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Exploring gout inflammatory pathways and potential novel treatment targets

Maartje C.P. Jacobs-Cleophas
Colofon

Exploring gout inflammatory pathways and potential novel treatment targets

The work presented in this thesis was carried out within the Radboud Institute for Molecular Life Sciences and the department of Experimental Internal Medicine, Radboud University Medical Center, Nijmegen, the Netherlands.

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Exploring gout inflammatory pathways and potential novel treatment targets

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General introduction, aim and outline of this thesis
CHAPTER 1

GENERAL INTRODUCTION

A historic perspective of gout

Gout has been known as a chronic debilitating disease for millennia. In 1859, the English physician Alfred Baring Garrod wrote (1): “Gout is a malady fairly entitled to boast of its great antiquity, for it was probably one of the earliest diseases to which flesh became heir when man began to participate in the luxuries of civilized life.” It was first identified by the ancient Egyptians in 2640 BCE (2) and many medical doctors and scientists have later described its excruciating pain and depicted it satirically in illustrations and paintings (Figure 1). In the fifth century BCE, Hippocrates described gout as “the unwalkable disease” (3). Sydney Smith (1771-1845), an English wit, writer and Anglican cleric, described gout itself as “that toe-consuming tyrant” and mentioned that the pain of gout was as if he was “walking on his eyeballs” (4,5).

Figure 1. The introduction of gout
A painting by George Cruikshank from 1818 depicting a wealthy man indulging in foods and drinks, while a little devilish figure is about to introduce him to the pain of acute gout (6).

The word gout is derived from the Latin word gutta, meaning drop, which originates from the humoral model of health and disease. This conception was first described in Hippocratic writings and remained influential until the eighteenth century. It describes that in health there is a balance of fluxes between the four fluids in the body: blood, choler (yellow bile), phlegm, and melancholy (black bile). In gout, it was thought that a morbid fluid would be distilled into the joint, drop by drop, and cause disease (1). The Hippocratic writings already describe gout as the result of a sedentary lifestyle and an excessive diet, causing ‘indigestion of the humours’ (5).

Since its origin, people have put their trust in therapies for gout. In Ancient Greek times it was already known that gout could be managed by a proper diet. Claudius Galenius, a medical doctor, would prescribe bland, easily digestible foods, such as barley bread, cabbage and vegetable soup. Fruits were only to be consumed in moderation. Another form of therapy, based on the humoral model, was the depletion of the morbid humours by means of vomiting, purging, diuresis or phlebotomy (5). The first long-lasting breakthrough in the treatment of gout originates from as early as as early as the 6th century: Alexander of Tralles, an Ancient Greek physician, advised the use of hermodactyl, which was derived from the plant Colchicum
**General introduction and outline of this thesis**

Interestingly, this is still widely used today for the treatment of acute gout in the form of colchicine (3,5).

Although people have been familiar with gout since the time of Ancient Egypt, the first known medical description of gout originates from a much later time. Thomas Sydenham, often referred to as the English Hippocrates (1), described gout as follows in his ‘Treatise of the gout and dropsy’ (7) in 1683: “The patient goes to bed, and sleeps quietly, till about two in the morning, when he is awakened by a pain, which usually seizes the great toe, but sometimes the heel, the calf of the leg, or the ankle. The pain resembles that of a dislocated bone, and ... is mild in the beginning, but grows gradually more violent every hour, and ... becomes so exquisitely painful, as not to endure the weight of the clothes, nor the shaking of the room from a person’s walking briskly therein.”

In 1679, only just before the treatise on gout by Sydenham, Antoni van Leeuwenhoek described the microscopic appearance of the chalk-like substance from a gouty tophus (8): “First of all I observed the solid matter which to our eyes resembles chalk, and saw to my great astonishment that I was mistaken in my opinion, for it consisted of nothing but long, transparent little particles, many pointed at both ends and about 4 ‘axes’ of the globules in length...”

A Swedish chemist, Scheele, was the first to identify the chemical structure of uric acid in crystals of a renal calculus in 1776 (3). It was not until the 19th century, however, that we learned more about the pathogenesis of gout. Sir Alfred Baring Garrod published his elaborate work ‘The Nature and Treatment of Gout and Rheumatic Gout’ in 1859, in which he describes a method for detecting and roughly quantifying uric acid in the blood. In addition, he states that the blood of gout patients is rich in ‘urate of soda’ in crystalline form, Furthermore, he states that “the deposited urate of soda may be looked upon as the cause, and not the effect, of the gouty inflammation” and that “the inflammation of gout tends to the destruction of the urate of soda in the blood of the part, and consequently of the system generally” (1).

**Epidemiology of gout**

Today, gout can no longer be described as an ailment of the elite. As was satirically stated in Punch magazine in 1964 (9): "In keeping with the spirit of more democratic times, gout is becoming less upper-class and is now open to all ... It is ridiculous that a man should be barred from enjoying gout because he went to the wrong school". Gout poses a large burden on the world’s population, currently affecting 1-4% of adults in Western society (10-12). It is known to affect men in particular and it is strongly associated with metabolic syndrome, including abdominal obesity, hypertension, and dyslipidemia, as well as with chronic kidney disease and type 2 diabetes (10,13-15).

Characteristic for gout are the recurrent and debilitating attacks of joint inflammation. The biggest risk factor for the development of gout is hyperuricemia. Urate is the breakdown product of purines, which are important building blocks of nucleic acids. Interestingly, humans are particularly prone to developing hyperuricemia compared to other species due to an inactive form of the uricase enzyme. In all other mammals except certain primates, uricase is responsible for breaking down uric acid into a more soluble end product, allantoin. The inactivation of uricase dates back millions of years to a series of genetic mutations, which
gradually reduced its activity in primates. Eventually, pseudeogenization events rendered the gene completely inactive (16).

Due to the non-functional uricase enzyme, humans inherently have higher levels of circulating urate in the blood. Hyperuricemia is defined as a urate concentration above 6.8 mg/dL. At this concentration, it saturates the blood and starts to precipitate in the form of monosodium urate (MSU) crystals (17). These crystals, which Garrod referred to as ‘urate of soda’ have long been known to be the direct cause of acute gouty arthritis (1). The presence of MSU crystals is the biological culprit for inducing the inflammatory response of acute gout. Therefore, hyperuricemia is a risk factor but in itself is not sufficient to cause gout. In fact, many people remain hyperuricemic asymptomatically for a large part of their lives. Several studies have shown that approximately 3-36% of hyperuricemic individuals developed gout within follow-up periods ranging from 5-10 years (18-21). Conversely, some patients show normal urate levels during an acute gout attack (22).

Besides the loss of uricase many other factors can influence urate levels and increase the risk of developing gout. One of these has been known since the time of Hippocrates: the influence of diet. Mainly foods rich in purines, particularly meat, seafood, beer and liquor, are known to elevate urate levels (23,24). Additionally, fructose has long been known to increase urate levels (25). With current soft drinks being mostly sweetened with fructose, it was also found that these products increase urate levels, whereas diet soft drinks did not (26,27). In contrast, dairy products and coffee have been shown to reduce urate levels (27-29).

Although hyperuricemia can be caused by overproduction, elevated urate levels are for the largest part caused by lower-than-normal excretion (30,31). This is also reflected by the single nucleotide polymorphisms (SNPs) that have been found to be associated with hyperuricemia and gout. Several genome-wide association studies combined currently identified 26 genome-wide significant SNPs associated with serum urate levels (32-37). The two SNPs most significantly associated with serum urate concentrations are located in genes coding for urate transporters, SLC2A9 and ABCG2. Together these SNPs account for an estimated 3.4% of the serum urate variance (35). SLC2A9 encodes the GLUT9 protein and is expressed in the apical and basolateral membranes of the proximal renal tubule. ABCG2 is expressed in the apical membrane of the proximal renal tubule, but is also abundantly present in the apical membrane of the small intestine endothelial cells (38).

Pathogenesis of acute gout

Although MSU crystals have been recognized as being the culprit of acute gouty arthritis for a long time (39), only little over 10 years ago it was found that MSU crystals induce gouty arthritis through activation of the NOD-like receptor pyrin domain-containing-3 (NLRP3) inflammasome (40), a protein scaffold that activates the caspase-1 enzyme. This was a finding of major importance, as caspase-1 in turn cleaves the inactive pro-interleukin-1β (pro-IL-1β) to active IL-1β, the crucial cytokine in the induction of gouty arthritis (Figure 2). The importance of IL-1β in gout has since been confirmed in several in vivo studies in which IL-1β was blocked, either by the recombinant IL-1 receptor antagonist (IL-1Ra) protein anakinra or by the anti-IL-1β monoclonal antibody canakinumab (41,42).
Pro-IL-1β is not constitutively expressed and therefore a first signal is required to induce transcription of the \textit{IL1B} gene. This signal can be provided by Toll-like receptor (TLR) ligands, such as \textit{E. coli}-derived lipopolysaccharide (LPS, TLR4 ligand) or Pam3CSK4 (TLR2 ligand), but also long-chain saturated fatty acids \textsuperscript{(43,44)}. In combination with MSU crystals and the subsequent activation of caspase-1, that represents the second signal leading to post-translational processing, this induces secretion of active IL-1β that acts on the IL-1 receptor (Figure 2). After the initial release of active IL-1β, other cytokines and chemokines are produced during the inflammatory response. Importantly, large numbers of neutrophils are recruited to the inflamed joint, where they sustain the inflammation and cause damage to surrounding tissues.

\textbf{Figure 2. Schematic overview IL-1β production: two-signal cascade}

For monosodium urate (MSU) crystals to induce active IL-1β, two signals are required. Signal 1 is a signal leading to the transcription of the pro-IL-1β gene. This signal can be provided by for example Toll-like receptor (TLR) 2 or TLR4 ligands, and by active IL-1β binding to the IL-1 receptor (IL-1R). The second signal, provided by the MSU crystals, leads to the assembly of the NLRP3 inflammasome and activation of caspase-1, and subsequently post-translational processing of the inactive pro-IL-1β into the active cytokine. A positive feedback loop is set up via excretion and binding to the IL-1R.
Currently recommended pharmacological treatment options for gout

There are many effective treatment options available for gout. In fact, gout is the only chronic form of arthritis that is curable. Its treatment mainly exists out of lowering serum urate levels below the target concentration of 6.0 mg/dL (45). Within urate lowering therapy (ULT) there are several drug options. The xanthine oxidase inhibitor allopurinol is the recommended first-line pharmacological treatment (45). As an alternative, in case of ineffectiveness, contraindication or intolerance, the xanthine oxidase inhibitor febuxostat may be used (45,46). Furthermore, in case of insufficient urate lowering effect of allopurinol alone, a uricosuric agent (such as benzbromarone or probenecid) in combination with allopurinol may be prescribed (45). Uricosuric agents are also recommended as monotherapy when there are contraindications or intolerance to at least one of the xanthine oxidase inhibitors (46). ULT could be started during an acute gout attack (alongside anti-inflammatory management) (46), and is indicated in patients with recurrent flares, tophi, other forms of urate arthropathy and/or the presence of renal stones (45). All ULTs are started at a low dose and are gradually titrated upwards under regular monitoring of serum urate levels (45,46).

Aside from urate-lowering therapy, there are several options to treat and prophylactically prevent acute gout flares. Flares should be treated as early as possible with colchicine, non-steroidal anti-inflammatory drugs (NSAIDs), oral corticosteroids, or articular injection of corticosteroids. In addition, gout attack prophylaxis in the form of low-dose colchicine or low-dose NSAIDs is recommended during the first 6 months of ULT (45,47), as ULT can lead to transiently increased serum urate levels through the dissociation of MSU depositions and an increased risk of gout flares. In patients with recurring flares and intolerance or contraindications to colchicine, NSAIDs and corticosteroids, IL-1 blocking agents can be considered to treat gout flares (45,47). Furthermore, an option for patients with severe refractory gout with a poor quality of life, in whom target serum urate cannot be reached with any of the other available ULTs, is pegloticase, a pegylated form of the uricase enzyme (45). With these available therapies, we should be able to reach target serum urate levels and prevent gout flares in the majority of the patients.

However, despite our relatively extensive knowledge on the pathophysiology of gout and the many effective treatment options, gout management remains suboptimal (11). A systematic review of several studies on gout management showed that generally the proportion of gout patients on ULT was low and that serum urate level was not monitored in most patients (48). In addition to poor adherence to ULT, another difficulty in gout management is the fact that many of the comorbidities associated with gout are known contraindications for its first-line treatment options, such as allopurinol, colchicine, glucocorticoids and NSAIDs (49). Finally, comorbidities and concomitant medications used in the general gout patient population increase the risk of adverse drug reactions in many of the anti-gout therapies (50,51). These considerations warrant the need for new inexpensive targeted treatment options that are well tolerated by the general gout patient population.

A role for histone deacetylases in inflammation

Histone deacetylases (HDACs) are enzymes that reversibly change the epigenetic landscape by removing acetyl groups from lysine residues on histones. The opposite action of transferring acetyl groups from acetyl-CoA to lysine residues is provided by histone acetyl transferases
(HATs). By modulating the level of acetylation on histones, the chromatin structure is altered and gene expression is regulated. Overall, the general consensus is that acetylation is an activation mark, leading to increased gene expression. Acetylation loosens up the way the DNA is wound around the histone proteins, making promoter and enhancer regions more accessible to transcription factors (Figure 3) (52).

HDACs are divided up into two families and four classes. Firstly, there is the classical histone deacetylase family, which consists of 11 members divided over three classes. The other family of HDACs is the Sir2 regulator family, consisting of seven sirtuins (SIRTs), which are grouped in the class III HDACs. The classical HDAC family is divided into three classes based on their sequence homology to yeast proteins (Figure 3) (52). Here, we will focus on this classical HDAC family.

Although histones were the first identified substrates for HDACs, we now know that HDACs can target many different proteins. In a study from 2009 by Choudhary and others, 3600 acetylation sites were identified on 1750 proteins. These proteins were mostly annotated as nuclear or mitochondrial, but also a vast number of cytoplasmic proteins were identified (53). In fact, many of the acetylation sites were identified in proteins important in several essential cellular processes, such as RNA splicing, cell cycle regulation, nuclear transport and tubulin formation (53). This shows that HDACs have widespread cellular effects besides chromatin remodeling. However, further research on substrate specificity and functionality of the specific classical HDACs has proven to be difficult. In particular the class I HDACs often function together in large multiprotein corepressor complexes and show functional redundancy to some extent (52).
The first identified HDAC inhibitor was n-butyrate, a short-chain fatty acid present in the gut. However, it is only effective at high concentrations and induced many other nonspecific effects. Another HDAC inhibitor, trichostatin A (TSA) was isolated from a Streptomyces strain and showed inhibition of HDACs at low nanomolar concentrations (54). Not much later, two other important HDAC inhibitors were discovered, suberoylanilide hydroxamic acid (SAHA, or vorinostat) and FK228 (romidepsin) (52,55,56). In 2006, vorinostat was FDA-approved for treatment of cutaneous T-cell lymphoma. The approval of romidepsin for the same indication followed in 2009 (52). Most well-known anti-cancer effects of HDAC inhibitors are their ability to induce cell cycle arrest and apoptosis (57). A theory of Dawson and Kouzarides proposes that certain cancer types show ‘epigenetic vulnerability’. They hypothesize that certain cancer cells rely on epigenetic regulators such as HDACs for maintaining the expression of genes involved in their survival and proliferation, unlike normal healthy cells (58).

Shortly after the discovery of the first HDAC inhibitors came the first reports of their anti-inflammatory effects. Interestingly, the concentrations required to achieve anti-inflammatory effects with HDAC inhibitors are much lower than those needed for anti-cancer effects (59). The main observed effects in numerous reports were suppression of cytokine and nitric oxide production, inhibition of inflammatory transcription factors such as NF-kB and STAT, and decreased proliferation and differentiation of immune cells (60). More recently, HDAC inhibitors were shown to possess anti-rheumatic effects. HDAC inhibition suppressed inflammatory cytokine production ex vivo in rheumatoid arthritis fibroblast-like synoviocytes and peripheral blood mononuclear cells (PBMCs) (61–63). Furthermore, several studies showed anti-rheumatic effects of HDAC inhibition in well-established experimental arthritis models in mice (64,65). The orally active pan-HDAC inhibitor givinostat (ITF2357) was even shown to be safe in healthy volunteers (66) and decreased disease activity in children with juvenile idiopathic arthritis (67). These studies provide a rationale for the potential use of HDAC inhibitors in chronic auto-inflammatory and arthritic diseases.

AIM AND OUTLINE OF THIS THESIS

In the present work, the aim was to investigate in the first place the anti-inflammatory effects of HDAC inhibitors in monosodium urate crystal-induced cytokine responses. In addition, several factors influencing gout susceptibility were studied.

In Chapter 2, the current knowledge and recent discoveries on factors affecting the immune response in acute gouty arthritis flares is reviewed. Chapter 3 goes deeper into the mechanistic role of soluble uric acid as a danger signal and an additional risk factor for gout susceptibility, independent of its formation into MSU crystals.

In Chapter 4, the potential of using HDAC inhibitors to fight acute gout is introduced. The effects of the broad-acting HDAC inhibitor sodium butyrate on MSU-induced cytokine production in PBMCs from healthy volunteers and gout patients were investigated. In addition, we aimed to identify the specific HDAC enzymes playing a role in the MSU-induced inflammatory response in vitro. In Chapter 5, the effects of 4-week oral butyrate supplementation on the inflammatory potential of circulating PBMCs and monocytes were assessed in healthy volunteers and obese insulin resistant males. Chapter 6 explores the further possibilities of using highly specific and
potent synthetic HDAC inhibitors to suppress MSU-induced cytokine production. Furthermore, we attempted to elucidate the mechanism by which inhibition of HDAC1 and HDAC2 suppresses MSU-induced cytokine production in vitro.

Chapter 7 reviews the current knowledge on ABCG2 polymorphisms in gout and how it increases disease susceptibility. Furthermore, we discuss the Q141K polymorphism and its role as a risk factor for gout, independent of hyperuricemia, and go deeper into the cellular mechanistic aspects of a defective ABCG2 transporter. Finally, chapter 8 introduces a novel copy number variable region influencing the risk of gout.
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General introduction and outline of this thesis


Factors modulating the inflammatory response in acute gouty arthritis

Maartje C.P. Cleophas, Tania O. Crișan, Leo A.B. Joosten

Current Opinion in Rheumatology 2017; 29(2): 163-170
CHAPTER 2

ABSTRACT

Gout is a common debilitating form of arthritis and despite our extensive knowledge on the pathogenesis its prevalence is still rising quickly. In the current review, we provide a concise overview of recent discoveries in factors tuning the inflammatory response to soluble uric acid and monosodium urate crystals.

It appears that soluble uric acid has a much larger role to play than just being a risk factor for gout. It may have widespread consequences for systemic inflammation and the development of metabolic syndrome. Additionally, a specific gout-related gut microbiome might not only provide us with a new diagnostic tool, but also highlights possible new therapeutic targets.

Furthermore, several recent publications further elucidated the roles of mitochondrial dysfunction, production of reactive oxygen species, autophagy, and AMP-dependent protein kinase in monosodium urate-induced NLRP3 inflammasome activation. Finally, neutrophils have been shown to be involved in both the promotion and resolution of gouty inflammation. A new alpha-1-antitrypsin fusion protein may limit the pro-inflammatory effects of neutrophil-derived serine proteases.

Together, these studies provide us with many new insights in the pathogenesis of gout as well as important new treatment targets, and a rationale to further study the role of soluble uric acid in inflammatory diseases.
INTRODUCTION

Acute gout is an ancient form of arthritis characterized by debilitating joint inflammation, commonly occurring in the first metatarsophalangeal joint. Despite the long history and the known pathogenesis of gout, its prevalence is rising at an alarming rate and currently affects 1-4% of the population in North America and Europe (1, 2).

Essential for the development of gouty arthritis is the deposition of monosodium urate (MSU) crystals, which form when the blood is saturated with uric acid. MSU crystals act as a danger signal, activating the nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain-containing 3 (NLRP3) inflammasome (3). This activates caspase-1, an enzyme capable of converting pro-interleukin-1β (pro-IL-1β) to mature IL-1β by proteolytic cleavage. Only in the presence of a secondary stimulus, like Toll-like receptor ligands or free fatty acids, pro-IL-1β is produced and can promptly be activated via cleavage by caspase-1 (4). The subsequent release of bioactive IL-1β is the key event for the development of acute gouty arthritis.

Although our knowledge on its pathogenesis has expanded tremendously in the past decades, gout is still gaining ground and it is essential to get more insight into this complex metabolic disease. In this review, we discuss the recent advances and new viewpoints in the field of gout, focusing on factors such as: the role of soluble uric acid; the effects of diet, gut microbiota and metabolites; and the intra- and extracellular responses in acute gouty inflammation.

NEW INSIGHTS INTO URICASE LOSS

The culprit for the formation of MSU crystals is hyperuricemia. The largest contribution to the elevated susceptibility to hyperuricemia and gout in humans comes from the fact that uricase, the enzyme responsible for the breakdown of uric acid to allantoin, is inactive. Recently, Kratzer et al. demonstrated that the uricase gene was subject to several mutations, which gradually reduced its enzymatic activity, before the eventual pseudogenization events that rendered the gene completely inactive (5). Tan et al. found that these events coincided with changes in the URAT1 transporter gene (6). The URAT1 transporter that was present in our ancestors 77 million years ago was a low affinity but high capacity transporter. Around 27 million years ago, the gene had mutated stepwise to a form of URAT1 that is characterized by a high affinity for uric acid but low transport capacity. Tan et al. show that the changes in URAT1 allowed for an increase in and better control of plasma urate levels. The persistence of the mutations in uricase and URAT1 indicates that increasing serum uric acid levels was a key event for the survival of the apes.

There are multiple theories about the possible evolutionary advantage provided by the loss of uricase. Some decades ago, it was proposed that uric acid acts as a scavenger for free radicals, protecting our ancestors from oxidative stress-induced damage (7). Another hypothesis postulates that uric acid stimulated intellectual evolution due to its structural similarity to caffeine and theobromine (8). Much more recently, Johnson and Andrews proposed a new hypothesis based on Neel’s thrifty gene hypothesis (9-11). They argue that early hominoids first migrated to Eurasia during a period of global cooling and experienced periods of famine due to fruit being unavailable in the winter months. The loss of uricase may have aided the apes to turn fructose from fruits into fat tissue in order to survive these periods of scarcity. Unlike
glucose, fructose leads to accumulation of triglycerides and uric acid. Uric acid was shown to induce hepatic lipogenesis through mitochondrial stress (12). Additionally, uric acid-dependent inhibition of AMP-dependent protein kinase (AMPK) not only further inhibits beta-oxidation and stimulates fat accumulation (13), but also stimulates gluconeogenesis (14). According to Johnson and Andrews, uric acid protected ancient apes from starvation by increasing fat stores and maintaining glucose levels in the blood, but may currently underlie the metabolic syndrome pandemic.

**SOLUBLE URIC ACID: THE PRIME SUSPECT**

Although many studies highlighted a strong association between uric acid and metabolic syndrome (15-17), there is also evidence in support of causative role of soluble uric acid. Liu et al. assessed the effects of allopurinol in addition to conventional treatment in hyperuricemic type II diabetes patients. They found that allopurinol treatment led to a larger reduction in uric acid, triglycerides, insulin resistance, and blood pressure, and led to a more significant improvement of renal function than conventional treatment (18). In line with this, a pilot study by Takir et al. reports that lowering uric acid with allopurinol in hyperuricemic subjects decreased fasting glucose levels, insulin levels, insulin resistance, and circulating CRP (19). Madero et al. found that allopurinol treatment in overweight prehypertensive subjects resulted in lower systolic blood pressure compared with a placebo group (20). These results suggest that uric acid may cause systemic inflammation and play a role in the development of obesity, diabetes and renal insufficiency. However, there is a need for bigger and longer prospective trials with urate lowering therapy to confirm this.

