest zonder metabole acidose) was 25% (14 van de 57 patiënten). Verder hebben we de AMCL test vergeleken met een meer patiënt vriendelijke test, welke gebruik maakt van de combinatie furosemide en fludrocortison (FF). Vergeleken met de AMCL test heeft de FF test een positief en negatief voorspellende waarde van respectievelijk 46% en 82. Daarom concluderen we dat de FF test de AMCL test niet kan vervangen. Gezien de redelijke negatief voorspellende waarde en betere verdraagbaarheid kan de FF test wel worden gebruikt als screeningstest. In **hoofdstuk 4** hebben we de associatie tussen dRTA en BMD onderzocht. In de literatuur hebben meerdere studies een verlaagde BMD in dRTA patiënten beschreven, echter andere studies beschrijven geen effect van dRTA op BMD. Daarom hebben we de BMD van de lage ruggenwervels (LR) en de heupkop (HK) vergeleken tussen pSS patiënten met- en zonder dRTA door gebruik te maken van een DXA-scan. We hebben aangetoond dat patiënten met een een positieve zuurbelastingstest (complete en incomplete dRTA gecombineerd) geen significant veranderde LR- en HK-BMD hebben in vergelijking met patiënten met een negatieve

zuurbelastingstest. Gezien er geen epidemiologische data is betreffende BMD en pSS, hebben we ook de BMD van de complete pSS cohort (patiënten met en zonder dRTA gecombineerd) vergeleken met een gezonde leeftijd- en geslacht overeenkomende controlegroep. Onverwachts vonden we dat pSS patiënten een significant hogere LR- en HK-BMD hadden in vergelijking met de gezonde controlegroep, wat significant bleef, na correctie voor roken en body-mass index. We hypothetiseren dat het gebruik van HCQ (69% in onze pSS cohort) een mogelijke verklaring voor de hogere BMD. Helaas kunnen we deze associatie niet in onze cohort analyseren omdat informatie over de duur en dosering van HCQ behandeling ontbreekt.

Deel 2 van dit proefschrift bevat de studies gedaan in het laboratorium waarbij we de effecten van HCQ op menselijke botcellen (in vivo in in vitro) beschrijven. In **hoofdstuk 5** hebben we de effecten van HCQ op menselijke osteoblasten (bot aanmakende cellen) *in vitro* bestudeerd. We hebben een significant verminderde differentiatie (ontwikkeling) en mineralisatie van de HCQ-behandelde osteoblasten aangetoond in vergelijking met de controles. Verder hebben we de mogelijke werkingsmechanismen van HCQ op osteoblasten onderzocht door middel van microarray analyse en PCR validatie. Hieruit bleek dat zowel de cholesterol biosynthese pathway als de lysosomale pathway significant actiever waren in de 5 μg/ml HCQ behandelde cellen in vergelijking met de controles. Gebaseerd op deze bevindingen speculeren we dat of 1) HCQ een direct positief regulerend effect heeft op de cholesterol synthese, of 2) HCQ veroorzaakt een intracellulaire cholesterol verlaging wat indirect leidt tot toename van de cholesterol synthese of toename van de cholesterol opname. In de literatuur is beschreven dat simvastatine (SIM, een cholesterol synthese remmer) gunstige effecten heeft op de osteoblast differentitatie en mineralisatie. Om deze reden hebben we onderzocht of SIM de effecten van HCQ teniet kan doen. Echter, we hebben ontdekt dat SIM de osteoblast differentitatie en mineralisatie significant verlaagd. Tevens was de combinatie van SIM en HCQ behandeling vergelijkbaar met alleen HCQ behandeling. We speculeren dat HCQ-geïnduceerde lysosomale membraan permeabilisatie een mogelijke verklaring is voor de verminderde osteoblast ontwikkeling en activiteit. Om de osteoblast functie te herstellen worden de cholesterol biosynthese pathway en de lysosomale pathway geactiveerd als een compensatiemechanisme. Uit onze resultaten kunnen we concluderen dat het positieve effect van HCQ op