The effects of soluble uric acid on systemic inflammatory responses were also studied in vitro (21). A 24-hour pre-incubation of PBMCs with soluble uric acid significantly increases IL-1β and IL-6 production in response to Toll-like receptor ligands alone and in combination with monosodium urate crystals. This coincides with a significant decrease in IL-1Ra production, the endogenous antagonist of IL-1β. The pro-inflammatory effects of uric acid could be restored with 5'-methylthioadenosine (MTA), a histone methyltransferase inhibitor, indicating that the responses are likely to be epigenetically regulated (Figure 1). Importantly, this study provides a first mechanistic insight into the immune-modulatory properties of soluble uric acid that could potentially link hyperuricemia to metabolic and autoimmune diseases.

**GUT MICROBIOME AND METABOLITES**

Recently, a study by Guo et al. identified an altered microbiome in gout patients in China (Figure 1) (22). With a model based on the 17 microbe genera that were differentially represented in the gut of gout patients, gout could be predicted in a validation cohort with an accuracy of 88.9%. This could represent a future tool for the early diagnosis of gout, although validation in bigger cohorts and in different populations is a first necessity.

Interestingly, Guo et al. report that the gout patient gut microbiome is characterized by a significantly impaired microbial butyrate synthesis. Underrepresentation of butyrate-producing bacteria in the gut microbiome has been observed previously in type 2 diabetes patients (23,
Factors modulating the inflammatory response in acute gouty arthritis

24) and beneficial effects of butyrate have been shown with monosodium urate stimulation experiments in vitro (25). Butyrate is a potent inhibitor of class I histone deacetylases (HDACs) and inhibits monosodium urate-induced cytokine production in PBMCs from healthy donors and crystal-proven gout patients (25). We envisage, however, that the potential use of sodium butyrate for treatment of systemic inflammatory diseases such as gout is limited due to the high concentration needed for HDAC inhibition. Similar cytokine-suppressive effects were observed with more specific synthetic HDAC inhibitors at low dose (25), which may represent a more targeted treatment option. Therefore, there is a need for the development of safe, highly specific, and orally active histone deacetylase inhibitors.

Vieira et al. used a murine model to assess the effects of the gut microbiome, high fiber diet, and short-chain fatty acids in gouty arthritis. Interestingly, they observed that the inflammatory response to an intra-articular injection of MSU crystals was dependent on microbial production of short-chain fatty acids in the gut. Adding acetate to the drinking water of germ-free mice restored this response. In addition, in vitro production of reactive oxygen species (ROS), caspase-1 activation and IL-1β secretion was dependent on GPCR43, a short-chain fatty acid receptor with highest affinity for acetate (26). In contrast, the same group reported that treatment with oral acetate, propionate and butyrate in mice promoted resolution of MSU-induced inflammation by inducing neutrophil death in the joints. Acetate was shown to accelerate caspase-mediated neutrophil apoptosis in a GPCR43-dependent manner (27). These studies indicate that gut microbiome and short-chain fatty acids are of vital importance in tuning the inflammatory response to MSU-crystals in mice. It remains to be determined how this translates to the human immune responses.
Figure 1. Intracellular effects of soluble uric acid and monosodium urate (MSU) crystals

Genetic mutations in uricase and URAT1, as well as current purine- and fructose-rich Western diets dramatically increase human susceptibility to development of hyperuricemia. High levels of uric acid can induce epigenetic reprogramming of monocytes, leading to a more proinflammatory state. An altered microbiome in gout patients, which is less functional in producing short-chain fatty acids.

Uric acid potentiates the lipogenic effects of fructose, possibly contributing to development of obesity, dyslipidemia and insulin resistance.

Altered microbiome in gout patients, which is less functional in producing short-chain fatty acids.

Expression of pro-IL-1β expression can be induced through signaling via Toll-like receptors (TLR) or the IL-1 receptor (IL-1R).
**INTRACELLULAR SENSORS**

The NLRP3 inflammasome is crucial for the initial IL-1β activation in response to MSU crystals (3), although there is still much debate on the mechanism (Figure 1). Recently, Nomura et al. demonstrated that MSU crystals induce K+ efflux, leading to a loss of mitochondrial membrane potential and decreased intracellular ATP levels. This increased IL-1β production independently of MSU crystal-induced mitochondrial ROS production (28). Ives et al. found increased xanthine oxidase (XOR) activity in response to NLRP3 activators. Pharmacological XOR inhibitors or XOR knockdown inhibited MSU-induced IL-1β. This effect was dependent on cytoplasmic XOR-derived ROS as well as on mitochondrial PI3K-AKT-mTOR signaling and ROS production (29).

The role of mitochondrial ROS in MSU-induced IL-1β production is also of relevance in the context of autophagy. Zhong et al. (30) reported that NF-kB negatively regulated NLRP3 activation by increasing the expression of p62, an important autophagy receptor. Upon stimulation with NLRP3 activators, p62, LC3-II and Parkin were recruited to damaged mitochondria, initiating their clearance via autophagy (mitophagy). The authors therefore propose that NF-kB-induced p62 expression negatively regulates NLRP3 activation by inhibiting the release of DNA and ROS from damaged mitochondria. Conversely, Jhang et al. (31) proposed that p62 accumulates in case of MSU crystal-induced lysosomal disruption and impairment of autophagy. They showed that MSU crystals caused p62 to bind the kelch-like ECH-associated protein 1 (keap-1), which normally acts as a transcriptional repressor by binding to nuclear factor E2-related factor 2 (Nrf2), a transcription factor involved in oxidative stress responses. The release of Nrf2 from keap-1 facilitated its translocation to the nucleus and induced transcription of heme oxygenase-1 and superoxide dismutase, which was shown to be required for MSU-induced NLRP3 activation. Another study by Kim et al. (32) observed a positive feedback loop between MSU-induced ROS production and p62 expression, both mediating an increase in caspase-mediated apoptosis and IL-1β production. Thus, the NLRP3 inflammasome can be negatively regulated by p62 when damaged mitochondria are effectively removed by mitophagy, whereas excessive p62 can also stimulate NLRP3 activation through Nrf2-mediated transcription and ROS production.

A second intracellular sensor important for the pathogenesis of gout is AMP-activated protein kinase (AMPK, Figure 1). Wang et al. (33) recently demonstrated that MSU crystals inhibit AMPK activity and AMPKa knockout mice showed a significantly increased inflammatory response in an MSU crystal air pouch model. Furthermore, colchicine was shown to induce AMPK phosphorylation in vitro, identifying a new molecular target of this ancient medicine against gout. Although it is of importance to validate these findings in human cells and patient cohorts, they do provide a rationale for further research to explore the possibility of using AMPK activators to treat gouty inflammation.

**EXTRACELLULAR PROCESSING**

During an attack of acute gouty arthritis, enormous amounts of neutrophils are recruited to the joint. Without any control, neutrophils would sustain the inflammatory response endlessly and damage the surrounding tissues. However, even without treatment gouty arthritis is self-limiting. In a study by Cumpelik et al., neutrophil-derived phosphatidylserine-expressing ectosomes were shown to play a role in the resolution of the inflammation by binding the
phosphatidylserine receptor MerTK, and inducing expression of suppressor of cytokine signaling (SOCS) 3. Independent of MerTK, the ectosomes induced tumor growth factor β (TGF-β) secretion (34) (Figure 2).

Figure 2. Neutrophil-mediated effects in acute gouty arthritis
Recruitment of neutrophils in the first place serves to promote the inflammation. Upon formation of neutrophil extracellular traps (NETs) the serine proteases are released that can activate pro-IL-1β, which is released upon cell death. Alpha-1-antitrypsin (AAT) inhibits serine protease-mediated IL-1β activation. In contrast, in case of high density of neutrophils, their NETs aggregate and have an anti-inflammatory function by capturing and inactivating proinflammatory mediators. Neutrophils have another role in resolution of the inflammation by secreting ectosomes. These express phosphatidylserine (PS), which can bind the MerTK receptor and induce the expression of suppression of cytokine signaling 3 (SOCS3).

Another neutrophil-specific process is the formation of neutrophil extracellular traps (NETs). Schauer et al. (35) reported that NETs aggregate when there is a high density of infiltrating neutrophils, as occurs in the synovial fluid during acute gout. Besides extracellular DNA, these aggregated NETs were shown to contain proteins from neutrophil granules, such as serine proteases, which were able to degrade secreted cytokines and limit the inflammation. In contrast, when the number of neutrophils is too low to induce aggregated NETs, the process of NETosis promotes inflammation (Figure 2). Recently, it was described that NET formation in response to MSU crystals is dependent on signaling via receptor-interacting protein kinase (RIPK)-1, RIPK-3, and mixed lineage kinase domain-like (MLKL) (36). The authors conclude that these signaling molecules may be interesting new therapeutic targets to prevent proinflammatory NET formation. However, it is crucial to approach this with care as inhibiting NETosis might also exacerbate inflammation through prolonged presence of neutrophils.
Importantly, neutrophil-derived serine proteases such as proteinase-3, neutrophil elastase, and cathepsin-G can cut precursor IL-1β, which is released upon cell death, very close to the caspase-1 cleavage site. In this way, secretion of neutrophil serine proteases mediates caspase-1-independent IL-1β activation (37, 38). A natural inhibitor of neutrophil serine proteases is alpha-1-antitrypsin (AAT). Recently, we reported on the therapeutic effects of a newly developed AAT-Fc fusion protein. In vitro this compound was shown to inhibit PR3-mediated activation of pro-IL1β and increased IL-1Ra production. Moreover, human AAT transgenic mice were protected from MSU-induced gouty arthritis (39) (Figure 2). Besides treatments inhibiting caspase-1 activation, this is a very promising additional treatment option to limit caspase-1-independent IL-1β activation by infiltrating neutrophils.

**SUMMARY AND FUTURE PERSPECTIVES**

In this review, we highlighted some of the important advances in the field of gouty arthritis. New light was shed on the importance of soluble uric acid. On the basis of recent results and new theories, urate-lowering therapy may prove to be a crucial aid in preventing not only gout, but also hyperuricemia-induced renal failure, hypertension, insulin resistance, and systemic inflammation in the future. Another important finding was a gout-specific microbiome, which might represent a new tool for the early diagnosis of gout or an increased risk of gout. New studies on the role of microbiome-derived short-chain fatty acids in gout warrant further research to the role of GPR43 in humans, as well as the possible therapeutic effects of highly specific histone deacetylase inhibitors.

The first step in acute gouty arthritis is the MSU-induced intracellular activation of NLRP3 and caspase-1. This was reported to be mediated for a large part through mitochondrial damage, highlighting the importance of restoring the cellular energy balance and mitochondrial homeostasis in acute gout. Furthermore, AMPK was proposed as a potential new therapeutic target in gout. Finally, the crucial role of neutrophils was discussed. Neutrophil-derived ectosomes were found to mediate anti-inflammatory effects, whereas NETs can have both proinflammatory and anti-inflammatory properties. Neutrophil serine proteases play an important role in activating IL-1β, thus promoting the proinflammatory feedback loop. In an attempt to limit caspase-1-independent IL-1β production, a new AAT fusion protein was found to have potent effects in limiting inflammation in gout patients.

Overall, the above recent results not only provide more insight in the pathogenesis of gout, but also propose several important new treatment targets. Additionally, it is important to realize that asymptomatic hyperuricemia possibly has much wider implications than just representing a risk factor for gout.
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Soluble uric acid primes TLR-induced proinflammatory cytokine production by human primary cells via inhibition of IL-1Ra

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ABSTRACT

Objectives

The study of the proinflammatory role of uric acid has focused on the effects of its crystals of monosodium urate (MSU). However, little is known whether uric acid itself can directly have proinflammatory effects. In this study we investigate the priming effects of uric acid exposure on the cytokine production of primary human cells upon stimulation with gout-related stimuli.

Methods

Peripheral blood mononuclear cells (PBMCs) were harvested from patients with gout and healthy volunteers. Cells were pretreated with or without uric acid in soluble form for 24 h and then stimulated for 24 h with toll-like receptor (TLR)2 or TLR4 ligands in the presence or absence of MSU crystals. Cytokine production was measured by ELISA; mRNA levels were assessed using qPCR.

Results

The production of interleukin (IL)-1β and IL-6 was higher in patients compared with controls and this correlated with serum urate levels. Proinflammatory cytokine production was significantly potentiated when cells from healthy subjects were pretreated with uric acid. Surprisingly, this was associated with a significant downregulation of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra). This effect was specific to stimulation by uric acid and was exerted at the level of gene transcription. Epigenetic reprogramming at the level of histone methylation by uric acid was involved in this effect.

Conclusions

In this study we demonstrate a mechanism through which high concentrations of uric acid (up to 50 mg/dl) influences inflammatory responses by facilitating IL-1β production in PBMCs. We show that a mechanism for the amplification of IL-1β consists in the downregulation of IL-1Ra and that this effect could be exerted via epigenetic mechanisms such as histone methylation. Hyperuricaemia causes a shift in the IL-1_/IL-1Ra balance produced by PBMCs after exposure to MSU crystals and TLR-mediated stimuli, and this phenomenon is likely to reinforce the enhanced state of chronic inflammation.
INTRODUCTION

Gout is one of the oldest described rheumatic diseases which affects approximately 1% of the world’s population (1) and reaches 2.5-3.9% prevalence in developed countries (2,3). Gout is characterized by painful, recurrent and initially self-limited attacks of acute inflammation with long-term progression towards chronic tophaceous gout in some patients (4). The biological culprit of gout is represented by monosodium urate (MSU) crystals (5), which are formed during hyperuricaemia and elicit inflammatory events (4). The main proinflammatory cytokine that has been proven to strongly mediate acute gouty inflammation is interleukin (IL)-1β (6-10). MSU crystals have been associated with the activation of the NLRP3 inflammasome (7). This effect is exerted via microtubule-mediated co-localisation of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) to the site of inflammasome formation (11). Nevertheless, second signals are equally required to induce pro-IL-1β and synergise with MSU crystals (9,10) in line with the clinical situation where gouty flares are precipitated in specific environmental situations, despite continuous deposition of MSU crystals in the joint (12).

The single factor that is significantly associated with gout susceptibility and represents a necessary cause in gout development is hyperuricaemia (13). This is the elevation of serum uric acid levels above the threshold of 0.36 mM (14) when uric acid crystallisation ensues. Uric acid is the end product of purine metabolism in humans and higher primates due to the evolutionary loss of uricase activity, an enzyme that metabolises uric acid to the more soluble product allantoin (15). Recently, it has been shown that the loss of uricase activity is gradually lost during evolution, allowing adaptation to less enzymatic activity and slow rise of uric acid levels in the blood (16). Additionally, several uric acid transporters are involved in the reabsorption of approximately 90% of the total urate that has been filtered via the glomeruli (17). On the one hand, these two mechanisms have led to several hypotheses of evolutionary advantage that might be conferred by higher serum urate levels in primates (18,19). On the other hand, they make humans more prone to develop hyperuricaemia and gout. Consistently with uric acid being the major risk factor for gout, the most clear genetic associations with gout susceptibility were obtained for genetic variations in genes encoding urate transporters (20,21).

Up to now, major lines of research investigating the proinflammatory effects of uric acid have focused on MSU crystal-induced processes (22). However, emerging data suggest that uric acid in soluble form might also have proinflammatory effects. It has been observed that hyperuricaemic mice have a higher cytokine production upon lipopolysaccharide (LPS) challenge compared with control animals (23), and different studies have shown nuclear factor-kB activation in renal and pancreatic tissue of mice receiving intraperitoneal injections with uric acid (24,25).

In this study, we hypothesise that uric acid might exert proinflammatory properties by predisposing to MSU crystallisation, tissue deposition and acute inflammation, and also through a direct effect on human primary peripheral blood mononuclear cells (PBMCs). Here, we show that cells originating from patients with gout differ in their cytokine production capacity from control volunteers and that these differences correlate with serum uric acid levels. Furthermore, we describe enhanced proinflammatory cytokine production by primary cells exposed to high uric acid concentrations in vitro and show that this effect is due to downregulation of IL-1Ra, the natural antagonist of the IL-1 receptor type I.
MATERIALS AND METHODS

A detailed Materials and methods version is provided in the supplementary information of the manuscript.

**PBMC isolation and stimulation**

Peripheral blood was drawn from healthy volunteers or patients with gout (for basic parameters see supplementary table S1), after informed consent. PBMCs were separated using Ficoll-Paque and suspended in Roswell Park Memorial Institute (RPMI) culture medium. Cells were stimulated for 24 h with Pam3Cys or C16:0 with or without MSU crystals. Separately, cells were incubated for 24 h with culture medium, uric acid or allantoin (priming). After priming, culture medium was removed and remaining adherent cells were re-stimulated with medium, Pam3Cys, LPS with or without MSU crystals.

**Flow cytometry**

Cells primed for 24 h uric acid were stained with annexin V-FITC (BioVision) and propidium iodide (PI) (Invitrogen) for assessment of cell death. Fluorescence was measured using Cytomics FC500 (Beckman Coulter).

**Cytokine measurements**

Cytokine concentrations were determined using ELISA kits for IL-1β, tumour necrosis factor α (TNFα), IL-1α, IL-1Ra (R&D Systems), IL-6 and IL-10 (Sanquin).

**Quantitative-PCR**

Samples stimulated for 4 h were treated with TRIzol Reagent (Invitrogen) for total RNA purification. Isolated RNA was transcribed into complementary DNA using iScript (Bio-Rad) followed by quantitative-PCR using Sybr Green.

**Statistical analysis**

Data were analysed using Kruskal-Wallis, Mann-Whitney or Wilcoxon signed rank test according to the number of datasets and experimental design, α<0.05.

RESULTS

**Enhanced cytokine production in PBMCs of patients with gout compared with healthy volunteers**

To assess the cytokine production of PBMCs of patients with gout compared with healthy controls, 24 h stimulations were performed using MSU crystals alone or in combination with TLR2 ligands Pam3Cys and saturated fatty acid palmitate (C16:0). MSU crystals alone did not induce IL-1β or IL-6 on their own, but significantly increased the effects of Pam3Cys or C16:0 in both patients and controls (figure 1A, B). Nevertheless, the overall cytokine levels observed in
PBMCs originating from patients with gout were higher compared with controls (figure 1A, B). Of high interest, a positive relation was observed between serum uric acid levels and ex vivo IL-1β secretion in cells from patients with gout: patients with hyperuricaemia (>0.36 mM) having a higher response than patients with normouricaemia (<0.3 mM) (Figure 1C, D, and supplementary figure S1).

Figure 1. Enhanced cytokine production in cells from patients with gout compared with healthy volunteers
Freshly isolated PBMCs from patients with gout (n=19) and healthy controls (n=7) were stimulated for 24 h with Pam3Cys (10 μg/ml) or C16:0 (200 μM) in the presence or absence of MSU crystals (300 μg/ml). C16:0 vehicle (albumin and ethanol) was added to controls. Cytokine measurements were performed by ELISA for IL-1β (A) and IL-6 (B). Cytokine response differences between low (n=9) and high (n=9) uric acid in patients with gout are shown for IL-1β (C) and IL-6 (D). Data are shown as mean±SEM, Mann-Whitney test *p<0.05. IL, interleukin; MSU, monosodium urate; PBMCs, peripheral blood mononuclear cells; RPMI, Roswell Park Memorial Institute.

Uric acid pretreatment enhances proinflammatory cytokine production in primary PBMCs of healthy volunteers
In order to mimic in vitro the effects of hyperuricaemia, cells were exposed to soluble uric acid or were left untreated for 24 h. Subsequently, they were stimulated with MSU crystals in the presence or absence of TLR2 ligand Pam3Cys or TLR4 ligand LPS. Uric acid alone after 24 hours priming did not induce detectable IL-1β or TNF concentrations but slightly increased IL-6 (Figure 2A-C). Nevertheless, after stimulation, significantly increased cytokine levels were measured in uric acid pretreated cells compared with RPMI pretreated cells, effects that occurred in a dose-dependent (figure 2A–E) and time-dependent manner (see supplementary figure S2A). To assess mRNA modifications, after the first 24 h priming with uric acid, cells were restimulated for another 4 h. Uric acid pretreated cells exhibited higher relative IL-1β mRNA compared with control conditions (Figure 2F) and this was also observed in a dose-dependent manner (see supplementary figure S2B). TNF and IL-6 mRNA levels were also enhanced (see supplementary
This effect of uric acid was most prominent at the highest concentration (50 mg/dl). Nevertheless, lower uric acid levels show the same tendency towards increased IL-1β production (see supplementary figure S3A). The effect on IL-1β was replicated in purified monocyte cell suspensions isolated from PBMCs (see supplementary figure S4).

**Figure 2. Uric acid pretreatment increases proinflammatory cytokines**

Peripheral blood mononuclear cells of healthy volunteers were treated for 24 h with culture medium (Roswell Park Memorial Institute (RPMI) with 10% human serum) or with increasing concentrations of uric acid, followed by removal of medium and restimulation with RPMI, Pam3Cys (10 μg/ml) or lipopolysaccharide (LPS) (10 ng/ml) in the presence or absence of monosodium urate (MSU) crystals (300 μg/ml). Cytokine production is shown in supernatants after the first 24 h of pretreatment (priming) and after the second 24 h stimulation for secreted interleukin (IL)-1β (A), IL-6 (B) and tumour necrosis factor (TNF) (C). Cells were lysed after stimulation by three sequential freeze-thaw cycles, and intracellular IL-1β (D) and IL-1α (E) were measured. Data represent mean±SEM of values observed in at least 5 to 37 volunteers, from at least three independent experiments. Cells exposed to control medium or uric acid (50 mg/dl) for 24 h were restimulated for 4 h, followed by mRNA isolation and qRT-PCR for IL-1β mRNA assessment. Data are shown as relative fold induction of IL-1β mRNA levels by uric acid in six volunteers from three independent experiments (F). Kruskall Wallis (A-C) or Wilcoxon (D-F) *p<0.05.
Uric acid specifically downregulates the production of the anti-inflammatory cytokine IL-1Ra

To further decipher the mechanism of uric acid induction of proinflammatory cytokines, the effect on anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra) and IL-10 was investigated. Of interest, the IL-1Ra concentrations were found to be significantly decreased in PBMCs treated with uric acid compared with medium pretreated cells (figure 3A), whereas IL-10 levels were not affected (figure 3B). This surprising effect of uric acid on IL-1Ra production was observed at the level of transcription after 4 h uric acid exposure (figure 3C) and after 24 h priming followed by 4 h stimulation (Figure 3D). This implies a specific modulation of uric acid on IL-1 natural inhibition. IL-1Ra downregulation was also obvious at the lower uric acid doses (see supplementary figure S3B) and was present in selected monocytes (see supplementary figure S4). Addition of exogenous IL-1Ra (recombinant protein) dose-dependently reversed the effects of uric acid (see supplementary figure S5).

**Figure 3. Specific downregulation of interleukin-1 receptor antagonist (IL-1Ra) due to uric acid exposure**

Peripheral blood mononuclear cells (PBMCs) of healthy volunteers were treated for 24 h with culture medium (Roswell Park Memorial Institute (RPMI) with 10% human serum) or with uric acid 50 mg/dl, followed by removal of medium and restimulation with RPMI, Pam3Cys (10 μg/ml), lipopolysaccharide (LPS) (10 ng/ml) in the presence or absence of monosodium urate (MSU) crystals (300 μg/ml). Cytokine production is shown in supernatants after the first 24 h of pretreatment (priming) and after the second 24 h stimulation for secreted IL-1Ra (n=13) (A) and IL-10 (n=5) (B). Data are shown as mean±SEM, Wilcoxon *p<0.05. PBMCs were treated with medium or uric acid for either 4 h (C) or for 24 h followed by 4 h restimulation (D) and were subjected to mRNA isolation and qRT-PCR for IL-1Ra transcription assessment. Data represent relative fold induction of IL-1Ra mRNA in four (C) or six (D) volunteers. Wilcoxon *p<0.05.
Modulation of immune responses by uric acid is independent of allantoin, myeloperoxidase inhibition or cell death

To determine whether the effect of uric acid is specific or whether it can also be induced by related metabolites, allantoin was used as control in the same experimental conditions as uric acid. Uric acid induced significantly higher IL-1β production and strongly suppressed IL-1Ra, which was not seen in allantoin-treated PBMCs (Figure 4A and supplementary figure S6A, B). Myeloperoxidase (MPO) inhibitor, 4-ABH, was used to assess whether oxidation products resulting after MPO-mediated oxidation of uric acid might play a role in the effects observed. However, the effect of uric acid on cytokine production was not modified by MPO inhibition (Figure 4B and supplementary figure S6C, D). Moreover, annexinV/PI staining after uric acid treatment did not show differences in cell death due to uric acid exposure (Figure 4C).

Figure 4. Uric acid effects are independent of similar or secondary metabolites or cell death

Peripheral blood mononuclear cells of healthy volunteers were treated for 24 h with culture medium, uric acid 50 mg/dl or equivalent concentrations of allantoin, followed by removal of medium and restimulation with Roswell Park Memorial Institute (RPMI) medium, Pam3Cys (10 μg/ml), lipopolysaccharide (LPS) (10 ng/ml) in the presence or absence of monosodium urate (MSU) crystals (300 μg/ml). Relative fold induction of interleukin (IL)-1β and IL-1 receptor antagonist (IL-1Ra) cytokines by uric acid or allantoin compared with medium control is shown (A). Data represent mean±SEM of data obtained in seven volunteers from four independent experiments, Wilcoxon *p<0.05. Priming with uric acid was performed in the presence or absence of myeloperoxidase (MPO) inhibitor, 4-aminobenzoic hydrazide (100 μM), and fold IL-1β and IL-1Ra induction of cytokines by uric acid compared to medium control is shown (n=4) (B). Cells were treated for 24 h with medium or increasing concentrations of uric acid and cell death was assessed by flow cytometry after annexin V/propidium iodide (PI) staining. Percentages of early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) cells are shown in samples derived from three volunteers (C).
PBMCs of patients with gout reveal to be less responsive to uric acid priming for IL-1β induction when compared with PBMCs from healthy controls.

Furthermore, we investigated whether uric acid priming is a possible mechanism explaining the higher cytokine production observed in cells of patients with gout (as described in figure 1). For testing this hypothesis, the experimental setup of 24 h uric acid priming and 24 h stimulation of PBMCs was replicated in a larger cohort of healthy controls and patients with gout aiming at overruling bias due to cytokine variation. Indeed, in consistency with previous data shown in figure 1, the absolute cytokine concentrations determined in cells from patients with gout were significantly higher than in controls (white bars, figure 5A).

Figure 5. Reduced priming effects in cells from patients with gout compared with healthy controls

Freshly isolated human peripheral blood mononuclear cells from 114 healthy volunteers and 42 patients with gout were primed with medium or uric acid 50 mg/dl for 24 h, followed by stimulation with medium, lipopolysaccharide (LPS) (10 ng/ml) or LPS+monosodium urate (MSU) (300 μg/ml) for another 24 h. Absolute values and relative fold of change due to uric acid priming were assessed for interleukin (IL)-1β (A and C) and IL-1 receptor antagonist (IL-1Ra) (B and C). CD14+ cells were positively selected using magnetic beads from three healthy volunteers and eight patients with gout and basal IL-1β mRNA levels were assessed in unstimulated CD14+ cells (E). Data are shown as mean±SEM, Mann-Whitney *p<0.05.
The enhancement of IL-1β (figure 5A) and suppression of IL-1Ra (figure 5B) due to uric acid were observed in both groups. However, the degree of IL-1β induction by uric acid was significantly lower in patients with gout, as observed by relative induction of IL-1β by uric acid (figure 5C). CD14+ monocytes of patients with gout also exhibited higher steady-state IL-1β mRNA levels than healthy controls in unstimulated conditions (figure 5D), suggesting that cells of patients with gout might have encountered factors in vivo inducing a facilitated state for IL-1β production. IL-1Ra production was significantly decreased upon uric acid priming, with a lower basal level of IL-1Ra in unstimulated cells from patients with gout compared with controls (figure 5B). However, the level of IL-1Ra inhibition by uric acid in patients with gout does not significantly differ from that of controls (figure 5C).

Pharmacological inhibition of histone-modifying enzymes reverse the priming effect of uric acid

Uric acid primes PBMCs for enhanced cytokine production at the transcription level, and we hypothesised that epigenetic modifications known to influence cytokine production, such as histone methylation and acetylation, are involved. In order to investigate the role of epigenetic histone modifications on uric acid-induced priming that results in aggravated IL-1β, histone methyltransferase inhibitor, MTA (5’-deoxy-5’-methylthio-adenosine), and histone acetyltransferase inhibitor, EGCG (epigallocatechin-3-gallate), were coincubated with uric acid. Of high interest, upon restimulation, the enhancement of IL-1β by uric acid was reversed by MTA (figure 6A) while staying unmodified by acetyltransferase inhibitor EGCG (figure 6B).

Figure 6. Reversal of uric acid effects by pharmacological inhibition of histone methyl transferases

PBMCs of healthy volunteers were primed with uric acid 50 mg/dl in the presence or absence of broad-spectrum histone methyltransferase inhibitor MTA (5’-deoxy-5’-methylthio-adenosine), 1 mM (A) or histone acetyltransferase inhibitor, EGCG (epigallocatechin-3-gallate), 30 μM (B). IL-1β levels were measured in the supernatants after 24 h priming followed by 24 h restimulation. Data represent mean±SEM of data obtained in six volunteers from three independent experiments, Wilcoxon *p<0.05. IL, interleukin; LPS, lipopolysaccharide; MSU, monosodium urate; PBMCs, peripheral blood mononuclear cells; RPMI, Roswell Park Memorial Institute.
DISCUSSION

In this study, we have revisited the hypothesis of uric acid acting as a possible proinflammatory agent, independently of its precipitated form in MSU crystals. As previously reported (26), here we confirm the finding that ex vivo stimulated PBMCs from patients with gout reveal enhanced inflammatory cytokine production and we show that this is linked with serum uric acid levels of the donors. Using primary human PBMCs as well as purified monocytes, we have performed an in vitro stimulation protocol mimicking hyperuricaemia for 24 h of initial treatment of the cells (priming) followed by restimulation with TLR ligands and MSU. These experiments revealed that significantly increased levels of proinflammatory cytokines (up to threefold more IL-1β) were produced by uric acid primed cells.

We next studied the molecular level at which uric acid exerts its effect: remarkably, we show that IL-1β production is enhanced at both intracellular and extracellular compartments, suggesting that enhanced secretion is probably not the explanation of these effects, but transcriptional upregulation of cytokines is the likely mechanism. Together with IL-1β, other proinflammatory cytokines like IL-6 and TNF were also increased, probably at least in part secondarily to IL-1β induction. However, most interestingly, a specific inhibition of IL-1Ra production was observed after uric acid pre-exposure, both at protein and transcription level. The increased IL-1β and decreased IL-1Ra levels were similarly observed in monocytes purified from the PBMC suspension. This finding is of high importance, as IL-1Ra is the natural inhibitor of IL-1RI, and it is known to be upregulated in parallel to IL-1β production in a feedback loop aiming to control IL-1β and IL-1α-driven inflammation (27). To our knowledge this is a unique finding of a signal that does not upregulate both IL-1β and IL-1Ra in the same direction, but rather decreases IL-1Ra production. Therefore, this promotes IL-1β-induced IL-1β production without the natural antagonist counterbalancing this pathway. Exogenous IL-1Ra addition to uric acid-treated samples during the first 24 h of priming re-established the IL-1Ra levels during the first 24 h. This, however, did not have lasting effects for the second 24 h stimulation on IL-1β (see supplementary figure S5A, B). Adding IL-1Ra, also at the moment of restimulation, dose-dependently restored IL-1Ra and diminished IL-1β (see supplementary figure S5C, D). This proves that IL-1Ra downregulation by uric acid is an important part of the mechanism of IL-1β enhancement, not only an associated phenomenon with no functional significance.

Cells isolated from patients with gout were found to be less prone to potentiate IL-1β induction after uric acid pre-exposure, compared with healthy volunteers. This effect was due to an already enhanced state of IL-1β production, most likely due to the effects of hyperuricaemia already present in these patients. In line with this, steady-state IL-1β mRNA levels appear to be higher in patients with gout than in controls and basal levels of IL-1Ra in unstimulated cells are lower than those observed in healthy volunteers. These observations suggest that the priming effects of soluble uric acid, which enhance IL-1β while decreasing IL-1Ra, are biologically relevant and present in vivo in patients with gout.

In experiments using PBMCs of patients with gout and healthy controls for in vitro priming, uric acid was not present during the time of restimulation with TLR ligands and/or MSU crystals. This implies a long-term consequence of uric acid exposure that induces an effect reminiscent of non-specific immunological memory (‘trained immunity’) of monocytes observed after infections and vaccinations (28,29). Long term adaptive effects on innate immune cells
resulting in enhanced cytokine production have been shown to be mediated by epigenetic reprogramming of cells (28,29). The increased long-term H3K4me1 induced by inflammatory stimuli has been reported to increase cell responsiveness, and these epigenetic marks have been termed ‘latent enhancers’ (30). Because uric acid exposure induces a long-term increase in IL-1β gene transcription (accompanied by less IL-1Ra), we hypothesised that this effect may be mediated by histone modifications. Using pharmacological inhibitors of either histone methylation or acetylation, two major histone modification marks with functional consequences on gene expression, we show that uric acid effects were abolished when histone methyl transferase inhibitor was used. This is the first indication that indeed uric acid might exert effects at the epigenetic level and that these effects could have long-term consequences on the individual cytokine profile. Future studies are warranted to assess the role of hyperuricaemia on the specific epigenetic marks and complete functional signature associated with high urate exposure.

A link between soluble uric acid and induction of inflammation has also been suggested by previous reports where uric acid has been identified as a danger signal released in the context of cell death-inducing inflammation (31), or adaptive immunity (32). Although the initial event in these studies is also the release of uric acid and formation of a high urate environment, the main inflammatory role in this context is still in fact attributed to MSU crystals (33). In contrast, data linking uric acid with higher LPS responses (23), and NF-kB activation (24,25), were obtained in mice receiving intraperitoneal uric acid injections. In this study, despite the high uric acid concentrations used (approximately eight times higher than the threshold for hyperuricaemia) we were unable to detect MSU crystals being formed over the 24 h of cell culture in the presence of uric acid, arguing for the stability of the solutions used for the time and conditions of our experimental setup. Moreover, MSU crystals and soluble uric acid have totally opposite effects on IL-1Ra levels; as shown in figure 3A and 5B, MSU crystals increase the levels of IL-1Ra secreted upon MSU stimulation alone, or in synergism with other ligands, while soluble urate significantly downregulates IL-1Ra.

The high concentrations of uric acid used can be a limitation of this study. Nevertheless, we provide evidence (see supplementary figure S3) that the same cytokine profile is observed also at lower uric acid levels, in the range of clinical hyperuricaemia and within the solubility limits of uric acid. The rationale of using a high dose was to have the ability to best observe these significant effects in an in vitro setting, allowing further experimental intervention. In figure 5, we show data supporting that similar features of uric acid priming were also present in patients with gout, sustaining the biological relevance of this finding. Further studies using in vivo models are needed to demonstrate this in more detail.

This mechanism of uric acid induced modulation of proinflammatory cytokines is of high relevance also for other metabolic diseases. There is large body of evidence that associates uric acid with metabolic syndrome, atherosclerosis, hypertension, type 2 diabetes and chronic kidney disease (34-38). In this context, mechanistic data on the proinflammatory effects of uric acid are likely to turn into a potential link between the diseases of modern society associated with chronic low-grade inflammation.

In conclusion, in this study we propose a mechanism through which high uric acid concentrations mediate the metabolic modulation of inflammatory responses by facilitating IL-1β production in
PBMCs. We show that a mechanism for the amplification of IL-1β is via the unique inhibitory effect on IL-1Ra that would normally counterbalance the effects of IL-1 and the IL-1β autoinduction loop. This effect is likely to be epigenetically mediated by histone marks that modify the degree of transcription for cytokine genes. Consequently, patients having hyperuricaemia could be at risk of increased reactivity of cells upon encounter of acute inflammatory stimuli (infectious or sterile danger signals) and this might induce enhanced states of (auto)inflammation. Thus, we provide here the first evidence that uric acid represents a silent modulator of cytokine production in primary human cells. This pinpoints to the role of metabolic triggers on the inflammatory properties of the circulating cells and could represent a relevant link for understanding the pathogenesis of gout and other metabolic diseases with inflammatory components.
REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Participants

Subjects to this study consisted in healthy volunteers and patients diagnosed with crystal-proven gout (by dr. Tim L. Jansen) being followed at the Rheumatology Department of the Radboud University Medical Center. Participants were enrolled after written informed consent and peripheral blood was collected on EDTA under sterile conditions. The patient study was approved by the Ethical Committee of the Radboud University Medical Center (registration number 2012/482). Blood from a total of 57 gout patients of Dutch nationality has been used in 24h stimulation experiments (n=19) or in 48h uric acid priming experiments (n=49). Mean age was 62.8 (range 29-85). A set of main clinical parameters is shown in Table S1. All patients were under either urate lowering therapy, anti-inflammatory therapy for control of the attacks, or both. Urate lowering therapy consisted mainly in allopurinol (or febuxostat when allopurinol was counterindicated), occasionally in combination with benz bromarone. Anti-inflammatory therapy consisted mainly in colchicine, with fewer cases on steroids or non-steroidal anti-inflammatory drugs.

Reagents

Uric acid, palmitic acid (C16:0), LPS (E. coli serotype 055:B5), allantoin, 4-aminobenzoic hydrazide (4-ABH), 5′-deoxy-5′ (methylthio) adenosine (MTA) and epigallocatechin-3-gallate (EGCG) were purchased from Sigma. Synthetic Pam3Cys was purchased from EMC Microcollections. Recombinant IL-1Ra was obtained from R&D. LPS was subjected to ultrapurification before cell culture experiments. In experiments using C16:0, albumin (Albuman, human albumin 20% for intravenous use, Sanquin) was used to initially conjugate fatty acids before applying to cells. Equal amounts of albumin and ethanol were added to controls. In experiments using 4-ABH and MTA equal amounts of DMSO vehicle were added to control wells.

Preparation of MSU crystals

A 0.03 M solution of MSU at a volume of 200 ml was prepared after diluting 1.0 gram of uric acid in 200 ml of sterile water containing 24 grams of NaOH. The pH was adjusted to 7.2 after the addition of HCl, and the solution became pyrogen-free after heating for 6 hours at 120°C. The solution was left to cool at room temperature and stored at 4°C. Crystals produced were 5–25 μm in length.

PBMC isolation and stimulation

Peripheral blood was harvested from the antecubital vein of healthy volunteers or gout patients, after obtaining informed consent. The PBMC fraction was obtained by differential centrifugation over Ficoll-Paque (Pharmacia Biotech). Cells adjusted to 5x10⁶ or 10x10⁶ cells/ml (depending on experiment setup) were suspended in culture medium RPMI (Roswell Park Memorial Institute) 1640, supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine and 1 mM pyruvate. PBMCs were plated at 5x10⁵ cells/well in 96-well plates (Corning). Cells were stimulated for 24h in round bottom plates with Pam3Cys (10 μg/ml) or C16:0 (200 μM) in the presence or absence of MSU crystals (300 μg/ml).
In different sets of experiments, cells were incubated in flat bottom 96-well plates for 24h with culture medium (RPMI with 10% human serum), uric acid 50 mg/dl or allantoin 50 mg/dl. In experiments investigating dose-dependent effects, lower uric acid concentrations were also used. Experiments investigating the effects of pharmacological inhibitors on uric acid priming consisted in 1 hour pre-incubation with or without inhibitor, followed by 24 hours exposure to uric acid. After this initial treatment with uric acid, culture medium was removed and the remaining adherent cells were stimulated for another 24 hours with RPMI medium, Pam3Cys (10 μg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 μg/ml). Cytokine induction experiments were performed in duplicate wells and the replicates were pooled before measurement.

**Monocyte isolation**

Purified monocyte suspensions were obtained from PBMCs using MACS beads according to manufacturer’s instructions (Miltenyi Biotec). CD14+ cells were isolated using CD14 microbeads followed by magnetic separation over selection columns. Negative selection of monocytes was performed after an initial monocyte-lymphocyte separation of PBMCs using high-density hyperosmotic Percoll solution, followed by MACS. This was carried out either by labeling CD3+, CD19+ and CD56+ cells using the corresponding microbeads and cell separation over depletion columns, or by using the pan monocyte isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. Monocyte suspensions were adjusted to 1x10^6 cells/ml and experiments were performed using 1x10^5 cells/well.

**Flow cytometry**

Cells treated for 24 hours with or without uric acid were stained with annexin V-FITC (BioVision) and propidium iodide (PI) (Invitrogen) for assessment of cell death. In brief, annexin V-FITC was diluted 1/100 in CaCl₂-enriched RPMI (6 μM), samples were stained with 100 ul annexin V staining solution and were incubated for 15 minutes on ice in the dark. This was followed by PI staining (1 mg/ml, 1.5 μl per sample) and 5 minutes incubation before measurement. Purity of cells obtained through MACS isolation was assessed by 30 minutes staining on ice in the dark of 1x10^5 freshly isolated cells with CD14-FITC, CD56-PE, CD19-ECD, CD3-PC5, CD45-PC7, followed by 1 washing step with 1% BSA. Fluorescence was measured using Cytomics FC500 (Beckman Coulter).

**Cytokine measurements**

Cytokine concentrations were determined using specific sandwich ELISA kits for IL-1β, TNFα, IL-1α, IL-1Ra (R&D Systems), IL-6 and IL-10 (Sanquin). Secreted cytokines were measured in the supernatant collected at the end of the experiment. For intracellular cytokine levels, after removal of supernatant, 200μl medium was added to the remaining cells and was subjected to three sequential freeze-thaw cycles. These induce the disintegration of the membrane and release of intracellular cytokine content into the medium.
Quantitative-PCR

Samples stimulated for 4 hours at 37°C were treated with TRIzol Reagent (Invitrogen) and total RNA purification was performed according to manufacturer’s instructions. Isolated RNA was subsequently transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad) followed by quantitative PCR using the Sybr Green method. The following primers were used in the reaction: IL-1β forward 5'-GCCCTAAACAGATGAAGTGCTC-3' and reverse 5'-GAACCAGCATCTTCCTCAG-3'; IL-1Ra forward 5'-GCCTCCGCAGTCACCTAAT-3' and reverse 5'-TCCCAGATTCTGAAGGCTTG-3', β2-microglobulin forward 5'-ATGAGTATGCTGCGTGTG-3' and reverse 5'-CCAAATGCGGCATCTTTCAAC-3' (Biolegio). Results are shown as fold change in mRNA levels in stimulated samples compared to controls.

Statistical analysis

Data was analysed using GraphPad Prism version 5.03 and SPSS version 20. The differences were analysed using Kruskal Wallis test, Mann-Whitney test or Wilcoxon signed rank test according to the number of datasets and experimental design. Correlation analysis was performed using Spearman’s correlation. Data was considered statistically significant at a p-value<0.05. Data is shown as cumulative results of levels obtained in all volunteers (means ± SEM).
SUPPLEMENTARY TABLES AND FIGURES

Table S1. Main clinical parameters of gout patients

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Figure S1. Freshly isolated PBMCs from gout patients (n=19) were stimulated for 24h with Pam3Cys (10 μg/ml) or C16:0 (200 μM) in the presence or absence of MSU crystals (300 μg/ml). C16:0 vehicle (albumin and ethanol) was added to control wells. Cytokine measurements were performed by ELISA and Spearman correlation was assessed between serum uric acid levels and cytokine production upon specific stimulations (Spearman r and p-values depicted in figure, α<0.05).
CHAPTER 3

Figure S2. Cells were exposed to control medium or uric acid (50 mg/dl) for 4, 8, 16 and 24h and cytokine production after re-stimulation was assessed. Relative fold induction of cytokines by uric acid compared to medium control in response to Pam3Cys+MSU or LPS+MSU is shown (n=2) (A). Cells were treated for 24h with culture medium (RPMI with 10% human serum) or with increasing concentrations of uric acid, followed by 4h re-stimulation for IL-1β mRNA assessment. Data is shown as relative fold induction of IL-1β mRNA levels observed in 2 volunteers (B). PBMCs were treated with medium or uric acid for either for 24h followed by 4h restimulation and were subjected to mRNA isolation and qRT-PCR. Data represents relative fold induction of IL-6 (C) and TNF (D) mRNA in 3 volunteers.

Figure S3. Cells were exposed to lower uric acid concentrations (5-20 mg/dl range) and compared to 50 mg/dl for 24h priming and 24h stimulation with the mentioned stimuli. Data represents cytokine levels in 4 volunteers for IL-1β (A) and IL-1Ra (B).
Figure S4. Monocytes were negatively or positively selected from the PBMC fraction using magnetic separation and were subjected to the priming protocol consisting of 24h incubation with or without uric acid 50 mg/dl followed by another 24h stimulation RPMI medium, Pam3Cys (10 μg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 μg/ml). Fold induction or suppression of cytokines by uric acid compared to medium control is displayed for the first 24h of priming and for the 24h restimulation in CD3+/CD19+/CD56+ depleted PBMCs (A), enriched monocytes after pan-monocyte isolation (B) or positively selected CD14+ cells (C). Data represents means ± SEM of at least 3 volunteers.
Figure S4. Monocytes were negatively or positively selected from the PBMC fraction using magnetic separation and were subjected to the priming protocol consisting of 24h incubation with or without uric acid 50 mg/dl followed by another 24h stimulation RPMI medium, Pam3Cys (10 μg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 μg/ml). Fold induction or suppression of cytokines by uric acid compared to medium control is displayed for the first 24h of priming and for the 24h restimulation in CD3+/CD19+/CD56- depleted PBMCs (A), enriched monocytes after pan-monocyte isolation (B) or positively selected CD14+ cells (C). Data represents means ± SEM of at least 3 volunteers.

Figure S6. Absolute individual values are shown for the cytokine production obtained by priming with uric acid, allantoin (n=7) (A, B) or priming with uric acid in the presence or absence of MPO inhibitor (n=4) (C, D). Restimulation followed with medium or LPS (10 ng/ml) in the presence or absence of MSU crystals (300 μg/ml).
Soluble uric acid primes human primary cells via inhibition of IL-1Ra
Suppression of monosodium urate crystal-induced cytokine production by butyrate is mediated by the inhibition of class I histone deacetylases

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ABSTRACT

Objectives

Acute gouty arthritis is caused by endogenously formed monosodium urate (MSU) crystals, which are potent activators of the NLRP3 inflammasome. However, to induce the release of active interleukin (IL)-1β, an additional stimulus is needed. Saturated long-chain free fatty acids (FFAs) can provide such a signal and stimulate transcription of pro-IL-1β. In contrast, the short-chain fatty acid butyrate possesses anti-inflammatory effects. One of the mechanisms involved is inhibition of histone deacetylases (HDACs). Here, we explored the effects of butyrate on MSU+FFA-induced cytokine production and its inhibition of specific HDACs.

Methods

Freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with MSU and palmitic acid (C16.0) in the presence or absence of butyrate or a synthetic HDAC inhibitor. Cytokine responses were measured with ELISA and quantitative PCR. HDAC activity was measured with fluorimetric assays.

Results

Butyrate decreased C16.0+MSU-induced production of IL-1β, IL-6, IL-8, and IL-1β mRNA in PBMCs from healthy donors. Similar results were obtained in PBMCs isolated from patients with gout. Butyrate specifically inhibited class I HDACs. The HDAC inhibitor, panobinostat, and the potent HDAC inhibitor, ITF-B, also decreased ex vivo C16.0+MSU-induced IL-1β production.