de BMD niet kan worden verklaard door een stimulerend effect van HCQ op de osteoblast. In **hoofdstuk 6** hebben we de effecten van HCQ op menselijke osteoclasten *in vitro* en *in vivo* bestudeerd. We hebben 63 patiënten met rheumatoïde arthritis (RA) zes maanden behandeld met HCQ of methotrexaat (MTX). We vonden dat de serum marker β-CTx (een botresorptie marker) significant was verlaagd na zes maanden HCQ behandeling, in vergelijking met de start van behandeling. Dit was niet het geval bij de MTX behandelde patiënten. Aangezien HCQ en MTX de ontstekingsreactie verminderen kan de daling van β-CTx verklaard worden doordat er minder bot door de ontsteking wordt geresorbeerd. Er was geen associatie tussen de HCQ-geïnduceerde daling van β-CTx en daling van CRP, wat suggereert dat HCQ een direct remmend effect heeft op de botresorptie. In tegenstelling tot HCQ, was de daling van CRP MTX wel geassocieerd met een daling van β-CTx. Daarnaast hebben we aangetoond dat osteoclasten na HCQ behandeling significant minder bot resorberen in vergelijking met de controles in vitro. Verder vonden we dat HCQ behandeling van osteoclasten leidt tot een significant lagere intracellulaire pH en een toename van cholesterol opname in vergelijking met de controles. Deze observaties worden ook beschreven in cellen met LMP. LMP kan resulteren in een verminderde botresorptie doordat de benodigde protonen en lysosomale enzymen minder naar de resorptie pit worden getransporteerd. Onze hypothese is dat HCQ-geïnduceerde LMP leidt tot verminderde botresorptie. Gebaseerd op deze bevindingen denken we dat de kwaliteit van het bot van patiënten met een verhoogd risico op osteoporose (botontkalking) en botbreuken, dus ook postmenopauzale vrouwen en patiënten met een inflammatoire ziekte zoals pSS en RA, kan worden verbeterd doordat HCQ botverlies helpt voorkomen.



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List of abbreviations

ACR	American College of Rheumatology
AE-1	Chloride-bicarbonate cotransporter
AECG	American-European consensus group
AIH	Autoimmune hepatitis
AIT	Autoimmune thyroiditis
ALP	Alkaline phosphatase
AMCL	Ammonium chloride
ANA	Anti-nuclear antibodies
ANOVA	one-way analysis of variance
AQP1	Aquaporin 1
BAFF	B cell activating factor
BAP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BTM	Bone turnover marker
CAII	Carbonic anhydrase type 2
CI	Confidence interval
CRP	C-reactive protein
CT	Computed tomography
CTSK	Cathepsin K
DMARDs	Disease-modifying antirheumatic drugs
dRTA	Distal renal tubular acidosis
DXA	Dual-energy X-ray absorptiometry
ENaC	Epithelium Na^+ channel
ERF	Erasmus Rucphen Family
ESSDAI	EULAR Sjögren syndrome Disease Activity Index
FF	Furosemide and fludrocortisone
FN	Femoral neck
GO	Gene ontology
GWAS	Genome-wide association studies
HCQ	Hydroxychloroquine

HDL	High density lipoprotein
HMCGR	3-hydroxy-3-methylglutaryl-CoA reductase
hMSCs	Human mesenchymal stromal cells
IFN	Interferone
IL	Interleukin
ILD	Interstitial lung disease
LDL	Low density lipoprotein
LDLR	LDL receptor
LMP	Lysosomal membrane permeabilization
LS	Lumbar spine
MALT	Mucosa-associated lymphoid tissue
MFI	Multidimensional fatigue inventory
MMP	Mitochondrial membrane permeabilization
MS	Multiple sclerosis
MTX	Methotrexate
NaPi	Sodium-phosphate cotransporter
NBCe-1	Sodium-bicarbonate cotransporter
NH_3	Ammonia
NH_4^+	Ammonium
NHE3	Na^+-H^+ exchanger isoform 3
NKCC2	Na^+-K^+-($2Cl^-$) cotransporter
NSIP	Nonspecific interstitial pneumonia
NTX	N-terminal crosslinking telopeptide of type I collagen
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PI3K	Phosphatidylinositol 3-kinase
PINP	N-terminal propeptide of type I procollagen
pSS	Primary Sjögren syndrome
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROMK	Ba^{2+}-sensitive K^+ channels