Conclusions

In agreement with the reported low inhibitory potency of butyrate, a high concentration was needed for cytokine suppression, while synthetic HDAC inhibitors showed potent anti-inflammatory effects at nanomolar concentrations. These novel HDAC inhibitors could be effective in the treatment of acute gout. Moreover, the use of specific HDAC inhibitors could even improve the efficacy and reduce any potential adverse effects.
INTRODUCTION

Gout is a crystal-induced disease with an increasing prevalence that currently affects up to 4% of adults in developed countries (1,2). Acute gout is characterised by recurrent self-limiting attacks of joint inflammation. A prerequisite for the acute joint inflammation is the presence of monosodium urate (MSU) crystals with additional inflammatory signals providing a second hit. Formation of MSU crystals is a result of chronic hyperuricemia in selected patients (3).

Acute gout attacks are dominated by the production of the classical proinflammatory cytokine interleukin-1 (IL-1β) (4,5), which is produced by monocytes as inactive pro-IL-1β. Pro-IL-1β can be cleaved to its mature form via activation of the NLRP3 inflammasome and caspase-1 or via other IL-1β-converting enzymes, such as proteinase-3 and elastase (6-8). MSU crystals are potent activators of the NLRP3 inflammasome and can mediate caspase-1-dependent activation of IL-1β (8). However, a second signal is required to induce the production of pro-IL-1β. Interestingly, such signal can be induced by saturated long-chain fatty acids (9), which are abundantly present in the blood.

In contrast to long-chain fatty acids, short-chain fatty acids have been reported to exert various opposite anti-inflammatory effects. They are produced in the colon by bacterial fermentation of indigestible dietary fibers. High-dose butyrate, in particular, was found to have immune-modulatory effects; it decreases lipopolysaccharide (LPS)-induced cytokine production and nuclear factor (NF)-κB activation in human peripheral blood mononuclear cells (PBMCs) (10-12).

One of the mechanisms by which butyrate exerts its anti-inflammatory effects is inhibition of histone deacetylases (HDACs) (13,14). Recently, the synthetic pan-HDAC inhibitor Givinostat was shown to have a broad anti-inflammatory activity with beneficial effects in experimental models of arthritis and even led to attenuation of clinical scores in a trial with patients with juvenile idiopathic arthritis (15,16). Inhibition of HDACs might therefore also have beneficial effects in acute gouty arthritis.

In this study, we explore the suppressive effects of butyrate on MSU crystal-induced cytokine production. We confirm that butyrate specifically inhibits class I HDACs and show that butyrate has highest specificity for HDAC8. In addition, we show the effects of the pan-HDAC inhibitors, givinostat and panobinostat, as well as those of a selective HDAC8 inhibitor and a potent HDAC inhibitor devoid of class IIa inhibitory activity, on LPS-induced and MSU crystal-induced cytokine production. With these results, we provide a rationale for further exploring the beneficial effects of specific HDAC inhibitors in gouty arthritis.
CHAPTER 4

METHODS

Human samples

Gout patients visited the outpatient Rheumatology department of the Radboud University Medical Center in Nijmegen, the Netherlands. All patients were diagnosed with crystal-proven gout by an experienced rheumatologist (TLJ). The gout patient cohort consisted of 117 volunteers. Written informed consent was received from all donors. Experiments with human blood were performed in accordance with the Declaration of Helsinki.

Reagents

Uric acid, butyric acid and palmitic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). *Escherichia coli* LPS was purchased from Invitrogen (Bleiswijk, the Netherlands) and sodium hydroxide from Merck (Darmstadt, Germany). Human albumin (Albuman) was purchased from Sanquin (Amsterdam, the Netherlands). Panobinostat was purchased from Selleckchem (Munich, Germany). Givinostat and other ITF compounds were kindly provided by Dr. Fossati. Synthetic HDAC inhibitors were dissolved in dimethyl sulfoxide, which was present in the cell culture at a maximal concentration of 0.01%.

Palmitic acid and albumin conjugation

Stock palmitic acid was dissolved in 100% ethanol. Palmitic acid (C16.0) and human albumin were conjugated by warming to 37°C in a water bath before adding together in a 1:5 ratio. The mixture was sonicated for 20-25 minutes and kept at 37°C until use. The vehicle control for 50 μM C16.0 consisted of 0.025% albumin and 0.025% ethanol.

Monosodium urate crystal formation

In 400 mL of sterile water, 1.0 gram of uric acid and 0.48 grams sodium hydroxide were dissolved. The pH was adjusted to 7.2 and the solution was sterilised by heating it for 6 h at 120°C. No LPS contamination was detected by Limulus amoebocyte lysate assay.

PBMC isolation and stimulation experiments

Venous blood was drawn from healthy donors and patients with gout. PBMCs were isolated after Ficoll-Paque density centrifugation and plated in a U-bottom 96-well plate at 5x10⁵ cells per well. They were cultured for 24 h with either culture medium, 10 ng/mL *E. coli* LPS, 50 μM C16.0, or a combination of C16.0 with 300 μg/mL MSU crystals. Cells were preincubated with butyrate or HDAC inhibitors for 1 h. In experiments with PBMCs from patients with gout, butyrate was added to the cells without preincubation.

Cytokine measurements

IL-1β, IL-1Ra, IL-6, IL-8, tumour necrosis factor-α, IL-10, and transforming growth factor-β1 protein concentrations were determined with commercially available ELISA kits (R&D Systems, Abingdon, UK; or Sanquin, Amsterdam, the Netherlands), according to the manufacturer’s protocol. Intracellular IL-1β was determined after lysing the cells via three freeze-thaw cycles.
RNA isolation, cDNA synthesis and qPCR

RNA was extracted by phase separation with 400 μL TRIzol reagent (Life Technologies, Paisley, UK) and 80 μL of chloroform (Merck, Darmstadt, Germany). RNA was precipitated using 200 μL 2-propanol (Merck, Darmstadt, Germany). Complementary DNA was obtained using iScript cDNA synthesis kit (Bio-Rad, Hercules, California, USA). qPCR was performed with SYBR Green PCR Master Mix (Life Technologies, Paisley, UK). Primers were as follows: IL-1β forward primer 5'-GCCCTAAACAGATTGAAGTGC-3', IL-1β reverse primer 5'-GAACCAGCATCTTCTCAG-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5'-AGGGGAGATTCAGTGTGGTG-3', and GAPDH reverse primer 5'-CGACCACCTTTGTCAAAGCTCA-3' (BioLegio, Nijmegen, the Netherlands).

Flow cytometry

Cell death was determined by means of flow cytometry. Cells were stained with Annexin V-FITC (BioVision, Milpitas, California, USA). Annexin V-FITC was diluted 1/100 in CaCl₂-enriched Roswell Park Memorial Institute medium (6 μM) and 100 μL of the diluted Annexin V staining was added to each sample. This was incubated for 15 minutes in the dark on ice. Fluorescence was measured with the Cytomics FC500 (Beckman Coulter, Woerden, the Netherlands).

HDAC activity assay

Soluble human recombinant HDAC enzymes were purchased (HDAC1-10: BPS Bioscience, San Diego, California; HDAC11: Farmingdale, New York, USA). Fluor de Lys deacetylase substrate (Enzo Life Sciences) was used to assay activity of HDAC1, HDAC3, HDAC6, HDAC10 and HDAC11. Fluor de Lys green deacetylase substrate (Enzo Life Science) was used for HDAC8, and Nε-Trifluoroacetyl-L-lysine was used to assay HDAC4, HDAC5, HDAC7, and HDAC9. Recombinant enzymes were preincubated with butyrate at 30°C in a total volume of 25 μL. Next, 25 μL of substrate was added. A fluorescent signal was generated after adding 50 μl Fluor de Lys developer (Enzo LifeScience) containing 2 μM trichostatin A (Sigma-Aldrich). Conditions were optimised for each assay. Positive control consisted of enzyme plus substrate. Fluorescence was measured using a Victor multilabel plate reader (Perkin Elmer, Waltham, Massachusetts, USA).

Statistics

In dose-response experiments with butyrate or synthetic HDAC inhibitors, experimental conditions were compared to the shaded control bar using with Friedman’s test followed by Dunn’s test for multiple comparisons. The same test was used for the gout patient analysis of different treatment groups and time points. All groups were compared to the total gout patient group or time point t=0, respectively. In gout patient stratification by serum urate levels, the two groups were compared with the Mann-Whitney U test. In all other graphs, mean values were compared with the Wilcoxon signed-rank test. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed with GraphPad Prism Software (v6.0f).
RESULTS

Butyrate suppresses C16.0+MSU-induced cytokine production in a dose-dependent manner

A combination MSU crystals and palmitic acid (C16.0) was used to induce potent production of active IL-1β. C16.0 alone induced the production of IL-1β and IL-6, and MSU crystals synergistically amplified this effect (figure 1A, D). Similar results were found for IL-8 (see supplementary figure S1). Butyrate suppressed C16.0+MSU-induced IL-1β production in a dose-dependent manner (figure 1B). A half maximum inhibitory concentration (IC50) of 0.485 mM was calculated based on figure 1B using non-linear regression. This effect of butyrate was specific for C16.0+MSU stimulation. Butyrate even increased the LPS-induced IL-1β production at 5 mM (figure 1C). Butyrate did not induce cell death in combination with C16.0+MSU (figure 1E) or LPS (figure 1F).

Figure 1. Butyrate differentially inhibits C16.0+MSU-induced and LPS-induced IL-1β production in a dose-dependent manner

(A) and (B) Freshly isolated PBMCs from healthy volunteers were stimulated for 24 h with 1 mM butyrate, C16.0, MSU crystals, or a combination of C16.0+MSU. (B), (C) and (F) PBMCs were preincubated for 1 h with butyrate, and subsequently stimulated for 24 h with LPS or C16.0+MSU. Release of IL-1β (A)-(C) and IL-6 (D) in the cell culture supernatant was measured by ELISA. Cell death (E, F) was determined by flow cytometry with Annexin V-FITC. Data in (A)-(D) are shown as individual points and median. Data in (E) and (F) are shown as mean±SD. Panels all include n≥6. IL, interleukin; LPS, lipopolysaccharide; MSU, monosodium urate; PBMCs, peripheral blood mononuclear cells; RPMI, Roswell Park Memorial Institute medium.

Butyrate decreases C16.0+MSU-induced IL-1β mRNA at 1 mM

Butyrate decreased C16.0+MSU-induced IL-1β, IL-6, and IL-8 production (figure 2A-C). This suppressive effect was not caused by an increased production of the IL-1 receptor antagonist (IL-1Ra), because the IL-1Ra release was also suppressed by butyrate. In addition, IL-10 or TGF-β1 production was not increased upon stimulation with C16.0+MSU crystals (supplementary figure S2).
No difference was observed in intracellular IL-1β between stimulation with C16.0 or C16.0+MSU (figure 2D). However, there was an increase in IL-1β mRNA and extracellular IL-1β with the combination of C16.0 and MSU crystals compared with C16.0 alone (figure 2A, F). Butyrate decreased C16.0+MSU-induced IL-1β mRNA levels, as well as intracellular IL-1β (figure 2D, E). PBMC stimulation with C16.0+MSU induced a dramatic increase of IL-1β mRNA, which reached a plateau at around 16 h of culture (figure 2F).

Butyrate inhibits C16.0+MSU-induced IL-1β production in PBMCs from patients with gout, regardless of treatment, uric acid level or phase of the disease.

The suppressive effects of butyrate were also studied in PBMCs of patients with gout. There are no differences in the effect of butyrate on C16.0+MSU-induced IL-1β or IL-6 production between healthy controls and patients with gout (figure 3A, D) nor when stratifying for medication, serum urate levels or time after last flare (figure 3B, C, E, F).
Butyrate inhibits C16.0+monosodium urate (MSU)-induced interleukin (IL)-1β in peripheral blood mononuclear cells (PBMCs) from patients with gout regardless of treatment, serum urate levels or phase of the disease

Freshly isolated PBMCs from patients with gout (n=117, mean age 62±14 SD, 85% men) and healthy controls (n=9, mean age 48±14 SD, 78% men) were stimulated with C16.0+MSU alone or in combination with 1 mM of butyrate. Release of IL-1β (A)-(C) and IL-6 (D)-(F) in the cell culture supernatant was measured by ELISA. Data are shown as percentage decrease in C16.0+MSU-induced IL-1β or IL-6 by butyrate in Tukey boxplots. Data are stratified by treatment (A) and (D), serum urate levels (B) and (E) or months after last gout flare (C) and (F). The treatment stratification groups in (A) and (D) include patients using only the indicated medication. The number of patients in each group is shown beneath the X-axis.

Butyrate specifically inhibits class I HDACs

Next, we determined the inhibitory effect of butyrate on the deacetylase activity of all class I, II and IV HDACs separately. Butyrate specifically inhibited class I HDACs with an IC50 between 100 and 700 μM (table 1). HDAC10 and HDAC11 were inhibited by butyrate at a much higher concentration, with IC50 values of 1.9 and 2.8 mM, respectively. These results are in agreement with previously published data on HDAC1-9 (17), although it is worth noting that IC50 values in this work are higher because of higher substrate concentrations used in the HDAC assay to assure proper enzyme saturation. Furthermore, our experimental conditions show that HDAC8 is the most sensitive isoform to butyrate inhibition.
Table 1. Inhibition of specific histone deacetylases by butyrate and synthetic HDAC inhibitors

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Data are presented as IC50 values. For butyrate, the values are in μM; > indicates more than 10 mM. For the synthetic HDAC inhibitors, IC50 values are in nM. ITF-A; > indicates more than 8000 nM. ITF-B; > indicates more than 400 nM.

Pan-HDAC inhibitor panobinostat decreases C16.0+MSU-induced IL-1β production

The effects of butyrate were compared with two well-known pan-HDAC inhibitors, givinostat and panobinostat. Givinostat did not decrease C16.0+MSU-induced IL-1β, but LPS-induced IL-1β production was significantly decreased with 25 nM of givinostat (figure 4A, B). Panobinostat showed effects similar to butyrate. It effectively decreased C16.0+MSU-induced IL-1β in a dose dependent manner. Conversely, it decreased LPS-induced IL-1β production only in the low concentration range (figure 4C, D). Givinostat and panobinostat do not affect PBMC viability after 24 h culture (see supplementary figure S3A, B).

Figure 4. C16.0+monosodium urate (MSU)-induced interleukin (IL)-1β is inhibited by the pan-histone deacetylase inhibitor panobinostat, but not by givinostat

Freshly isolated peripheral blood mononuclear cells from healthy volunteers were preincubated for 1 h with culture medium, givinostat (A) and (B) or panobinostat (C) and (D) and subsequently stimulated with C16.0+MSU (A) and (C) or lipopolysaccharide (LPS; B) and (D) for 24 h. Release of IL-1β in the cell culture supernatant was measured by ELISA. Data are shown as individual points and median. All panels include n≥6.
**HDAC inhibitor ITF-B decreases C16.0+MSU-induced IL-1β production**

Because butyrate most strongly inhibits HDAC8, we studied the effects of a highly specific HDAC8 inhibitor, ITF-A (table 1). Addition of ITF-A did not significantly decrease C16.0+MSU-induced IL-1β production (figure 5A). However, it decreased LPS-induced IL-1β in a dose-dependent manner (figure 5B). Next, we tested an HDAC inhibitor (ITF-B) that resembles butyrate in that it inhibits HDAC1, 2, 3, 8, 10 and 11. Its effects on C16.0+MSU-induced and LPS-induced IL-1β are similar to butyrate. ITF-B inhibits C16.0+MSU-induced IL-1β production in a dose-dependent manner. In contrast, LPS-induced IL-1β production is only inhibited by ITF-B at 10 nM, and in some donors even increases IL-1β production at higher concentrations. ITF-A and ITF-B do not induce cell death in human PBMCs in a 24 h culture (see supplementary figure S3C, D).

![Graphs](image)

**Figure 5.** C16.0+monosodium urate (MSU)-induced interleukin (IL)-1β is inhibited by histone deacetylase (HDAC) inhibitor ITF-B, but not by HDAC8 inhibition alone

Freshly isolated peripheral blood mononuclear cells from healthy volunteers were preincubated for 1 h with culture medium, HDAC8 inhibitor ITF-A (A) and (B) or HDAC inhibitor ITF-B (C) and (D) and subsequently stimulated with C16.0+MSU (A) and (C) or lipopolysaccharide (LPS; B) and (D) for 24 h. Release of IL-1β in the cell culture supernatant was measured by ELISA. Data are shown as individual points and median. All panels include n≥6.
DISCUSSION

In the present study, we have explored the capacity of butyrate and synthetic HDAC inhibitors to suppress ex vivo cytokine production. To study MSU crystal-induced cytokine production, a priming stimulus is needed for transcription of pro-IL-1β. In all our experiments, we used a combination of C16.0 and MSU crystals. MSU crystals alone did not result in cytokine production, but C16.0+MSU induced a synergistic increase in IL-1β, IL-8 and IL-1Ra production. This is in line with previously reported synergy of MSU crystals with stearic acid (9).

Butyrate inhibited C16.0+MSU-induced cytokine production in a dose-dependent manner. Furthermore, we have shown that butyrate decreased both IL-1β mRNA and intracellular IL-1β. Based on this, we can conclude that butyrate exerts its effects at the level of transcription or translation and does not result from inhibition of activation or excretion of IL-1β. In contrast, LPS-induced IL-1β was significantly increased with 5 mM butyrate.

Butyrate is known to inhibit HDACs. In agreement with published results (17), we show that butyrate inhibited class I HDACs specifically, with highest specificity for HDAC8. In addition, the IC50 of butyrate with regard to suppression of C16.0+MSU-induced IL-1β is in the same range as the IC50 values for inhibition of the different class I HDACs, suggesting that this effect of butyrate is indeed mediated though class I HDAC inhibition.

Currently, 11 human HDACs and 7 sirtuins have been identified, which are classified according to their homology to different yeast HDACs (18). The molecular mechanism by which HDAC inhibitors elicit anti-inflammatory effects remains unknown. Lysine acetylation modulates both histone and non-histone proteins and affects a wide range of cellular processes (19). The role of each individual HDAC in inflammation is still largely unknown. However, different HDACs may have opposing effects in inflammatory pathways, depending on the stimulus, cell type and site of lysine deacetylation (20). For this reason, it is crucial to identify the effects of individual HDACs and to develop safe specific HDAC inhibitors, reducing the adverse effects of inhibiting other HDACs.

Although butyrate effectively inhibits C16.0+MSU-induced cytokine production ex vivo, its use as possible treatment for gout has to be considered carefully. Butyrate concentrations used in our experiments are higher than its physiological concentration in peripheral blood, which lie around 4 μM (21). Butyrate is naturally present mainly in milk products, but the majority of butyrate is produced endogenously via bacterial fermentation of indigestible fibres in the gut. Foods that are high in indigestible fibres are mainly whole grains and beans. To our knowledge, it is unknown to what extent a high-fibre diet can increase human plasma butyrate levels. Although it is possible to enhance serum butyrate concentration in subjects by oral intake of the prodrug tributyrin (22), no data are yet available for the validity of this approach. However, synthetic specific HDAC inhibitors are potent at extremely low concentrations, making them attractive therapeutic agents for many different inflammatory diseases.
The pan-HDAC inhibitor panobinostat (23) effectively decreased C16.0+MSU-induced IL-1β, whereas LPS-induced IL-1β was only decreased at 2 nM. These dose-dependent effects of panobinostat closely resemble the effects of butyrate, suggesting that a similar mechanism is involved. Addition of givinostat, which is a well-known pan-HDAC inhibitor that was reported to have beneficial effects in several forms of arthritis in vivo (15,16), decreased LPS-induced IL-1β at 25 nM. This is consistent with a previous study in human PBMCs (24). However, givinostat had no significant suppressive effect on C16.0+MSU-induced IL-1β.

The HDAC inhibitor ITF-B resembles butyrate in its specificity for HDACs and also in its effects on LPS-induced and C16.0+MSU-induced IL-1β. At the concentrations used in our experiments, ITF-B has higher specificity for HDAC10 and HDAC11 than butyrate. However, the resemblance between their effects on cytokine production is a clear indication that specific class I HDAC inhibition is the causative mechanism. ITF-A, a highly specific HDAC8 inhibitor, had no significant effect on C16.0+MSU-induced IL-1β production, suggesting that sole HDAC8 inhibition is insufficient to account for the cytokine-suppressing effect of butyrate. These findings with specific HDAC inhibitors indicate that the suppressive effects of butyrate are caused by class I HDAC inhibition and, in particular, via inhibition of HDAC1, HDAC2 and/or HDAC3. This conclusion is supported by the observation that panobinostat strongly inhibited IL-1β production at 200 nM. At this concentration, class IIa HDACs should be minimally affected by this inhibitor, whereas class I, IIb and IV should be well inhibited. Because butyrate and panobinostat show a similar effect and the former inhibits HDAC10 and HDAC11 at high concentrations, inhibition of class I appears to be necessary for the inhibition of IL-1β induced by C16.0+MSU. Inhibition of HDAC10 and 11 might also play a role, but should be minor in the case of butyrate because it almost totally inhibited IL-1β at 1 mM, well below the IC50 for HDAC10 and 11 inhibition (table 1).

The exact mechanism by which HDAC inhibitors suppress cytokine production is subject to much debate. An obvious candidate for the observed general cytokine suppression is NF-κB. NF-κB signalling can be regulated by acetylation at multiple levels (25,26), but the functional effects of acetylation appear to be highly dependent on the protein and specific lysine residue that is targeted, the proinflammatory stimulus used and the cell type. HDAC1, HDAC2 and HDAC3 have all been reported to target NF-κB subunits. Both HDAC1 and HDAC2 have been described to suppress NF-κB signalling (27-29). For HDAC3, both inhibitory (30) and stimulatory (31,32) effects on NF-κB signalling have been described. Next to direct deacetylation of NF-κB subunits, HDAC3 can also potentiate NF-κB activation through repression of nuclear receptors such as peroxisome proliferator-activated receptor γ (33), which has been implicated as a mechanism in the anti-inflammatory effects of butyrate (34-36).

Opposing effects of HDACs within inflammatory signalling pathways make it difficult to pinpoint the exact mechanism of butyrate. After identification of the specific HDAC(s) involved, experiments with highly specific synthetic HDAC inhibitors should be performed to study mechanisms behind the cytokine-suppressing effect. In addition, finding anti-inflammatory genes that are upregulated by butyrate would warrant us to study the epigenetic profiles within these genes.

In conclusion, this study shows that butyrate inhibits C16.0+MSU-induced cytokine production at the mRNA level. This effect is most likely caused by inhibition of HDAC1, HDAC2 and/or HDAC3. However, these three HDACs might distinctly regulate cytokine production in LPS-stimulated
Butyrate suppresses MSU-induced cytokine production by inhibiting class I HDACs. Therefore, inhibition of all three HDACs may explain the different effects between the two stimuli. Although this study warrants further research on the function of each HDAC in gout, lysine acetylation is an important regulatory mechanism in inflammation and targeting specific HDACs could be a promising treatment option for gouty arthritis.
REFERENCES

25. Ashburner BP, Westerheide SD, Baldwin AS, Jr. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate
Butyrate suppresses MSU-induced cytokine production by inhibiting class I HDACs


Supplementary Figure S1. The combination of C16.0 and MSU crystals produce a synergistic IL-8 response in human PBMCs

Freshly isolated PBMCs from healthy volunteers were stimulated with 1 mM butyrate, 300 μg/mL MSU crystals, 50 μM C16.0 or a combination of C16.0+MSU for 24 hours. Release of IL-8 in the cell culture supernatants was measured by ELISA. Data are shown as individual points with a bar representing the median. **p<0.01; n=9.

Supplementary Figure S2. Butyrate inhibits C16.0/MSU-induced IL-1Ra

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium or 1 mM butyrate and subsequently stimulated with 50 μM C16.0 or a combination of 50 μM C16.0 and 300 μg/mL MSU crystals for 24 hours. Release of IL-1Ra, IL-10, and TGF-β1 in the cell culture supernatant was measured by ELISA. Data are shown as individual points with a bar representing the median. **p<0.01; n=9 in A and n=5 in B and C.
Supplementary Figure S3. HDAC inhibitors in combination with C16.0+MSU do not cause cell death in PBMCs after 24h culture

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium or a synthetic HDAC inhibitor (Givinostat, Panobinostat, ITF-A or ITF-B) and subsequently stimulated with a combination of 50 μM C16.0 and 300 μg/mL MSU crystals for 24 hours. Cell death was assessed by flow cytometry. Cells were stained with Annexin V-FITC and presented as percentage of live cells (FITC-negative population). All conditions include n=4, except ITF-B 25 and 50 nM (n=2).
Effects of oral butyrate supplementation on inflammatory potential of circulating peripheral blood mononuclear cells in healthy and obese males

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ABSTRACT

Objective

Sodium butyrate is well-known for its immune-modulatory properties. Studies until now only focused on the in vitro effects of butyrate or assessed local effects in the gut upon butyrate administration. In this trial, we studied the systemic anti-inflammatory effects induced by sodium butyrate supplementation in humans.

Methods

Nine healthy (Lean) and ten obese (metabolic syndrome group, MetSyn) males were given 4 grams sodium butyrate daily for 4 weeks. PBMCs were isolated before and after supplementation for direct stimulation experiments and induction of trained immunity by oxidized low-density lipoprotein (oxLDL), β-glucan, or Bacillus Calmette-Guérin vaccine (BCG).

Results

Butyrate supplementation moderately affected some of the cytokine responses in the MetSyn group. In the direct stimulation setup, effects of butyrate supplementation were limited. Interestingly, butyrate supplementation decreased oxLDL-induced trained immunity in the MetSyn group for LPS-induced IL-6 responses and Pam3CSK4-induced TNF-α responses. Induction of trained immunity by β-glucan was decreased by butyrate in the MetSyn group for Pam3CSK4-induced IL-10 production.

Conclusion

In this study, while having only limited effects on the direct stimulation of cytokine production, butyrate supplementation significantly affected trained immunity in monocytes of obese individuals with metabolic complications. Therefore, oral butyrate supplementation may be beneficial in reducing the overall inflammatory status of circulating monocytes in patients with metabolic syndrome.
INTRODUCTION

The sophisticated symbiosis between humans and colonizing microbes in the gut has been studied intensively during the last decades. Extensive research in the last years has shown that the microbial community living in our gut plays a significant role in maintaining our health. Its main functions include harvesting energy from dietary components, maintaining the intestinal mucosal barrier, and regulating immune responses (1,2). One important microbial process that has been linked to all of these functions is represented by the production of short-chain fatty acids (SCFAs), a biological process taking place in the large intestine (3).

For the digestion of the complex carbohydrates mainly found in fruit, vegetables and whole grains, we rely on fermentative metabolism by a group of anaerobic gram-positive bacteria in the colon. This process yields energy and SCFAs; mainly acetate, propionate and butyrate. Despite the fact that these metabolites consist of only a few carbon atoms, they have been shown to play a substantial role in health and disease. Butyrate in particular not only constitutes the main source of energy for colonocytes (4,5), it has also been the focus of research due to its potent immune-regulating effects. Many studies report inhibition of pro-inflammatory cytokines, NF-κB activation, and nitric oxide production by butyrate in vitro, although the exact effects appear to depend on cell type, stimulus and concentration of butyrate (6-10).

Two main mechanisms have been suggested to mediate the effects of butyrate. Firstly, butyrate was reported to be a broad histone deacetylase (HDAC) inhibitor, inhibiting most isoforms of class I and II (11-14). This leads to hyperacetylation of both histone and non-histone proteins such as transcription factors, altering gene expression patterns. Secondly, butyrate binds G protein-coupled receptors 41 and 43, which were later renamed to fatty acid receptors FFA3 and FFA2, respectively. FFA2 is found predominantly on peripheral blood mononuclear cells (PBMCs), monocytes, and neutrophils (15,16), but its exact role in regulating the immune system is still a topic of debate. Both protective and detrimental effects of FFA2 are reported in murine models of inflammatory diseases (17,18). In addition, it is unknown whether FFA2 is able to directly affect cytokine production or which pathways are involved in this.

The concentrations of butyrate required for HDAC inhibition and FFA2/FFA3 activation both exceed physiological levels in the peripheral blood, but are well within range of butyrate concentrations in the gut (12,15-19). We therefore envisage that monocytes can encounter high concentrations of butyrate in the gut and change their inflammatory potential to mediate systemic effects. Previous studies in mice have shown that oral butyrate inhibits cytokine production in peritoneal macrophages (20), protects from atherosclerotic lesions in the aorta (21), prevents diet-induced obesity, and increases insulin sensitivity and mitochondrial function (22). These results suggest that butyrate supplementation may be beneficial for the treatment of metabolic diseases in which exaggerated inflammation plays an important role such as diabetes, atherosclerosis and gout.

Although oral butyrate supplementation in humans has been shown to ameliorate clinical symptoms of inflammatory bowel disease (23,24), data on its effects on the inflammatory potential of circulating monocytes are lacking. In the present pilot study, healthy and obese subjects received 4 grams of butyrate daily for 4 weeks. We assessed the effects of butyrate supplementation on the capacity of circulating monocytes to produce innate immune
cytokines directly in response to a range of endogenous and pathogenic stimuli. Furthermore, we investigated whether oral butyrate influences the capacity of monocytes to be trained by oxLDL or microbial components to respond more vigorously to a secondary unrelated stimulus, a process described previously as trained innate immunity (25-27).

METHODS

Study setup

The ex vivo experiments performed in this study are part of the larger OBUGAT study (Effect of Oral BUTyrate on human Glucose and brown FAT metabolism), which has been performed in the Academic Medical Center in Amsterdam, the Netherlands (personal correspondence with prof. Nieuwdorp). In this study, participants received oral butyrate supplementation of 4 grams daily for 4 weeks. Before and after treatment, venous EDTA blood was drawn for ex vivo experiments, and short-chain fatty acid concentrations were determined in plasma and feces. This study was approved by the medical research ethics committee of the Academic Medical Center in Amsterdam (METC number 2013_229) and conducted according to the principles in the Declaration of Helsinki. All volunteers gave informed consent. The study was registered in the WHO International Clinical Trial Registry Platform on 9 January 2014 under the reference number NTR4392.

Subjects

In total 10 healthy lean males and 10 obese males were included in the study. To be eligible for inclusion in the study, subjects in the Lean group must be over 18 years of age and have a body mass index (BMI) of 19-25 kg/m². Exclusion criteria were smoking, medication use, and an estimated glomerular filtration rate of <60. For inclusion in the obese (MetSyn) group, subjects must be over 18 years of age, have a body mass index (BMI) of >25 kg/m², and possess 3 of the 5 criteria for metabolic syndrome. These have been described by the American Heart Association and National Heart, Lung, and Blood Institute as fasting plasma glucose ≥ 5.6 mmol/L, blood pressure ≥ 130/85 mmHg, triglycerides ≥ 1.69 mmol/L, high-density lipoprotein cholesterol < 1.03 mmol/L, or a waist circumference ≥ 102 cm (36). Exclusion criteria for this group are smoking and medication use.

SCFA measurement in plasma and feces

Acetate, propionate and butyrate concentrations were determined in overnight fasted plasma samples and feces by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (37). Feces were collected during 24 hours before study visits at baseline before butyrate supplementation and at 4 weeks after supplementation regime. Subjects kept the samples in the fridge at 3-4°C before the visit and transported it on ice packs.

PBMC isolation and direct stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Ficoll-Paque and resuspended in Dutch modified RPMI culture medium (Invitrogen)
supplemented with 50 µg/mL gentamycin (Centrafarm), 2 mM GlutaMAX and 1 mM pyruvate (Life Technologies). For the first stimulation experiments, the cells were cultured for 24 hours in the presence of 1x10⁶ CFU/mL heat-killed Escherichia coli (ATCC 35218, in house), 1x10⁶ CFU/mL Staphylococcus aureus (ATCC 29213, in house), 1x10⁶ CFU/mL Bacteroides fragilis (ATCC 25285, in house), 1 µg/mL Mycobacterium tuberculosis (H37Rv, in house), 1x10⁶ CFU/mL Borrelia burgdorferi (ATCC 35210), or 1x10⁶ Candida albicans conidia (UC820, in house). Secondly, in another experiment cells were cultured for 24 hours with 1 µg/mL Pam3CKS4 (EMC Microcollections), 1 ng/mL E. coli lipopolysaccharide (LPS, Sigma), or 50 µM palmitate (C16.0, Sigma, conjugated to human albumin as described in 12) alone or in combination with monosodium urate (MSU) crystals (made in house with uric acid, Sigma, and sodium hydroxide, Merck, as described in 12).

**Trained immunity experiments in adherent monocytes**

For the training experiment, PBMCs were plated in a flat-bottom 96-well plate and left to adhere at 37°C 5% CO₂ for 1 hour, after which the cells were gently washed three times with warm phosphate-buffered saline (PBS). Subsequently, the cells were cultured for 24 hours with 1 µg/mL β-glucan (kindly provided by professor David Williams, TN, USA), 10 µg/mL Bacillus Calmette-Guérin vaccine (BCG, Statens Serum Institute) or 10 µg/mL oxidized LDL (prepared in house as described before in 38). The cells were washed once with PBS at 37°C and left to rest for 5 days, replenishing culture medium with 10% human pooled serum at day 3. At day 6, the cells were restimulated for 24 hours with 10 µg/mL Pam3CSK4 or 10 ng/mL E. coli LPS (see figure 3).

**Cytokine measurements and blood cell counts**

After culturing the cells, supernatants were collected from the experiments. Interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1Ra), and tumor necrosis factor-α (TNF-α) concentrations in the supernatant were determined with commercially available ELISA kits from R&D Systems, and the ELISA kits for IL-6 and IL-10 were purchased from Sanquin. Blood differential test was performed with whole EDTA blood by the clinical diagnostic laboratory of the Radboudumc in Nijmegen, the Netherlands.

**Statistical analyses**

The Lean and MetSyn groups of the study were compared using the Mann-Whitney U test and time points within one group were compared with the Wilcoxon signed rank test, both performed with Graphpad Prism software version 6.0. Due to the small group numbers in this pilot study no correction for multiple testing was applied. The number of subjects per group may differ for the different ex vivo experiments due to exclusion of donors giving a cytokine response to RPMI controls. Exact numbers per experiment are stated in the figure legends.
RESULTS

Baseline characteristics

Ten lean males and ten obese males were included in the analysis. At baseline, the obese (MetSyn) group was characterized by a higher age, BMI, diastolic blood pressure, fasting plasma glucose, plasma low-density lipoprotein cholesterol, plasma triglycerides, and neutrophils counts, as well as lower plasma high-density lipoprotein cholesterol (Table 1). General data and metabolic syndrome parameters in table 1 have also been published as baseline characteristics in a previous publication on the same population (table 1 in 28). There is a slight difference with the numbers in the previously published table due to the exclusion of one donor in the other paper (28).

<table>
<thead>
<tr>
<th>General</th>
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<th>MetSyn group</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age</td>
<td>25 ± 2.4</td>
<td>42 ± 2.4 ***</td>
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Metabolic syndrome parameters

<table>
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<tbody>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.0 ± 2.3</td>
<td>33.2 ± 3.6 ***</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 ± 9</td>
<td>139 ± 16</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>75 ± 6</td>
<td>82 ± 6</td>
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<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>4.4 ± 0.3</td>
<td>4.9 ± 0.4</td>
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Circulating lipids

<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.2 ± 0.8</td>
<td>4.9 ± 0.7</td>
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<td>High-density lipoprotein cholesterol (mmol/L)</td>
<td>1.4 ± 0.2</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>Low-density lipoprotein cholesterol (mmol/L)</td>
<td>2.3 ± 0.6</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.8 ± 0.3</td>
<td>1.8 ± 0.3</td>
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Blood cell differentiation

<table>
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<th>MetSyn group</th>
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<tbody>
<tr>
<td>Neutrophil count (x10⁹/L)</td>
<td>2.49 ± 0.70</td>
<td>3.23 ± 0.31</td>
</tr>
<tr>
<td>Lymphocyte count (x10⁹/L)</td>
<td>1.94 ± 0.65</td>
<td>1.92 ± 0.52</td>
</tr>
<tr>
<td>Monocyte count (x10⁹/L)</td>
<td>0.48 ± 0.09</td>
<td>0.56 ± 0.12</td>
</tr>
</tbody>
</table>

At baseline, various parameters were measured to define the two subject groups. Statistically significant differences between the two subject groups are represented as follows: *p<0.05, **p<0.001, ***p<0.0001. Data are presented as mean ± standard deviation. Part of data from this table is also published in (28).

Short-chain fatty acid concentrations in plasma and feces

The effects of 4 weeks oral sodium butyrate supplementation were assessed by measuring SCFA levels in the plasma and feces and were compared between volunteers in the Lean and MetSyn group. At baseline, the MetSyn group showed a different composition of plasma SCFA, with a lower percentage of acetate and higher percentages of propionate and butyrate (Table 2) compared to the Lean group. There were no baseline differences in fecal SCFA concentrations between the two groups (Table 3). In contrast, butyrate supplementation affected the fecal SCFA concentrations to a greater extent than plasma levels. Only the plasma propionate concentration was decreased in the MetSyn group after treatment compared to baseline. In feces, total SCFA, acetate, and propionate concentrations were significantly lower after 4-week butyrate supplementation in both the Lean and the MetSyn group. Fecal butyrate concentrations were only significantly decreased after butyrate supplementation in the MetSyn group.
Table 2. SCFA concentrations in plasma before and after oral butyrate supplementation

<table>
<thead>
<tr>
<th>Group</th>
<th>Time point</th>
<th>Total SCFA (µM)</th>
<th>Acetate (µM)</th>
<th>Acetate (%)</th>
<th>Propionate (µM)</th>
<th>Propionate (%)</th>
<th>Butyrate (µM)</th>
<th>Butyrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>0</td>
<td>112.3 ± 45.5</td>
<td>101.1 ± 43.3</td>
<td>89.5 ± 3.4</td>
<td>81 ± 3.3</td>
<td>7.6 ± 2.9</td>
<td>31 ± 0.9</td>
<td>28 ± 0.7</td>
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<tr>
<td>Lean</td>
<td>4w</td>
<td>114.2 ± 38.3</td>
<td>103.0 ± 34.2</td>
<td>90.4 ± 2.7</td>
<td>70 ± 3.7</td>
<td>6.1 ± 2.3</td>
<td>43 ± 3.4</td>
<td>35 ± 18</td>
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<tr>
<td>MetSyn</td>
<td>0</td>
<td>79.9 ± 23.1</td>
<td>68.6 ± 20.7</td>
<td>85.6 ± 2.8*</td>
<td>8.2 ± 2.7</td>
<td>10.4 ± 2.8*</td>
<td>32 ± 10</td>
<td>40 ± 0.8**</td>
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<tr>
<td>MetSyn</td>
<td>4w</td>
<td>67.7 ± 28.2</td>
<td>59.0 ± 25.5</td>
<td>86.9 ± 5.0</td>
<td>5.6 ± 2.6*</td>
<td>8.6 ± 3.9</td>
<td>31 ± 28</td>
<td>45 ± 3.2</td>
</tr>
</tbody>
</table>

Short chain fatty acid (SCFA) concentrations in plasma were measured before and after butyrate supplementation in healthy lean males (Lean) and obese males (MetSyn). * represents a statistically significant difference at baseline between the Lean and MetSyn groups, *p<0.05. **p<0.01. # represents a statistically significant difference between time point 0 and time point 4w (4 weeks), p<0.05. Data are presented as mean ± standard deviation.

Table 3. SCFA concentrations in feces before and after oral butyrate supplementation

<table>
<thead>
<tr>
<th>Group</th>
<th>Time point</th>
<th>Total SCFA (µM)</th>
<th>Acetate (µM)</th>
<th>Acetate (%)</th>
<th>Propionate (µM)</th>
<th>Propionate (%)</th>
<th>Butyrate (µM)</th>
<th>Butyrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>0</td>
<td>344.2 ± 220.6</td>
<td>213.6 ± 146.1</td>
<td>61.7 ± 3.8</td>
<td>73.0 ± 61.9</td>
<td>20.1 ± 41</td>
<td>57.6 ± 29.4</td>
<td>18.2 ± 5.6</td>
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<tr>
<td>Lean</td>
<td>4w</td>
<td>186.1 ± 79.8*</td>
<td>114.4 ± 49.5*</td>
<td>62.1 ± 10.2</td>
<td>41.0 ± 22.0*</td>
<td>21.6 ± 4.5</td>
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<td>16.3 ± 8.0</td>
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<tr>
<td>MetSyn</td>
<td>0</td>
<td>340.6 ± 141.9</td>
<td>208.3 ± 97.6</td>
<td>60.1 ± 7.9</td>
<td>78.3 ± 36.9</td>
<td>23.8 ± 6.8</td>
<td>54.0 ± 23.9</td>
<td>16.2 ± 4.1</td>
</tr>
<tr>
<td>MetSyn</td>
<td>4w</td>
<td>226.9 ± 129.0**</td>
<td>139.8 ± 85.3**</td>
<td>61.9 ± 9.7</td>
<td>44.5 ± 25.9**</td>
<td>20.3 ± 5.9</td>
<td>42.5 ± 34.8**</td>
<td>17.9 ± 8.5</td>
</tr>
</tbody>
</table>

Short chain fatty acid (SCFA) concentrations in feces were measured before and after butyrate supplementation in healthy lean males (Lean) and obese males (MetSyn). * represents a statistically significant difference between time point 0 and time point 4w (4 weeks), *p<0.05 **p<0.01. Data are presented as mean ± standard deviation.
**Effects of sodium butyrate treatment on total blood cell counts**

To study the possible effects of sodium butyrate supplementation on white blood cell counts, blood differential tests were performed before and after butyrate supplementation. As mentioned above, only neutrophil counts differed significantly at baseline between the lean and obese groups (Table 1). No differences were found in percentages or absolute counts of neutrophils, lymphocytes, or monocytes in whole blood between the time points before and after supplementation (Supplemental Figure 1).

**Cytokine production of circulating peripheral blood mononuclear cells**

To assess the effects of oral sodium butyrate supplementation on the cytokine-producing capacity of PBMCs, we stimulated the cells with a range of heat-killed human pathogens. An overall glance on the heatmap (Figure 1A) representing the mean fold increase or decrease in cytokine production after butyrate supplementation, shows that the differences are mostly small. The following panels in figure 1 show the absolute cytokine production of stimuli-cytokine combinations that have a statistically significant effect or show larger effects in the heatmap. Baseline comparison between the groups showed that PBMCs from MetSyn volunteers were only less capable of mounting an IL-6 response to stimulation with *Bacteroides fragilis* compared to volunteers in the Lean group (Figure 1B). After 4 weeks of oral butyrate supplementation, PBMCs from MetSyn subjects produced less IL-1β in response to *Candida albicans* (Figure 1F) compared to baseline. As shown by the IL-10 production in response to *Escherichia coli* (Figure 1A, J), there is sometimes large donor variation, causing the heatmap to show somewhat biased differences.

Because gouty arthritis is highly associated with metabolic syndrome, PBMCs were also stimulated with a combination of monosodium urate crystals (MSU) and Toll-like receptor ligands to induce a potent cytokine response. No differences in cytokine production were observed between the Lean and MetSyn groups at baseline (Figure 2). The heatmap even shows mainly increases in IL-1β and IL-6, and decreases in the anti-inflammatory cytokine IL-1Ra (Figure 2A). Surprisingly, the IL-1β production in response to a combination of MSU crystals and palmitic acid (C16:0) was significantly increased in the MetSyn group after oral butyrate supplementation (Figure 2B).
Figure 1. Effects of 4-week butyrate supplementation on PBMC cytokine responses to pathogens

Freshly isolated PBMCs from healthy lean volunteers (Lean) and metabolic syndrome patients (MetSyn) were cultured for 24 hours in the presence of pathogens before and after 4-week supplementation with 4 grams oral butyrate daily. (A) A heatmap shows the mean fold decrease or increase in cytokine production after supplementation in both groups. (B-J) Stimuli-cytokine combinations showing a substantial decrease or increase in panel A are highlighted in separate graphs to show the absolute cytokine values at baseline (BL) and 4 weeks (4w). Lean n=9, MetSyn n=10.
Figure 2. Effects of 4-week butyrate supplementation on PBMC cytokine responses to monosodium urate crystals
Freshly isolated PBMCs from healthy lean volunteers (Lean) and metabolic syndrome patients (MetSyn) were cultured for 24 hours in the presence of monosodium urate crystals in combination with inflammatory stimuli before and after 4-week supplementation with 4 grams oral butyrate daily. (A) A heatmap shows the mean fold decrease or increase in cytokine production after supplementation in both groups. (B-G) Stimuli-cytokine combinations showing a substantial decrease or increase in panel A are highlighted in separate graphs to show the absolute cytokine values at baseline (BL) and 4 weeks (4w). Lean n=10, MetSyn n=7.

Training capacity of innate immune cells
One additional feature of monocytes is their capacity to be trained to produce more cytokines upon secondary stimulation with an unrelated stimulus (29). This process is mediated through epigenetic reprogramming and metabolic rewiring of the macrophages and can, among others, be achieved by oxidized low-density lipoprotein (oxLDL), β-glucan, or Bacillus Calmette-Guérin vaccine (BCG) (25,27,30). To study the effect of butyrate supplementation
on this process, we measured the training capacity of adherent monocytes before and after the 4-week supplementation according to the schedule in figure 3. The heatmap (Figure 4A) shows a few decreases after butyrate production in the MetSyn group. The three strongest specific comparisons (Figure 4B, E, and G) yield statistical significance. After 4 weeks of butyrate supplementation, training induced by β-glucan upon restimulation with Pam3CSK4 was decreased for IL-10 production in the MetSyn group (Figure 4B). Additionally, monocytes isolated from volunteers in the MetSyn group displayed a decreased training capacity by oxLDL after butyrate supplementation. Upon restimulation with Pam3CSK4, the fold increase in TNF-α production was decreased compared to baseline (figure 4E), and upon restimulation with LPS there was a significantly reduced up-regulation of IL-6 (Figure 4G). There were no differences in baseline training capacity between the Lean and the MetSyn group.

**Figure 3. In vitro innate immune training setup**

In freshly isolated adherent monocytes from healthy lean volunteers (Lean) and metabolic syndrome patients (MetSyn) training was induced by 24-hour culture with β-glucan, Bacillus Calmette-Guérin vaccine (BCG) or oxidized low-density lipoprotein (oxLDL). The training stimulus was washed out with warm phosphate-buffered saline and cells were rested for 5 days in medium with 10% pooled human serum. On day 6, cells were restimulated with 10 μg/mL Pam3CSK4 or 10 ng/mL E. coli lipopolysaccharide (LPS) for 24 hours, after which supernatant was collected for cytokine ELISAs.
Figure 4. Effects of 4-week butyrate supplementation on monocyte training capacity

In freshly isolated adherent monocytes from healthy lean volunteers (Lean) and metabolic syndrome patients (MetSyn) training was induced as depicted in figure 3. This experiment was performed before and after 4-week supplementation with 4 grams oral butyrate daily. (A) A heatmap shows the mean fold decrease or increase in cytokine production with training after supplementation in both groups. (B-G) Stimuli-cytokine combinations showing a substantial decrease or increase in panel A are highlighted in separate graphs to show the fold change with training at baseline (BL) and 4 weeks (4w). Lean n=7, MetSyn n=7.