SEM	Standard error of the mean
SIM	Simvastatin
SLE	Systemic lupus erythematosus
SMR	Standardized mortality ratio
Th1	T-helper 1 cell
Th17	T helper 17 cell
TLRs	Toll-like receptors
TRAP	Tartrate-resistant acid phosphatase
Treg	Regulatory T cells
ß-CTx	Beta C-terminal telopeptide

Curriculum Vitae

Tim Both was born on November 1st, 1989 in Gouda. After graduating from high school in 2008 (Gymnasium Beta, Goudse Scholengemeenschap Leo Vroman, Gouda) he entered Medical School at the Medical Faculty, Erasmus University in Rotterdam. In 2010 he started at the Department of Internal Medicine, division of clinical immunology, under supervision of Dr. P.L.A. van Daele with literature research concerning fertility problems in colchicine-treated FMF patients. This research led to his first publication in Nederlands Tijdschrift van Geneeskunde.

In 2011 he started with his PhD research described in this thesis at the Department of Internal Medicine, division of clinical immunology, at the Erasmus Medical Centre under supervision of Prof. dr. P.M. van Hagen and Dr. P.L.A. van Daele. In January 2016, Tim graduated from Medical School and continued his PhD research at the Bone and Calcium Laboratory of the Department of Internal Medicine at the Erasmus Medical Centre under supervision of Dr. B.C.J. van der Eerden.

In September 2016 he started working at Ikazia Hospital in Rotterdam as resident Internal Medicine. In January 2017 he began with his specialty training in Internal Medicine in Reinier de Graaf Gasthuis at Delft.

Dankwoord

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Onderzoek doen en artikelen schrijven doe je niet alleen en daarom wil ik alle co-auteurs bedanken die de afgelopen jaren hebben meegeschreven. Een aantal wil ik specifiek benoemen. Beste **Prof. dr. Hoorn**, beste **Ewout**, graag wil ik ook jou bedanken voor je hulp bij het analyseren van de data en schrijven van mijn eerste original article. Verder wil ik je bedanken voor je hulp bij het schrijven van de aanvraag voor de toegekende subsidie bij de nierstichting. Beste **Dr. Versnel**, beste **Marjan**, bedankt voor je kritische blik op de immunologische stukken van het proefschrift. Beste **Dr. Dalm** en **Dr. van Laar**, beste **Virgil** en **Jan**, bedankt voor het meeschrijven aan de stukken maar zeker ook voor de gastvrijheid en gezelligheid tijdens mijn verblijf op de D-vleugel. Verder wil ik jullie bedanken voor het meekijken en meedoen op de poliklinieken, ik heb hier veel van de klinische immunologie geleerd. Beste **Prof. dr. van Leeuwen**, beste **Hans**, bedankt voor de besprekingen waarbij weer met een frisse blik naar de vergaarde data werd gekeken resulterend in nieuwe experimenten. Beste **Dr. van de Peppel**, beste **Jeroen**, bedankt voor het helpen met schrijven van het laatste stuk voor het boekje. Jouw hulp bij het uitvoeren van de microarray en vervolgens de analyses van de data was onmisbaar. Verder wil ik **Dr. Lam** uit het Sint Franciscus Gasthuis en **Dr. Weel** uit het Maasstad ziekenhuis bedanken voor hun hulp bij het rekruteren van patiënten en het nakijken van het manuscript.