In figure 5, we show the absolute cytokine responses of the training experiments in which butyrate supplementation had a statistically significant effect. When looking at absolute cytokine levels instead of fold change with training, we observe no significant effects in the IL-10 production after β-glucan training and Pam3CSK4 restimulation (Figure 5A). However, the TNF-α production upon training with oxLDL and restimulation with Pam3CSK4 is significantly lower compared to the absolute cytokine levels before butyrate supplementation (Figure 5B). The same is observed with IL-6 production after training with oxLDL and restimulation with LPS (Figure 5C).
Effects of oral butyrate supplementation on PBMC inflammatory potential in healthy and obese males

Figure 5. Effects of 4-week butyrate supplementation on absolute cytokine production in trained monocytes

This figure depicts the absolute cytokine responses in trained monocytes of those experimental setups in figure 4 that were significantly affected by butyrate supplementation. (A) shows the IL-10 responses in both groups at baseline (BL) and 4 weeks (4w) after Pam3CSK4 stimulation with and without β-glucan training. (B) shows the absolute TNF-α responses in both groups after Pam3CSK4 stimulation with and without oxLDL training. (C) shows the IL-6 responses in both groups after LPS stimulation with and without oxLDL training. Time points within one group of volunteers were compared with a Mann-Whitney U-test. Lean n=7, MetSyn n=7.
DISCUSSION

In the present study we assessed the effects of 4 weeks oral sodium butyrate supplementation on the inflammatory potential of PBMCs in healthy lean and obese male human volunteers. To our knowledge this is the first clinical trial studying the influence of butyrate supplementation on systemic inflammatory responses in humans.

We show here that in comparison to the potent anti-inflammatory effects of butyrate observed predominantly in *in vitro* studies, the effects of oral butyrate supplementation on *ex vivo* cytokine production by PBMCs in this study were limited. Direct stimulation of PBMCs resulted in decreased IL-1β production upon stimulation with *Candida albicans* conidia, but increased C16.0+MSU-induced IL-1β after butyrate supplementation in the MetSyn group. This is in contrast to the direct and potent anti-inflammatory effect of butyrate on C16.0+MSU-induced *in vitro* cytokine production (12). However, in the latter setup relatively high concentrations of butyrate were required to suppress histone deacetylases and the subsequent production of pro-inflammatory cytokines.

More consistent effects were found when assessing the impact of butyrate supplementation on the induction of trained innate immunity. Here, butyrate supplementation decreased oxLDL-induced trained immunity in the MetSyn group for LPS-induced IL-6 responses and Pam3CSK4-induced TNF-α responses. This effect was also seen when looking at absolute cytokine levels instead of training-induced fold increases. In addition, training with β-glucan was decreased for Pam3CSK4-induced IL-10 production. Interestingly, all effects observed after oral butyrate supplementation are limited to the MetSyn group. In the context of metabolic syndrome, especially oxLDL-induced trained innate immunity has been implicated to play a role in the development of atherosclerosis (31). Short-term exposure of monocytes to oxLDL was shown to induce long-lasting epigenetic modifications that subsequently lead to a more pro-inflammatory macrophage phenotype (27). Therefore, oral butyrate supplementation may be beneficial in preventing oxLDL-induced training of macrophages in patients with metabolic syndrome, possibly slowing down the process of vascular wall inflammation and the development of atherosclerosis.

Within the scope of the current study, we can only speculate on the mechanism of the effects of oral butyrate supplementation on systemic inflammatory responses. Our results suggest that the effects of oral butyrate supplementation are limited when looking at direct cytokine responses, but it may be able to inhibit the induction of trained innate immunity. From previous *in vitro* work we have seen that high concentrations of butyrate can induce inhibition of class I histone deacetylases, leading to inhibited cytokine production in PBMCs (12). Possibly, butyrate concentrations after supplementation are not high enough, or its effects on histone deacetylases do not last long enough to inhibit cytokine responses upon direct stimulation of PBMCs.

The long-lasting effects of trained innate immunity are mediated through epigenetic reprogramming at the level of histone methylation (25). Additionally, trained macrophages are characterized by a metabolic shift from mitochondrial oxidative phosphorylation to aerobic glycolysis that is mediated through mammalian target of rapamycin (mTOR) activation (26). In contrast, butyrate has been shown to induce mitochondrial respiration and fatty acid oxidation in various *in vitro* and murine studies (4,22,32). Based on this, it can be hypothesized that oral
butyrate supplementation leads to high local concentrations of butyrate in the gut, where it can affect cellular metabolism of circulating monocytes, making them less susceptible to training. Furthermore, our observation that only the training capacity of PBMCs from obese males is affected by butyrate supplementation suggests there might be a role for microbiome or diet in this group, or that PBMCs from these subjects are more susceptible to the changes induced by butyrate.

The current study has several limitations. Firstly, this was a pilot study with small numbers in each group, providing only limited power to detect differences in cytokine production as these already show high variability among donors. Secondly, we were unable to detect any increases in butyrate concentration in the feces or plasma after 4 weeks of supplementation. Most likely, this is because butyrate is very quickly utilized as an energy source by colonocytes. With only an estimated 5% of SCFAs being excreted through feces (33) and almost all of them present in circulation being metabolized by the liver (34), neither plasma nor fecal SCFA concentration are a good measure of the transiently increased SCFA concentrations in the gut. In a recent paper, Canfora et al. shows that the increase in plasma butyrate concentration after colonic administration of sodium butyrate is very quick and already undetectable at 2 hours after colonic infusion (35). The 4 weeks of butyrate supplementation did not lead to a stable increase in plasma SCFA levels, due to which we were unable to correlate changes in cytokine production directly to plasma SCFAs. After 4 weeks of butyrate supplementation we even detected decreases in plasma propionate, and fecal total SCFAs, acetate, propionate and butyrate. Within the scope of this study, we unfortunately have no method of exploring why this is occurring. Possibly, the addition of oral butyrate induced flux changes for acetate and propionate as well.

In conclusion, twice daily intake of 2 grams of sodium butyrate for 4 weeks most likely only transiently increased butyrate concentration in the gut. We found that this leads to a decreased training capacity of monocytes by oxLDL and β-glucan in the group of obese males. Oral butyrate supplementation may therefore be beneficial to reduce the overall inflammatory phenotype of circulating monocytes in metabolic syndrome patients and possibly even slow down the development of vascular wall inflammation and atherosclerosis. However, this will need to be studied in larger groups while closely looking at influences of the microbiome and diet on SCFA fluxes, and extensively examining vascular wall inflammation and atherosclerotic plaque formation.
REFERENCES

Effects of oral butyrate supplementation on PBMC inflammatory potential in healthy and obese males


Supplemental Figure 1. Blood cell counts before and after butyrate supplementation
Absolute counts and percentages of monocytes, lymphocytes and neutrophils were measured before and after butyrate supplementation. Baseline (BL) neutrophil counts were significantly higher in the MetSyn group compared to the lean individuals (panel E). No statistical significance was detected when comparing values before (BL) and after butyrate supplementation (4w).
Romidepsin suppresses monosodium urate crystal-induced cytokine production through upregulation of suppressor of cytokine signaling 1 expression


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ABSTRACT

Background

Acute gouty arthritis currently is the most common form of inflammatory arthritis in developed countries. Treatment is still suboptimal. Dosage of urate-lowering therapy is often too low to reach target urate levels, and adherence to therapy is poor. In this study, we therefore explore a new treatment option to limit inflammation in acute gout: specific histone deacetylase (HDAC) inhibition.

Methods

Peripheral blood mononuclear cells (PBMCs) were cultured with a combination of monosodium urate crystals (MSU) and palmitic acid (C16.0) in order to activate the NLRP3 inflammasome and induce IL-1β production. HDAC inhibitors and other compounds were added beforehand with a 1-h pre-incubation period.

Results

The HDAC1/2 inhibitor romidepsin was most potent in lowering C16.0+MSU-induced IL-1β production compared to other specific class I HDAC inhibitors. At 10 nM, romidepsin decreased IL-1β, IL-1Ra, IL-6 and IL-8 production. IL-1β mRNA was significantly decreased at 25 nM. Although romidepsin increased PTEN expression, PBMCs from patients with germline mutations in PTEN still responded well to romidepsin. Romidepsin also increased SOCS1 expression and blocked STAT1 and STAT3 activation. Furthermore, experiments with bortezomib showed that blocking the proteasome reverses the cytokine suppression by romidepsin.

Conclusions

Our results show that romidepsin is a very potent inhibitor of C16.0+MSU-induced cytokines in vitro. Romidepsin upregulated transcription of SOCS1 which was shown to directly target inflammatory signaling molecules for proteasomal degradation. Inhibiting the proteasome therefore reversed the cytokine-suppressive effects of romidepsin. HDAC1/2 dual inhibition could therefore be a highly potent new treatment option for acute gout, although safety has to be determined in vivo.
INTRODUCTION

Acute gouty arthritis is an agonizingly painful and debilitating disease and is currently the most common form of inflammatory arthritis in developed countries. Its prevalence has been rising steadily over the past decades (1,2), and it is currently estimated to affect 1-4% of the general population in most countries in Europe and in the USA (3-6).

The most important risk factor for the development of acute gout is hyperuricemia. Urate is a breakdown product of purine metabolism and its levels can be elevated in the blood due to several causes, including genetic variations in urate transporter genes or a purine-rich diet. When urate reaches supersaturation in the biologic fluids or tissues, it precipitates as monosodium urate crystals (MSU) (7). Interestingly however, the majority of hyperuricemic individuals stay asymptomatic throughout their life (2), and some patients have normal urate levels during an acute gout attack (8), indicating that induction of clinical symptoms by MSU crystals is a process that is dependent on many factors.

Nevertheless, the main prerequisite for development of acute gout are MSU crystal deposits in the joint. It has been known for about a decade now that MSU crystals trigger inflammatory arthritis through activation of the nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3 (NLPR3) inflammasome (9). Assembly of this protein scaffold activates procaspase-1, which in turn can cleave inactive pro-IL-1β into its bioactive form. However, for the initial production of pro-IL-1β, an additional signal is required, such as IL-1β binding to the IL-1 receptor, or Toll-like receptor (TLR) ligands. Saturated long-chain fatty acids, such as palmitic acid (C16.0) and stearic acid (C18.0) can also provide this first signal and synergize with MSU crystals to produce large quantities of active IL-1β (10,11).

Despite the elaborate knowledge on the pathophysiology of gout and the fact that it is curable, treatment is still suboptimal in many cases. Dosage of urate-lowering therapy is often too low to reach target urate levels, and adherence to therapy is poor among gout patients (12). Furthermore, many of the comorbidities associated with gout actually result in contraindications for the currently available anti-inflammatory medication used in acute gout (13). Biologics (recombinant antibodies) blocking IL-1β are advised in case NSAIDs and colchicine are contraindicated (12), but they are very costly (14). In the current study, we investigated the potential of specific histone deacetylase (HDAC) inhibitors to suppress MSU-dependent cytokine production.

HDACs are enzymes capable of removing acetyl groups from protein lysine residues. With this ability, they can alter gene expression (when deacetylating histone lysines) or affect the function of non-histone proteins such as transcription factors or other enzymes (15). The HDAC enzymes are divided into four classes based on phylogenetic analysis: class I yeast Rpd3-like deacetylases (HDAC1, HDAC2, HDAC3, and HDAC8), class II yeast Hda1-like deacetylases (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10), class III yeast Sir2-like sirtuins (SIRT1-7), and class IV deacetylase (HDAC11). The classical HDACs of classes I, II and IV, and the sirtuins of class III make up two distinct families (16,17). Together, these enzymes can elicit widespread effects through changing gene transcription and posttranscriptional modification of proteins.
Next to several naturally occurring HDAC inhibitors, such as the short-chain fatty acid butyrate and Streptomyces-derived trichostatin A (16), a range of synthetic inhibitors has been developed, with the primary aim of treating malignancies. The rationale behind this use is the HDAC-mediated suppression of genes involved in apoptosis, cell cycle arrest and tumor suppression (18). However, in recent years research has revealed a potential use of HDAC inhibitors to treat inflammatory diseases.

In the current study, we attempt to pinpoint the individual HDACs that could play a role in acute gouty arthritis by assessing the effects of specific HDAC inhibitors on C16.0+MSU-induced cytokine production by human PBMCs in vitro.

**MATERIALS AND METHODS**

*Ethics approval and consent to participate*

Buffy coats from healthy donors were obtained after written informed consent from Sanquin Blood Bank, Nijmegen, the Netherlands. Blood collection from Cowden syndrome patients was approved by the accredited medical research and ethics committee of the region Arnhem/Nijmegen in the Netherlands (reference 2014/147). Written informed consent was obtained before inclusion. All experiments with human material were performed according to the declaration of Helsinki.

*Reagents and inhibitors*

MSU crystals were produced in-house from urate (Sigma) and sodium hydroxide (Merck) as described previously (11) Palmitic acid (Sigma) dissolved in 100% ethanol and human albumin (Albuman 200g/L, Sanquin, Amsterdam) were conjugated as described previously (11). Romidepsin, entinostat, santacruzamate A, RGFP966, and bortezomib were purchased from Selleckchem. Etomoxir was purchased from Sigma. HDAC6 inhibitor ITF3107 was kindly provided by Italfarmaco SpA, Milan, Italy.

*PBMC stimulation experiments*

Venous blood was drawn from healthy donors or Cowden syndrome patients. PBMCs were isolated by means of Ficoll-Paque (GE Healthcare) density gradient centrifugation. Cells were plated at 0.5x10^6 cells per well in a U-bottom 96-well plate with Dutch Modified RPMI 1640 medium (Life Technologies) supplemented with 50 μg/mL Gentamycin (Centrafarm), 2 mM GlutaMAX and 1 mM pyruvate (Life Technologies). Cytokine responses to mimic gout in vitro were induced by adding a combination of 300 μg/mL MSU crystals and 50 μM C16.0. For all experiments the cells were cultured for 24 h.
Cytokine measurements

Commercially available ELISA kits (R&D Systems) were purchased for IL-1β, IL-1Ra, IL-6, IL-8, and IL-10, which were used according to the manufacturer’s protocol. Intracellular IL-1β and IL-1Ra were determined in the supernatant of a cell lysate. Cells were lysed using 0.5% Triton X-100 (Sigma).

Quantitative PCR

RNA was isolated using a phase separation method with TRizol reagent (Life Technologies) and chloroform (Merck) in a 5:1 ratio. Subsequently, the RNA was precipitated with 2-propanol (Merck). Reverse transcription into cDNA was performed using iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was done using a SYBR Green PCR master mix (Life Technologies).

Flow cytometry

Cells were fixed and permeabilized with FIX & PERM Cell Permeabilization Kit (Invitrogen) and subsequently stained with CD45-KO (Beckman Coulter) and p-STAT1-PE or p-STAT3-PE (eBioscience). Samples were measured on the Beckman Coulter CytoFLEX. During analysis, cells were gated on being CD45+ to eliminate debris. Gating on monocyte and lymphocyte populations was done in the forward versus side scatter plot.

Statistics

All figure panels include ≥6 donors divided over at least two experiments. Conditions were compared to C16.0+MSU alone (unless stated otherwise in the figures) with a Wilcoxon signed rank test.
RESULTS

Effects of specific HDAC inhibitors on decreasing C16.0+MSU-induced IL-1β.

Because the first production of IL-1β is of crucial importance for the development of acute gouty arthritis, we tested the ability of several specific HDAC class I inhibitors to decrease its production in PBMCs in response to a combination of C16.0 and MSU (Figure 1). In a previous study, we have shown that broad class I HDAC inhibition suppresses C16.0+MSU-induced cytokine production, but specific HDAC8 inhibition did not have a cytokine-suppressive effect (11). Here, both romidepsin and entinostat significantly reduced C16.0+MSU-induced IL-1β production (Figure 1a, b). However, the effect of romidepsin was significantly more potent, decreasing up to 80% of the IL-1β production, whereas entinostat inhibited up to 34%. The other specific HDAC inhibitors did not have any effect on C16.0+MSU-induced IL-1β production.

![Figure 1. Screening of different class I HDAC inhibitors for their suppressive effect on C16.0+MSU-induced IL-1β](image-url)

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 h with different HDAC inhibitors: a romidepsin (Romi, HDAC1/2 inhibitor), b entinostat (Entino, HDAC1/3 inhibitor), c Santacruzamate A (SCZM, HDAC2 inhibitor), d RGFP966 (RGFP, HDAC3 inhibitor), and e ITF3107 (ITF, HDAC6 inhibitor). IL-1β production was induced by adding a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU) for 24 h. Data are represented as percentage change compared to the IL-1β production with C16.0+MSU alone.
HDAC1/2 inhibitor romidepsin strongly inhibited C16.0+MSU-induced cytokines

Due to the potent cytokine-suppressive effects observed with HDAC1/2 inhibitor romidepsin, we decided to further examine its effects. As shown in figure 2, already at a dose as low as 10 nM romidepsin significantly inhibited IL-1β, IL-6, IL-8, and IL-1Ra production in response to C16.0+MSU stimulation in PBMCs. In addition, intracellular IL-1β levels were decreased significantly as well (Figure 2d). C16.0 alone only slightly increased the production of anti-inflammatory IL-10 (Additional figure S1), and there is an insignificant trend towards IL-10 decrease when romidepsin is added.

Figure 2. Suppressive effects of Romidepsin on C16.0+MSU-induced cytokine production in human PBMCs
Freshly isolated PBMCs were pre-incubated with different concentrations of romidepsin for 1 h, after which cytokine production was induced via addition of a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU). The supernatant was collected for extracellular cytokines (a-c, f). Cells were lysed with 0.5% Triton X-100, and subsequently, the supernatant of the lysate was collected for measurement of intracellular cytokines (d-e).
Effects of HDAC1/2 inhibition on cell viability and transcriptional levels

After determining that romidepsin effectively inhibits C16.0+MSU-induced cytokine production, we also examined the transcription of IL-1β and inflammasome-related genes. In line with previous observations (11), we see that the combination of C16.0+MSU induces a similar amount of IL-1β as is induced by C16.0 alone (Figure 3a). This supports the current theory of the two signals that are required to induce active IL-1β, as is described in the introduction. Whereas MSU crystals induce NLRP3 assembly and caspase-1 activation, it has no effect on transcription of pro-IL-1β. This signal is provided by C16.0.

In figure 3a, we show that IL-1β mRNA transcription was induced dramatically by C16.0 stimulation and was almost brought back to baseline levels by romidepsin. mRNA levels of NLRP3 inflammasome components were not as consistently modified by romidepsin. The lowest concentration of romidepsin of 10 nM significantly decreased CASP1 and increases NLRP3 transcription (Figure 3b, d). The higher concentrations of romidepsin increased transcription of adaptor protein ASC (PYCARD) in comparison to C16.0+MSU alone, but not in comparison to the medium control (Figure 3e). Following the drastic decreases in cytokine production upon addition of romidepsin, we wanted to ensure that cells were still viable after incubation by means of flow cytometry with Annexin V (AnV) and propidium iodide (PI) staining. Neither stimulation with C16.0+MSU, nor addition of romidepsin affected the percentage of live (AnV and PI negative) cells (Figure 3c). When looking at the stratification of early (AnV+PI-) and late (AnV+PI+) apoptotic cells, only the percentage of late apoptotic cells was increased with 50 nM romidepsin (Figure 3f).
Figure 3. Effects of romidepsin on cell viability and transcription of IL-1β and NLRP3 inflammasome components

Freshly isolated PBMCs from healthy donors were pre-incubated with different concentrations romidepsin for 1 h, after which a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU) was added to induce cytokine production. After 24 h of culture, mRNA was measured for IL1B (a), CASP1 (b), NLRP3 (d), and PYCARD (e). Percentages of Annexin V+ and PI+ were measured by flow cytometry to determine cell viability (c, f).
In the context of cancer, many research groups have associated HDAC inhibition with upregulation of tumor suppressor phosphatase and tensin homologue (PTEN) and subsequent inhibition of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (19-22). As this pathway can play an important role in cellular metabolic and inflammatory status (23-25), we assessed whether romidepsin affected PTEN expression levels. As shown in figure 4a, romidepsin significantly increased PTEN expression. In addition, mRNA levels of carnitine palmitoyltransferase IA (CPT1A) were also significantly elevated by romidepsin (Figure 4b). CPT1A shuttles long-chain fatty acids, such as C16.0, into the mitochondria, comprising the rate-limiting step in the process of fatty acid oxidation. This process can be in turn regulated via the Akt signaling pathway (26,27). Pre-incubating the cells with etomoxir, an irreversible CPT1 inhibitor, which inhibits fatty acid oxidation, increased the IL-1β production in response to C16.0+MSU (Figure 4c). To assess if PTEN upregulation mediates the cytokine-suppressive effects of romidepsin, we compared its effects in PBMCs from healthy individuals to the effects in PBMCs isolated from Cowden syndrome patients, who have a loss of function in the PTEN protein due to germline mutations. Loss of function in PTEN, however, did not reverse IL-1β suppression by romidepsin.
Romidepsin inhibits MSU-induced cytokine production via upregulation of SOCS1 expression

Romidepsin inhibits MSU-induced cytokine production via upregulation of SOCS1 expression

Figure 4. Romidepsin-induced increased expression of PTEN and CPT1A is independent of cytokine suppression

PBMCs from healthy volunteers or Cowden syndrome patients were pre-incubated for 1 h with several concentrations of romidepsin (Romi) or etomoxir. Cytokine production was induced by adding a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU). After 24 h, PTEN (a) and CPT1A (b) mRNA expression was determined by qPCR. IL-1β production was measured by ELISA after addition of etomoxir (c) or romidepsin in healthy volunteers and Cowden syndrome patients (d).

Romidepsin induced SOCS1 expression and inhibited activation of STAT1 and STAT3

As HDAC1 and HDAC2 act primarily by deacetylating accessible chromatin, we envisaged that the cytokine-suppressive effects may be mediated through upregulation of anti-inflammatory genes. Suppressor of cytokine signaling (SOCS)1 and SOCS3 are important negative regulators of inflammation and their genetic codes both include binding sites for HDAC1 and HDAC2. (Figure 5a). Romidepsin effectively upregulated expression of SOCS1 (Figure 5b), but not that of SOCS3 (Figure 5e). Furthermore, romidepsin inhibited activation of the inflammatory transcription factors signal transducer and activator of transcription (STAT1) (Figure 5c, d) and STAT3 (Figure 5f, g).
Figure 5. Romidepsin induced transcription of SOCS1 and inhibited activation of STAT1 and STAT3

Schematic representations of the SOCS1 and SOCS3 genome and their binding sites for class I HDACs were retrieved from the USCS Genome Browser by means of the track “Transcription Factor ChIP-seq (161 factors) from ENCODE with Factorbook Motifs” (a). PBMCs from healthy volunteers were pre-incubated with romidepsin, after which a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU) was added. After 24 h, mRNA was isolated and qPCR was performed for SOCS1 and SOCS3 (b, e). Cells were stained extracellularly with CD45-KO and intracellularly with either p-STAT1-PE (c, d) or p-STAT3-PE (f, g) for flow cytometry. Gating on CD45+ lymphocytes and monocytes was performed in the forward versus side scatter graph.
Romidepsin inhibits MSU-induced cytokine production via upregulation of SOCS1 expression

SOCS1 is known to induce degradation of inflammatory signaling molecules through the ubiquitin-proteasome pathway. In particular, it has been shown to induce proteasomal degradation of JAK2, p65, and TIRAP, which makes it an important negative regulator of TLR signaling pathways (28-30). The inhibition of cytokine production by romidepsin could therefore be induced through proteasomal degradation. In figure 6, we show that the proteasome inhibitor bortezomib reverses the romidepsin-induced suppression IL-1β.

Figure 6. Proteasome inhibitor bortezomib reverses cytokine suppression by romidepsin
PBMCs were isolated from healthy volunteers and were pre-incubated for 1 h with bortezomib. Then romidepsin was added for 1 h pre-incubation, after which a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU) was added for another 24 h. The supernatant was collected for extracellular IL-1β (A). Cells were lysed with 0.5% Triton X-100 and subsequently the supernatant of the lysate was collected for intracellular IL-1β measurement (B).
DISCUSSION

In this study, we assessed the effects of several specific HDAC inhibitors in order to pinpoint the HDACs that could play a role in acute gouty arthritis. In contrast to the use of HDAC inhibitors in cancer treatment, the anti-inflammatory effects of HDAC inhibitors are observed at very low concentrations (31). The HDAC inhibitors givinostat (ITF2357), suberoylanilide hydroxamic acid (SAHA), and trichostatin-A have been shown to inhibit joint swelling and cell influx in several different animal models of arthritis (32-34). More importantly, oral administration of givinostat was well-tolerated and was shown to reduce disease activity in patients with juvenile idiopathic arthritis (35).

However, most of the natural and synthetic HDAC inhibitors are broad-acting, blocking most of the classical HDACs with varying affinity. This may result in opposing effects on the immune system and cytokine production, or lead to unwanted side effects. To move forward in the field of HDAC research it is therefore important to elucidate the effects of the individual HDACs and to develop safe and orally active specific HDAC inhibitors (36,37).

In the current study, we made use of several specific synthetic HDAC inhibitors rather than HDAC gene knockdown for several reasons. Firstly, full genetic knockout of HDAC1 or HDAC2 in mice has been shown to be lethal (38, 39). For HDAC1- or HDAC2-specific genetic knockdown, we would have to use cell lines. This is much further from the in vivo situation in humans than we would like to be. Furthermore, expression patterns of HDACs are highly tissue-specific (40), which we envisage will lead to differential effects of HDAC inhibition in other cell types. Secondly, knockdown generally does not lead to a 100% inhibition. The synthetic HDAC inhibitors we use in this study are very potent and more likely induce HDAC inhibition to a larger extent than could be achieved by knockdown.

Here, we identify the simultaneous inhibition of HDAC1 and HDAC2 as a possible new treatment option in acute gouty arthritis. In previous experiments, we have shown that class I HDAC inhibition is effective in suppressing MSU-induced cytokine production. In addition, we showed that specific HDAC8 inhibition had no effect (11), leaving HDAC1-3 as possible mediators of the observed cytokine-suppressive effects. As a single HDAC enzyme can elicit a wide range of cellular effects, narrowing down on a specific HDAC to inhibit could be important to limit side effects. To do this, we tested several specific HDAC inhibitors. We observed that romidepsin (HDAC1/2 inhibitor) and entinostat (HDAC1/3 inhibitor) effectively reduced the production of MSU+C16.0-induced IL-1β. In contrast, single inhibition of HDAC2 or HDAC3 did not affect IL-1β levels. Finally, we tested a specific HDAC6 inhibitor. Although this is not a class I HDAC, it has been shown to associate with microtubules and could interfere with NLRP3 inflammasome assembly (41). Our finding that it does not affect IL-1β production is in line with a previous paper showing that the HDAC6 inhibitor tubastatin did not induce migration of ASC on mitochondria towards NLRP3 on the endoplasmic reticulum (42). Altogether, these data suggest that HDAC1 inhibition is capable of blocking IL-1β production. Due to the more potent effect of romidepsin compared to entinostat, we decided to continue with romidepsin alone.

At a concentration as low as 10 nM romidepsin already reduced C16.0+MSU-induced inflammatory cytokines. Furthermore, intracellular levels of IL-1β and IL-1Ra, as well as IL-1β mRNA levels were decreased. We ruled out the possibility of cytokine suppression due to cell
death by means of flow cytometry with Annexin V and propidium iodide staining. The slight increase in late apoptotic cells with 50 nM of Romidepsin is likely caused by early apoptotic cells dying, as there is no decrease in the percentage of live cells. Although the transcriptional levels of NLRP3, CASP1 and PYCARD seemed to be affected by romidepsin, the effects are small and do not follow the striking dose-response as is seen in the cytokine production. There could still be post-transcriptional changes in activity of the NLRP3 inflammasome. However, we envisage that this is unlikely, as we see no accumulation of intracellular IL-1β upon addition of romidepsin, and because HDAC1 and HDAC2 mostly affect transcriptional activity in the nucleus. These results suggest that romidepsin blocks IL-1β production at the level of pro-IL-1β transcription.

A likely candidate to mediate this effect would be the NF-κB transcription factor. In the canonical pathway, activation of the RelA protein is required to induce pro-inflammatory gene transcription. However, several studies have shown that acetylation of RelA in fact causes increased activation of NF-κB (43–46). Inhibition of HDACs would therefore lead to activation of this transcription factor. This does not fit with the significant decrease in cytokine production upon addition of romidepsin. Therefore, this pathway was not explored further within the scope of this manuscript.

In the context of cancer, romidepsin has been shown to interfere with the PTEN/PI3K/Akt pathway (19-22). This pathway plays a key role in the control of autophagy and cellular metabolism, and can indirectly affect the inflammatory status of the cell (25,47). In the current study, romidepsin indeed increased expression of PTEN, and also of CPT1A, suggesting a decreased activation of the PI3K/Akt pathway. However, in the absence of functional PTEN in Cowden syndrome patients, romidepsin still potently suppressed IL-1β production, indicating that the cytokine-suppressive effects of romidepsin are independent of PTEN.

In our search for a possible mechanism of romidepsin-mediated cytokine suppression we found that both SOCS1 and SOCS3 genes possess a binding site for HDAC1 and HDAC2. Subsequently, we showed that romidepsin significantly increases gene expression of SOCS1, but not of SOCS3. In addition, a significant decrease in STAT1 and STAT3 phosphorylation was observed in both monocytes and lymphocytes. Naturally, the decreased activation of STAT1 could be the result of SOCS1 upregulation. The decrease in STAT3 activation, however, is independent of SOCS3 and may be mediated via a romidepsin-induced decrease in IL-6 production. Several research groups recently established an interesting direct link between SOCS1 and IL-1β. This link involves targeting several inflammatory signaling proteins for proteasomal degradation (28-30). To test this pathway, we studied the effect of proteasome inhibitor bortezomib on romidepsin-induced cytokine suppression. We found that indeed bortezomib was able to reverse the suppression of IL-1β by romidepsin, indicating that proteasomal degradation is a key mechanism by which romidepsin exerts its effects.

Romidepsin (Istodax®) was FDA-approved in 2009 for treatment of cutaneous T cell lymphoma and is administered intravenously at 14 mg/m2 over a 4-h period on days 1, 8, and 15 of a 28-day cycle (48). We envisage that a much lower concentration could be used to treat gouty arthritis. Future in vivo studies will have to show whether this is a potential treatment option in gouty arthritis. Due to the potent anti-inflammatory effects, HDAC inhibition may be used in the future to treat other auto-inflammatory diseases as well. Additionally, it would be very valuable to
study the effects of romidepsin mechanistically by linking acetylation events in specific genetic loci to altered inflammatory gene transcription by means of chromatin immunoprecipitation and genetic sequencing (ChIP-seq). We have not performed this in the current study due to the high costs related to such methods. Alternatively, ChIP-PCR would lack sensitivity for several reasons. Firstly, the current view that HDAC inhibitors increase histone acetylation and increase gene transcription is most likely oversimplified. Genome-wide analyses revealed increased broad deacetylation conferred by HDAC inhibitors in vascular endothelial cells, mediated by the loss of EP300/CREBBP binding (49). Furthermore, only one target can be studied at a time with ChIP-PCR. HDACs have low substrate specificity, and there are several histone lysines known to be subject to acetylation (50). Studying histone acetylation is further complicated by the fact that HDAC1 and HDAC2 are present together in several repressing complexes (NuRD, Sin3a and co-REST complexes). Finally, there is abundant cross-talk between different histone modifications, affecting one another (50).

**Conclusion**

Taken together, we can conclude that inhibition of HDAC1 and HDAC2 by romidepsin effectively decreases C16:0+MSU-induced cytokine production. Its effects are most likely mediated via increased acetylation and subsequent increased expression of the SOCS1 gene. SOCS1 is able to directly target inflammatory signaling molecules for proteasomal degradation, which could prevent the initial transcription of IL-1β. Although romidepsin is a very potent inhibitor in vitro, more studies are required before we can achieve HDAC1/2 inhibition during a gout flare in patients.

**Acknowledgements**

We thank Italfarmaco SpA, Cinisello Balsamo, Italy, for providing us with the specific HDAC6 inhibitor, ITF3107.
Romidepsin inhibits MSU-induced cytokine production via upregulation of SOCS1 expression

REFERENCES

Supplemental Figure 1. Effects of Romidepsin on C16.0+MSU-induced IL-10 production in human PBMCs
Freshly isolated PBMCs were pre-incubated with different concentrations of Romidepsin for 1 hour, after which cytokine production was induced via addition of a combination of 50 μM palmitic acid (C16.0) and 300 μg/mL monosodium urate crystals (MSU). The cells were cultured for 24 hours. IL-10 concentration was measured in the supernatant.
ABCG2 polymorphisms in gout: insights into disease susceptibility and treatment approaches


Pharmacogenomics and Personalized Medicine 2017; 10: 129-142
ABSTRACT

As a result of the association of a common polymorphism (rs2231142, Q141K) in the ATP-binding cassette G2 (ABCG2) transporter with serum urate concentration in a genome-wide association study, it was revealed that ABCG2 is an important uric acid transporter. This review discusses the relevance of ABCG2 polymorphisms in gout, possible etiological mechanisms, and treatment approaches.

The 141K ABCG2 urate-increasing variant causes instability in the nucleotide-binding domain, leading to decreased surface expression and function. Trafficking of the protein to the cell membrane is altered, and instead, there is an increased ubiquitin-mediated proteasomal degradation of the variant protein as well as sequestration into aggresomes. In humans, this leads to decreased uric acid excretion through both the kidney and the gut with the potential for a subsequent compensatory increase in renal urinary excretion.

Not only does the 141K polymorphism in ABCG2 lead to hyperuricemia through renal overload and renal underexcretion, but emerging evidence indicates that it also increases the risk of acute gout in the presence of hyperuricemia, early onset of gout, tophi formation, and a poor response to allopurinol. In addition, there is some evidence that ABCG2 dysfunction may promote renal dysfunction in chronic kidney disease patients, increase systemic inflammatory responses, and decrease cellular autophagic responses to stress. These results suggest multiple benefits in restoring ABCG2 function.

It has been shown that decreased ABCG2 141K surface expression and function can be restored with colchicine and other small molecule correctors. However, caution should be exercised in any application of these approaches given the role of surface ABCG2 in drug resistance.
INTRODUCTION

Gout is an inflammatory arthritis caused by an extremely painful but self-limiting innate immune response to monosodium urate (MSU) crystals deposited in synovial fluid (1). Without effective management, in some individuals, gout can become chronic with the development of tophi (organized lumps of urate and immune cells) (2) and permanent bony erosion and disability. Gout is also comorbid with other metabolic-based conditions such as heart and kidney disease and type 2 diabetes (3). An elevated concentration of urate (hyperuricemia) is necessary, but not sufficient, for the development of gout. Host-specific and environmental factors are required for the progression from hyperuricemia to gout. Approximately 30 genetic loci, including \textit{ABCG2}, influence serum urate concentrations (4), but less is understood about the genetic control of the formation of MSU crystals and the subsequent inflammatory response (5). Urate-lowering therapy is a cornerstone of gout management; however, for various reasons, it is often not effective (1). Despite the availability of a range of cost-effective urate-lowering drugs, there is a need for the development of better therapies for acute gout.

The ATP-binding cassette G2 (ABCG2) protein first came to biomedical attention through its contribution to an ATP-dependent multidrug resistance phenotype in a breast cancer cell line (6). It is one of a superfamily of 48 human ABC transporters that, in general, transport a wide array of substrates and are dependent on ATP binding to activate transport. The ABCG2 transporter is one of three well-studied multidrug transporters (others are multidrug resistance protein 1 and P-glycoprotein encoded by the \textit{ABCC1} and \textit{ABCB1} genes, respectively). These transporters export a wide range of compounds, including in the case of ABCG2, uric acid from cells. ABCG2 is a “half transporter” ABC protein, containing one membrane-spanning domain of six transmembrane helices, a N-terminal single intracellular portion containing a nucleotide-binding domain (NBD), and a short intracellular C-terminal tail (Figure 1). “Half transporters” like ABCG2 need to homodimerize to function in vitro but more likely exist in higher-order tetrameric or greater assemblages (7,8).

ABCG2, also known as breast cancer resistance protein, is overexpressed in human and animal cell lines resistant to chemotherapeutic drugs (9,10) and transports anticancer drugs such as mitoxantrone and doxorubicin (6, reviewed in reference 11). ABCG2 is important in stem cell biology (12,13). Also, the wide expression pattern in normal tissues (Figure 2) suggests that ABCG2 may fill an important physiological role as an excretor of environmental and endogenous toxins, for example, heme (14). This assertion is supported by the susceptibility of ABCG2 knockout mice to diet-induced phototoxicity and protoporphyria (15). It is expressed in the small intestine (excretion and limiting absorption), blood–brain and blood–placental barriers (mediating distribution), liver and kidney (elimination and excretion), and mammary gland (transporting into milk) (16). The pattern of ABCG2 expression is consistent with its role as a uric acid efflux transporter (reviewed in reference 17).

The ABCG2 transporter was identified as a uric acid efflux transporter only after it was associated with serum urate concentrations in a large genome-wide association study (18,19). This review explores the effects of several polymorphisms in the \textit{ABCG2} gene on susceptibility to hyperuricemia, gout, and chronic kidney disease. Furthermore, the role of ABCG2 in inflammatory responses and the possibility of ABCG2 as a treatment target are discussed.
Figure 1. Schematic representation of gout- and hyperuricemia-related polymorphisms in ABCG2

The ABCG2 protein consists of 655 amino acids and can be divided into an intracellular N-terminal domain, a nucleotide binding domain, six transmembrane domains, and a C-terminal end. The nucleotide-binding domain (amino acids 44-288) contains several signature sequences, named Walker A (A), Walker B (B), and the ABC signature sequence (C). The beginning and end amino acid position numbers are given for the signature sequences and the transmembrane domains. The third extracellular loop contains two cysteine residues connected by a disulfide bridge, and a glycosylated asparagine residue, both of which influence stability of the receptor. The cysteine residue at position 603 is important for homodimer formation. The V12M polymorphism is located in the N-terminal domain. The Q126X and Q141K polymorphisms are both located in the nucleotide-binding domain between the Walker A and the ABC signature sequences.
Figure 2. Tissue expression of ABCG2 in humans
Generated from the Genotype Tissue Expression (GTeX) database, derived from deceased people (organ and tissue donors) and surgical donors. RPKM: reads per kilobase of exon per million reads mapped.
MAJOR ABCG2 GENETIC VARIANTS

The ABCG2 transporter, expressed on erythrocytes, is the basis of a recently explained blood group system, Junior (Jr) (20,21). Individuals null for ABCG2 produce an ABCG2 alloantibody upon blood transfusion that can cause transfusion reactions, in extreme cases leading to fatal hemolytic disease of the fetus and newborn. The Jr(a-) phenotype is most prevalent in Japan with the most common Jr(a-) mutations being Q126X (Japanese) and R246K (Europeans) (19,20).

There are only two common (≥1%) missense genetic variants in the ABCG2 gene (V12M; rs2231137 and Q141K; rs2231142) and one common nonsense variant (Q126X; rs72552713) that is specific to South East Asian populations (Figure 1). The Q141K and Q126X variants influence expression levels of ABCG2 and contribute risk to phenotypes such as serum urate levels, gout, and response to the urate-lowering drug allopurinol (Table 1). Numerous other rare (<1%) variants are identified (http://www.ensembl.org), some of which have been phenotyped (22-28). The most widely studied variant is Q141K, a variant that is clinically relevant and has significant impact on ABCG2 characteristics. It is prevalent in most major population groups with the 141K allele frequency varying between 1% in African and 29% in South East Asian populations. There is consistent evidence for the 141K variant reducing the amount of ABCG2 protein expressed both in vitro (19,22-25, 28, 29) and, in vivo, from human liver samples (30).

Residue 141 is located in the E-helix (31) of the NBD of ABCG2, an area critical for the packing of the soluble portion of the protein. The 141K variant reduces ATPase activity (26,27), uric acid transport activity, and total and surface abundance (Table 1 and Figure 3A). These defects are believed to be caused by reduced NBD stability (32). Although proteolysis and thermo-stability as assessed by melting point curves of the isolated 141K NBD showed only small differences from the wild type (33), work done on the whole protein shows evidence of increased NBD instability in mammalian expression systems. Stabilizing the 141K variant protein with either suppressor mutations or with small molecules that may bind the NBD directly (34) rescues the trafficking, function, and abundance of 141K ABCG2 (32). Furthermore, recent modeling of the 141K NBD suggested that a loop adjacent to the Q141K substitution seems to be shifted outward, caused by a potential clash with a histidine residue (H155) at the top of the loop. This potential packing error in the NBD seems to be corrected by a H155A substitution on the 141K background as evidenced by rescued protein abundance (35). The decreased NBD stability of the 141K variant increases the interaction of the protein with the endoplasmic reticulum-associated degradation pathway and subsequent ubiquitin-mediated proteasomal degradation (29). There is also strong evidence that the 141K variant protein may accumulate in aggresomes prior to its degradation (36). In addition, protein expression is also influenced by microRNAs (37, and references therein). In one example, the 3′ untranslated region truncation allows increased expression of ABCG2, removing an hsa-miR-519c binding site that blocks translation (38). The 141K variant enhances the hsa-miR-519c-mediated repression of translation (37). Interestingly, the ABCG2 F142 residue is in an analogous position to the ΔF508 cystic fibrosis-causing mutation in the cystic fibrosis transmembrane receptor (encoded by ABCC7) (32). In vitro deletion of F142 from ABCG2 leads to major processing and folding defects with no surface expression (32,33), marking the Q141 and F142 regions as a more general ABC transporter mutational ”hot spot.”
### Table 1: Major genetic variation in ABCG2 and impact on protein expression and phenotype

<table>
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</thead>
<tbody>
<tr>
<td>V12M (rs2231137)</td>
<td>12M: 0.06/0.33/0.15/0.06</td>
<td>Unchanged (22-26,28)</td>
<td>Same (25, 27, 30)</td>
<td>Same (25)</td>
<td>Same (27)</td>
<td>Same (25)</td>
<td>Same (25)</td>
<td>None (25)</td>
<td>Same (26, 27)</td>
<td>Same (22)</td>
<td>Same (23, 27, 28)</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>Increased risk (70, 73)</td>
</tr>
<tr>
<td>Q141K (rs2231142)</td>
<td>141K: 0.09/0.29/0.10/0.01</td>
<td>Reduced (22-25, 28-30, 32, 36)</td>
<td>Same (26)</td>
<td>Reduced (26, 27, 36)</td>
<td>Same (26)</td>
<td>Reduced (26, 27, 36)</td>
<td>Increased (26, 27)</td>
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<td>Same (26)</td>
<td>Increased (23, 26-28)</td>
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<tr>
<td>rs10011796</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Increased risk (4)</td>
</tr>
</tbody>
</table>

Phenotype and prevalence information is given to the minor (alternative) allele. All phenotypes are compared to wild-type. Study systems: Naturally occurring humans (4, 30, 58, 67, 69, 70, 73, 82, 86, 87, 117, 130-132); HEK293 (human embryonic kidney) and derivative cells (22,25, 27-29, 36) ; LLC-PKI (porcine epithelial kidney cells) (25, 26); Sf-9 insect cells (26, 27, 33); PA317 mouse fibroblast cells (23); Xenopus oocytes (32). The Sf-9 cells were used for ATPase activity assays. *Meta-analyses.
Figure 3. Schematic overview of the roles of ABCG2 in hyperuricemia and gout
(A) The ABCG2 transporter is highly expressed in the gut, where it regulates ATP-dependent uric acid excretion. The 141K ABCG2 variant leads to decreased surface expression, targeting of the misfolded protein to aggresomes and proteasomal degradation. In addition, the polymorphism increases miRNA-mediated degradation of mRNA. The resulting increase in circulating urate not only increases the risk of gout but can also induce a more pro-inflammatory state in monocytes due to epigenetic reprogramming (129). Colchicine and HDAC inhibitors (HDACi) can restore 141K surface expression and function. (B) Monosodium urate crystals can induce mitochondrial damage, leading to the release of mitochondrial reactive oxygen species and DNA (mtROS, mtDNA), which in turn can activate the NLRP3 inflammasome. This can be limited through autophagy-mediated clearance of damaged mitochondria. ABCG2 plays a role in this process. In defective or diminished autophagy, p62 can accumulate, bind to keap-1, and induce translocation of Nrf2 to the nucleus. Nrf2 induces transcription of heme oxygenase-1 and superoxide dismutase (HO-1, SOD), which can also induce NLRP3 inflammasome activation. MSU, monosodium urate; HDACi, histone deacetylase inhibitors; TLR, Toll-like receptor; NLRP3, NOD-like receptor pyrin containing.

REGULATION OF ABCG2 EXPRESSION

The expression of ABCG2 is regulated by several transcription factors and hormones. A large number of cis-regulatory regions have been identified in or near the promoter region of ABCG2 (reviewed in 39). Hypoxia-inducible factor-1 (HIF-1α) stabilizes under hypoxic conditions and has been found to bind to a hypoxia-responsive element in the ABCG2 promoter and increase expression (40,41). Upstream of HIF-1α, signaling via phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K) and protein B kinase (Akt) regulates ABCG2 expression on the cell membrane (42-45). Furthermore, nuclear factor E2-related factor 2 (Nrf2), a transcription factor involved in activating oxidative stress responses, can induce ABCG2 transcription (46) in addition to inducing the expression of peroxisome proliferator-activated receptor gamma (PPARγ) (47,48). Finally, the hormone estrogen has been found to up-regulate ABCG2 gene expression (49-51). To our knowledge, however, no polymorphisms in any of these regulatory genes have been associated with serum urate levels or gout. However, a variant in PPARGC1B (rs45520937) has been associated with gout in Han Chinese (52). PPARGC1B encodes the PGC1β transcriptional repressor, a coactivator for PPARγ that is involved in estrogen signaling (53,54) in addition to a key role in maintaining mitochondrial biogenesis (55). However, the association of PPARGC1B with gout has not yet been replicated and it is unknown if rs45520937 is itself associated with...
ABCG2 expression. Since reduced mitochondrial DNA copy number is associated with gout (56) it is possible that systematically linked changes in PGC1β and associated molecules (including perhaps ABCG2) are factors connecting mitochondrial dysfunction and gout (57).

**ABCG2, URATE CONTROL, AND RISK OF GOUT**

An individual’s serum urate level, like other complex traits, is a function of a suite of inherited genetic variants and environmental exposures (5). A genome-wide association study identified the Q141K variant in the ABCG2 gene as associated with serum urate concentration in people of European ancestry, with the 141K allele associated with increased urate concentration (18). This genome-wide significant level of association has been consistently replicated in other genome-wide association studies in people of European and East Asian ancestry (4,58) but not in people of African–American ancestry (49,60). Lack of association in African–Americans in GWAS is likely because of the reduced power because of lower allele frequency in African–Americans (≤3%) – there is nominal evidence for the association of the 141K variant with increased serum urate levels in African–Americans (18) and the increased risk of gout (19). The Q141K variant (and other variants) has been associated with serum urate levels or hyperuricemia in other populations, for example, Tibetan (61) and Korean (62). Uric acid transport assays in *Xenopus* oocytes showed that ABCG2 transports uric acid with the 141K variant causing ~50% reduced ability to secrete uric acid (19). The regulation of ABCG2 protein in epithelial cells is largely unknown. However, similar to other uric acid renal influx transporters, ABCG2 co-immunoprecipitates with the scaffold protein PDZK1 (63), suggesting a possible common regulatory pathway.