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List of publications

1. Both, T., van Laar, J. A. M., Bonte-Mineur, F., van Hagen, P. M. & van Daele, P. L. A. [Colchicine has no negative effect on fertility and pregnancy]. Ned. Tijdschr. Geneeskd. 156, A4196 (2012).

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5. Both, T. et al. Hydroxychloroquine affects bone resorption both in vitro and in vivo. J. Cell. Physiol. (2017). doi:10.1002/jcp.26028

6. Both, T. et al. Hydroxychloroquine decreases human MSC-derived osteoblast differentiation and mineralization in vitro. J Cell Mol Med. Accepted in 2017

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PhD PORTFOLIO SUMMARY

Name of PhD student: T. Both	PhD period: January 2011 – November 2017	
Erasmus MC Department: Internal Medicine	Promotor: Prof. dr. P.M. van Hagen	

1. PhD training		
	Year	**Workload Hours**
General academic skills		
Basic course Organization Clinical research	2012	20
Research Integrity	2016	8
Research skills		
Biostatistical Methods I: Basic Principles [CC02]	2013	40
In-depth courses (e.g. Research school, Medical Training)		
Masterclass on Future Therapeutics in Immune-Mediated Diseases - *IMID*	2012	36
Molecular Immunology – *EMC*	2013	24
Presentations		
Distal renal tubular acidosis in primary Sjögren syndrome - *IMID*	2012	Poster
Distal renal tubular acidosis in primary Sjögren syndrome - *Wetenschapsdagen*	2013	Poster
Distal renal tubular acidosis in primary Sjögren syndrome - *research meeting immunology*	2013	Oral
Distal renal tubular acidosis in primary Sjögren syndrome	2014	Poster
Bone mineral density in Sjögren syndrome patients with and without distal renal tubular acidosis - *Wetenschapsdagen*	2016	Poster
Bone mineral density in Sjögren syndrome patients with and without distal renal tubular acidosis – *ECTS*	2016	Poster
Beyond the dryness – *Reference meeting clinical immunology and allergology, EMC*	2016	Oral
Meer dan alleen droogteklachten - *Research meeting Internal Medicine, EMC*	2016	Oral
Bone involvement in pSS patients: effects of HCQ - *Research meeting Calcium and Bone lab and Orthopedics lab, EMC*	2016	Oral
Sjögren syndroom: meer dan alleen droogte – *Annual patients meeting NVSP*	2016	2016
International conferences		
Seventh European Workshop on Immune-Mediated Inflammatory Diseases Noordwijk aan Zee, Netherlands	2012	20
Ninth European Workshop on Immune-Mediated Inflammatory Diseases, Amsterdam, Netherlands	2015	20
Wetenschapsdagen Antwerpen, Belgium	2012	16
Wetenschapsdagen Antwerpen, Belgium	2016	16

Benlysta Sjogrens Syndrome Investigator Meeting Barcelona, Spain	2016	16
European Calcified Tissue Society Congress Rome, Italy	2016	32
American College of Rheumatology Congress Washington DC, USA	2016	32

Seminars and workshops		
Disturbances in elektrolyte regulation Course	2012	8
Symposium sarcoïdosis & IPF	2012	8
National Sjögren Patient Society – Nomination Sjögren Award	2013	8
National Sjögren Patient Society		8
Winterschool Kidney foundation	2014	36
Allergology and Clinical Immunology Congress	2014	8
National Sjögren Patient Society - Nomination Sjögren Award	2016	8

2. Teaching activities

Lecturing		
Renal involvement in primary Sjögren syndrome (oral at Research Master Infection and Immunity)	2014	8
Extraglandular manifestations of primary Sjögren syndrome (oral at Research Master Infection and Immunity)	2015	8
Bone and renal involvement in primary Sjögren syndrome (oral at Research Master Infection and Immunity)	2016	8
Diagnosis of thyroid disorders (Medical students)	2016	8