The magnitude of association of Q141K with serum urate levels is the second strongest genome-wide behind SLC2A9, which is consistently the strongest in Europeans and East Asians. Each copy of the 141K allele associates with a 0.22 mg/dL increase in serum urate in Europeans (4) and an 0.14 mg/dL increase in East Asians (58). The magnitude is stronger in men than women and is modulated by body mass index (BMI), with the magnitude being stronger in lean people than those who are overweight and obese. The sex difference is most marked in lean people (64). The sex difference in association with serum urate levels is an interesting observation given the regulation of ABCG2 expression by estrogen (49-51). Compared to other typical genetic variants controlling complex phenotypes, Q141K has a very large magnitude of association, explaining ~1% of variance in serum urate levels. This compares with ~0.3% for the strongest genetic association with BMI at the FTO/IRX3 locus (65).

When associations of other SNPs within the ABCG2 locus with serum urate levels are tested conditionally on Q141K genotype, there is evidence for a second independent association at the ABCG2 locus, marked by intronic SNP rs2622629 (66). This second association is of clinical relevance as the rs2622629-correlated SNPs (ie those SNPs in linkage disequilibrium) include rs10011796 (r²=0.84 in Europeans). Rs10011796 is also implicated in gout and in allopurinol response (Table 1) (67). The molecular mechanism whereby rs2622629 (or tightly correlated variants) influence serum urate levels is not known. However, it maps within a DNaseI hypersensitivity cluster of ~150 bp (genome.ucsc.edu) identified by the Encode project (www.encodeproject.org) consistent with the effect of the association being mediated through the control of gene expression and/or mRNA editing. Interestingly, Q141K was not associated with gout in a Han Chinese study of 143 patients (68), whereas other variants in ABCG2 were, further supporting the existence of multiple gout-associated variants in ABCG2.
Aside from Q141K, the only other common (>1%) missense variant in ABCG2 is V12M situated in a short N-terminal section of ABCG2 prior to the structurally well-defined NBD (Figure 3). This variant is genetically independent of both Q141K ($r^2=0.002$ in Europeans) and rs2622629 ($r^2=0.009$ in Europeans). However, the impact on ABCG2 characteristics are, compared to Q141K, very moderate and inconsistent (Table 1). There are some data on association with serum urate and gout, suggesting an independent role for V12M in urate control and risk of gout (given the genetic independence from Q141K and rs2622629). Hence, V12M can be considered a third independent effect on gout in ABCG2, after Q141K and rs10011796/rs2622629. The largest genome-wide association study in serum urate in Europeans reported an increase in serum urate of 0.077 mg/dL (4) and in East Asians an increase of 0.108 mg/dL (58) per copy of the 12V allele. In gout, the common 12V allele consistently confers risk in five separate samples drawn from four ancestral groups: Europeans within the UK Biobank (odds ratio [OR] =1.24 [T Merriman, unpublished data, October 2016]), Taiwanese Aborigines (OR =1.36) (69), Han Chinese in Taiwan (OR =1.33) (69), Han Chinese in Shanghai (OR =1.82) (70), and New Zealand Māori and Pacific (Polynesian) (OR =1.31 [T Merriman, unpublished data, October 2016]). Any impact of this variant on the uric acid transport activity of ABCG2 is currently unknown. A combined meta-analysis is shown in figure 4. Based on the impact of the V12M variant on ABCG2 characteristics (Table 1), it is not obvious how the 12V allele increases serum urate levels and risk of gout. It is possible that the V12M variant is itself not causal and in genetic correlation (linkage disequilibrium) with a separate causal variant (which is not Q141K or rs10011796/rs2622629).

The kidney is the major regulator of serum urate levels, which are determined by the balance of secretion and reabsorption. This is controlled by reuptake transporters such as GLUT9 (SLC2A9) and URAT1 (SLC22A12) and secretory transporters ABCG2 (71) and NPT1/NPT4 (SLC17A1, SLC17A3) (72), all of which are genetically associated with serum urate concentration (4). Expression of most of these transporters is relatively high in the kidney or, for URAT1, restricted to the kidney. However, expression of ABCG2 is also relatively high in the gut (Figure 2) (71). Recent work in a Japanese gout cohort has demonstrated the role of ABCG2 in uric acid excretion in both the gut and kidney. In this well-defined Japanese population sample, it was possible to create grades of ABCG2 dysfunction based on Q141K and Q126X genotype combinations. Individuals positive for the dysfunctional variants 126X and 141K had the highest serum urate levels and highest

<table>
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<th>Population</th>
<th>Coefficient</th>
<th>Std Error</th>
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<th>95%-CI</th>
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<td>-0.274</td>
<td>0.0977</td>
<td>0.761</td>
<td>[0.628; 0.921]</td>
<td>21.3%</td>
</tr>
<tr>
<td>UK Biobank</td>
<td>-0.217</td>
<td>0.0933</td>
<td>0.805</td>
<td>[0.670; 0.966]</td>
<td>23.4%</td>
</tr>
<tr>
<td>Overall (95% CI)</td>
<td></td>
<td></td>
<td>0.732</td>
<td>[0.670; 0.800]</td>
<td>100%</td>
</tr>
</tbody>
</table>

Heterogeneity: Q=6.3, df=4, p=0.181
Test for overall effect: p<0.0001

Figure 4. Meta-analysis of association of ABCG2 V12M (rs2231137) with gout
Han Chinese (China) (70), Han Chinese (Taiwan) (69), Taiwanese Aboriginal (69), New Zealand Polynesian (982 cases and 941 controls), and the UK Biobank (2,422 cases and 152,249 controls). Effect is shown to the minor allele (12M). Variant rs4148153 is a surrogate marker for rs2231137 in people of Polynesian ancestry. ABCG2, ATP-binding cassette G2; OR, odds ratio; CI, confidence interval; Std, standard.
ABCG2 polymorphisms in gout

risk for gout, and those homozygous for 141Q and 126Q had the lowest serum urate levels and lowest risk for gout (73). ABCG2 dysfunction has been shown to influence extra-renal excretion by correlating with an increase in total urinary uric acid excretion. This results in a renal overload (ROL) type of hyperuricemia (74). The presence of 141K (and 126X) alleles reduces excretion through the gut and adds to the circulating urate, overloading the kidney excretion system, resulting in increased urinary uric acid levels. Further study also revealed that ABCG2 dysfunction resulted in a renal under-excretion (RUE) type of hyperuricemia consistent with a critical role in ABCG2-mediated uric acid secretion in the kidney (75). Other studies support a primary role for ABCG2 in excretion of uric acid by the gut. A Spanish study demonstrated considerably stronger association of the 141K allele with gout in patients classified as normo-excretors than in those classified as under-excretors (76), and two New Zealand studies demonstrated 141K-positive individuals not to be renal under-excretors of uric acid (77,78).

Predictably, therefore, ABCG2 Q141K associates with gout in most, but not all, ancestral groups analyzed – European, Han Chinese, Japanese, Korean, African American, Taiwanese Aborigine, and Pacific Islanders but not New Zealand Māori (69,79–84). The 126X variant, specific to East Asian populations, confers a higher risk to gout than 141K in Han Chinese and Japanese sample sets (73). In the context that the magnitude of association of ABCG2 on serum urate concentration is less than that of SLC2A9 (4), it is interesting that the magnitude of association of ABCG2 Q141K on the risk of gout is consistently larger than that of SLC2A9 (4,5,85). This does not equate with the hypothesis that ABCG2 influences gout solely through contributing to hyperuricemia. Emerging evidence (described in the ‘ABCG2: a regulator of gouty inflammation?’ section) indicates that ABCG2 has pleiotropic effects in gout, contributing to the etiology of gout in the presence of hyperuricemia.

**ABCG2: A REGULATOR OF ACUTE GOUTY INFLAMMATION?**

The key initial checkpoint in gout is hyperuricemia (1). Further checkpoints are formation of MSU crystals, the acute inflammatory response to these crystals (gout flare), and tophus formation. A gout flare typically presents as an acutely inflamed and extremely painful lower limb joint. The symptoms peak over the first 24 h and then gradually resolve over the next 10–14 days. Flares are usually recurrent, and the patient is well in between flares. In some patients with prolonged hyperuricemia, tophi can develop both in the joint and non-articular tissues; these are collections of MSU crystals surrounded by a chronic inflammatory granulomatous response (2).

As described earlier, it is well established from genome-wide association studies that ABCG2 variants are associated with elevated serum urate concentrations. However, as pointed out above, these studies show that the influence of ABCG2 variants on serum urate is lower than SLC2A9 variants. In contrast, ABCG2 variants are more strongly associated with risk of gout than SLC2A9. Köttgen et al (4) reported ORs for gout of 1.73, \(P=1.7\times10^{-39}\) for ABCG2 and 1.56, \(P=1.9\times10^{-31}\), for SLC2A9. In a more recent analysis (86), ABCG2 was associated with an increased risk of gout; compared with asymptomatic hyperuricemia controls, the OR for gout was 1.83, \(P=2.6\times10^{-14}\), in European people, and was 2.35, \(P=3.9\times10^{-5}\), in Polynesian people. In contrast, SLC2A9 variants were not associated with increased risk of gout when compared with asymptomatic hyperuricemia controls (86). These are intriguing findings that suggest a role for ABCG2 in developing gout once hyperuricemia is established. They do, however, require replication in other sample sets.
ABCG2 variants have also been associated with tophaceous disease in people with gout. In a Taiwanese study of people with gout, 141K was associated with tophi in both Han and Aboriginal Taiwanese populations (OR 1.51 and 1.50, respectively, for the 141K allele, pooled OR 1.55, \( P=7.8 \times 10^{-5} \)) (69). Recently, a New Zealand study of people with gout has reported that risk alleles for two ABCG2 SNPs, Q141K and rs10011796, were associated with tophi (compared with gout patients without tophi), with effects observed in participants of Western Polynesian (Samoan, Tongan, Niuean, and Tokelaun) ancestry (OR for rs2231142 1.71, \( P=0.017 \) and for rs10011796, 3.76, \( P=0.002 \)) but not in people of other ancestries (European and Eastern Polynesian [NZ Māori, Cook Island Māori]) (87). The ABCG2 associations persisted in Western Polynesian people even after accounting for serum urate and disease duration in the regression models. Given that only two studies have thus far associated ABCG2 with tophus in gout, further studies are required to substantiate this relationship.

These genetic data suggest a role of ABCG2 in the presentation of gout that is, at least in part, independent of hyperuricemia. Supporting this, cyclosporine inhibits ABCG2 in vitro (88). hyperuricemia and gout can be induced by cyclosporine (89), and there is an interaction between cyclosporine and colchicine (90) (colchicine is commonly used to treat gout attacks and is able to correct the dysfunctional internalization of ABCG2 141K) (36). Collectively, these observations suggest that ABCG2 may be influenced by off-target effects of cyclosporine. It is possible that ABCG2 influences other checkpoints, such as MSU crystal formation or the inflammatory response to these crystals. Although the mechanisms of MSU crystal formation are incompletely understood, it is conceivable that ABCG2 variants lead to preferential transport of urate into the joint (or reduced efflux out of the joint), resulting in higher saturating urate concentrations and MSU crystal formation.

**MECHANISTIC ASPECTS OF ABCG2 IN INFLAMMATION**

A schematic diagram of the role of ABCG2 in inflammation is shown in figure 3B. NLRP3 (NBD, leucine-rich family [NLR], pyrin containing 3) inflammasome activation leading to mature interleukin (IL)-1β release through caspase-1 is a key step in the initiation of the acute gout flare (91). An initial signal (signal 1) is required to stimulate the expression of pro-IL-1β and inflammasome components (92). Signal 1 includes Toll-like receptor-4 (TLR4) and TLR2 ligands, as well as unsaturated long-chain free fatty acids (93,94), and can be inhibited by omega-3 fatty acids and HDL-cholesterol (95,96). MSU crystals act as signal 2, inducing NLRP3 inflammasome complex oligomerization and activation of caspase-1 (91). Genes in this system have been associated with gout. A variant in TLR4, that likely influences TLR4 expression, has been associated with gout in two studies (97,98). Two SNPs in NLRP3-related genes have been associated with gout (99-101). The first one is a nonsense variation in the caspase recruitment domain-containing protein 8 (CARD8, rs2043211), a negative regulator of the NLRP3 inflammasome. One of the studies also found a multiplicative interaction between this SNP and an IL-1β polymorphism (rs1143623) (101) consistent with a pathogenic model whereby greater inflammasome activity from reduced CARD8 expression, combined with higher levels of pro-IL-1β expression, leads to increased production of mature IL-1β and an amplified immune response. Furthermore, the PPARGC1B SNP associated with gout (rs45529037) upregulates the expression of NLRP3 and IL-1β (52).
The role of ABCG2 in regulating inflammation has been described in nonarticular cells. In HEK293 cells cultured with H$_2$O$_2$, ABCG2 inhibited reactive oxygen species generation, enhanced anti-oxidant capacity of the cells, and protected cells from reactive oxygen species-induced toxicity (102). This may be mediated by the role of ABCG2 in exporting urate, an established pro-oxidant in the intracellular setting (103) by mechanisms that include promotion of mitochondrial dysfunction (104). In animals, ABCG2 also inhibited NF-κB and the expression of the inflammatory gene IL-8 induced by reactive oxygen species (102). In rheumatoid arthritis, a chronic autoimmune inflammatory arthritis, ABCG2 is highly expressed in the intimal lining layer and on macrophages and endothelial cells in the synovial sub-lining layer (105). The synovium is also a very important tissue in gouty arthritis. In several cell culture systems, the expression and function of ABCG2 is reduced when stimulated with pro-inflammatory cytokines (106-108). Collectively, these results suggest both a suppressive effect of ABCG2 on pro-inflammatory signaling pathways and that ABCG2 is itself a target of pro-inflammatory cytokines, where its expression and activity is suppressed. To date, the influence of ABCG2 variants on the inflammatory response to MSU crystals has not been reported, although genetic and other data suggest a role for the 141K variant in promoting gout once hyperuricemia is established (see the ‘ABCG2: a regulator of gouty inflammation?’ section).

**ABCG2 AND AUTOPHAGY**

In addition to its effects through increased serum urate concentration and the inflammatory responses described earlier, ABCG2 has been reported to play a role in autophagy. In a study by Ding et al (109), ABCG2 overexpressing cell lines were more resistant to stressors such as nutrient deprivation due to increased autophagy. The authors observed more degradation of autophagy receptor p62 and increased accumulation of LC3-II, a protein essential for autophagosome formation and function. Knockdown of ABCG2 in these cells subsequently abolished these effects (109). It was shown previously that effective autophagy is essential in acute gouty arthritis to clear mitochondria that have been damaged by MSU crystals. This prevents the release of mitochondrial DNA and the production of reactive oxygen species, which can activate the NLRP3 inflammasome (110,111). When autophagy is not effective, p62 accumulates, causing nuclear factor E2-related factor 2 (Nrf2), a transcription factor involved in oxidative stress responses, to be released from its repressor, kelch-like ECH-associated protein 1 (keap-1), and translocate to the nucleus. Previously, Nrf2 has been shown to induce transcription of heme oxygenase-1 and superoxide dismutase, which can in turn also activate the NLRP3 inflammasome (112). Finally, ABCG2 expression has been shown to be upregulated by Nrf2, which promotes cancer stem cell survival (46). Taken together, ABCG2 plays a role in autophagy and its expression is induced through Nrf2. Decreased or a lack of ABCG2 function could therefore induce or prolong NLRP3 inflammasome activation in acute gout through defective autophagy of damaged mitochondria in response to MSU crystals.
ABCG2 AS A DRUG TARGET

Restoring the expression and function of ABCG2 molecules that harbor detrimental genetic polymorphisms may be an important next step to limit urate levels and inflammatory responses. Recently, several papers have shown that histone deacetylase (HDAC) inhibitors can restore the function of the 141K ABCG2 variant. Woodward et al demonstrated that the HDAC inhibitor 4-phenylbutyrate restores trafficking and dimer expression of 141K ABCG2 (32). Basseville et al showed that several different HDAC inhibitors, as well as colchicine, inhibit targeting of 141K ABCG2 to the aggresome and promote relocalization on the cell surface (36). Colchicine is an anti-inflammatory agent that acts by inhibiting microtubule polymerization through binding to tubulin. This suggests that HDAC inhibitors, as does colchicine, inhibit trafficking of ABCG2 to the aggresome along microtubules. Finally, in addition to restoring surface localization of 141K ABCG2, the HDAC inhibitor romidepsin was shown to increase gene transcription of ABCG2 by activating the aryl hydrocarbon pathway (113). Recently, small molecules that are effective at rescuing the trafficking, abundance, and function of the 141K variant protein have been found. The compounds VRT-325 and 4a, both from the corrector class of molecules found in efforts to rescue mutant CFTR protein, are effective rescue agents for mutant ABCG2 as well (32). These results should encourage further research into the use of less toxic small molecules than HDAC inhibitors to restore defective ABCG2 function in patients with the 141K polymorphism.

ABCG2 AND ALLOPURINOL RESPONSE

Allopurinol is the most commonly used urate-lowering therapy for people with gout. It is rapidly metabolized to oxypurinol, which is responsible for the majority of the urate-lowering effect through the inhibition of xanthine oxidase. Despite widespread use of allopurinol, many people fail to achieve the recommended target serum urate of <0.36 mmol/L. For example, in the Febuxostat versus Allopurinol Controlled Trial, only 21% of those receiving allopurinol 300 mg daily achieved the primary endpoint of the last three serum urates being ≤0.36 mmol/L (114). Although poor adherence and restricted dosing based on kidney function are frequently associated with failure to reach target serum urate, genes encoding the enzymes involved in the metabolism, mechanism of action, and excretion of allopurinol may also have a role (115). Most studies related to the pharmacogenetics of allopurinol have focused on adverse events rather than predicting efficacy (116). However, two recent studies have highlighted a potential role for ABCG2 in allopurinol response. A significant association between ABCG2 141K and reduced allopurinol response was initially identified in a genome-wide association study (67). However, the definition of response used in this study was allopurinol-related change in serum urate, and there was only assessment of adherence with allopurinol using administrative data. A subsequent study, in which poor response was defined as serum urate ≥0.36 mmol/L despite allopurinol >300 mg daily, and with adherence confirmed by plasma oxypurinol concentrations, replicated the finding with 141K conferring a significantly increased risk of poor response to allopurinol (OR 2.71 (1.70–4.48), \( P=6.0\times10^{-5}\)) (117). This effect remained significant after adjustment for various variables that influence serum urate and allopurinol response including age, sex, BMI, ethnicity, eGFR, diuretic use, and serum urate off urate-lowering therapy. The association between ABCG2 Q141K and allopurinol response is a very interesting observation that should be further replicated in further studies.
The mechanism by which ABCG2 modulates allopurinol response is not clear. Wen et al (67) reported ABCG2 to be an efficient allopurinol and oxypurinol efflux pump. HEK293 cells transfected with 141K had significantly impaired ABCG2 function leading to intracellular accumulation of allopurinol and oxypurinol (67). These authors suggested that the ABCG2 141K variant might impair allopurinol response by increasing allopurinol and oxypurinol concentrations in renal tubular cells and decreasing concentration in the tubule fluid, thereby reducing the inhibition of uric acid influx pumps within the kidney. However, this theory is unproven, and modeling the complexity of the relative contribution of reabsorbed allopurinol (118) versus nonsecreted allopurinol is extremely difficult; therefore, in vivo experiments are required to further explore this possible mechanism.

**ABCG2 AND CHRONIC KIDNEY DISEASE**

It is clinically established that chronic kidney disease is a cause of hyperuricemia and gout. However, the physiological response to disrupted renal uric acid handling in kidney disease is a question of clinical interest. Bhatnagar et al (119) addressed this question by evaluating the strength of genetic association with urate of variants in a suite of genes encoding uric acid transporters (including SLC2A9, ABCG2, SLC17A1-A3, and SLC22A11-A12) in 3,598 individuals with chronic kidney disease. In contrast to the situation in healthy individuals where SLC2A9 has the strongest magnitude of association on serum urate levels (0.37 mg/dL increase in urate per risk allele compared with 0.22 mg/dL for ABCG2 in sex-adjusted analysis) (4), each ABCG2 risk allele increased serum urate by 0.68 mg/dL in people of European ancestry, compared with 0.30 mg/dL for SLC2A9 in sex-adjusted analysis (119). Although this finding requires replication, it does suggest that the extra-renal (gut) urate handling of ABCG2 necessarily compensates in renal injury (119). This is consistent with the observation, in nephrectomized rats, of increased gut expression of ABCG2 and maintenance of normal serum urate concentration (120). Bhatnagar et al (119) evoke the “remote sensing and signaling” hypothesis whereby the action of multi-specific SLC and ABC transporters is critical in disrupted homeostasis resulting from organ injury (121). The hypothesis posits that the transporters cooperate in sensing and controlling fluctuations in important substances, with the ABC (and SLC) transporters facilitating interorgan communication through small molecules (121), including those important in oxidant status such as urate (122). One specific example of this is the ability of ABCG2 to transport and transfer heme, through an extracellular loop, to albumin (123).

In a study of fructose-induced hyperuricemia in humans, the urate-increasing 141K allele was associated with a lower increase in serum urate but greater increase in fractional excretion of uric acid when compared with the 141Q allele (78), consistent with the previously reported ROL type of hyperuricemia. Supporting ABCG2 as a “remote sensor and signaler,” the 141K allele was associated with a lower glucose response to the fructose load (78). Whether or not the urate and glucose effects are related is unclear; however, the remote sensing hypothesis could relate to differential ATP utilization by the 141Q and 141K alleles given that the 141K variant has significantly reduced ATPase activity (32).

Hyperuricemia predicts reduced renal function and chronic kidney disease, independent of measured confounders, in observational studies (124). However, whether or not urate is causal of reduced renal function is unresolved. Mendelian randomization studies, in which urate-associated genetic variants inherited at conception are used as an un-confounded
CHAPTER 7

proxy for exposure to urate (125), suggest that urate is not causal of reduced renal function in healthy people (126) but could promote progression of declining renal function in chronic kidney disease (127). Based on the observation in mice that uric acid may compete with toxins as an efflux substrate of ABCG2, Dankers et al postulate that, in the kidney, hyperuricemia promotes reduced renal ABCG2-mediated secretion of toxic molecules (128).

CONCLUSION

The ABCG2 transporter is an important molecule in urate excretion. Decreased ABCG2 expression and function due to genetic polymorphisms leads to both ROL hyperuricemia and RUE hyperuricemia. The most extensively studied genetic variant is Q141K. Besides significantly increasing serum urate concentration, the 141K ABCG2 variant has also been associated with acute gout, tophaceous gout, and poor allopurinol response. In addition, 141K-induced hyperuricemia may lead to excessive inflammatory responses and decreased ABCG2 function may cause defective autophagy. All of these effects warrant further research to the restoration of 141K ABCG2 function and surface expression, for example, by small molecules.
ABCG2 polymorphisms in gout

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ABCG2 polymorphisms in gout


Copy number variation of tubulin polymerization-promoting protein is associated with gout risk in Polynesian people


Manuscript in preparation
ABSTRACT

Background

Gout is a complex disease caused by precipitation of excess urate as monosodium urate (MSU) crystals, which can elicit acute arthritis. Genome-wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) have mainly identified genetic loci associated with serum urate concentrations. However, little is understood about the genetic influence on inflammatory responses to MSU crystals. Our aim was to evaluate by GWAS the role of copy number variation in gout.

Methods

For identification of copy number variable regions (CNVRs) from Illumina Immunochip-derived SNP intensity data, a gout patient cohort (n=468) of European descent from New Zealand and Australia was used and was compared to 1000 randomly chosen individuals from the WTCCC 1958 Birth Cohort. For validation of the identified CNVR by PCR, we selected four cohorts: Two cohorts of individuals of European ancestry from New Zealand and the Netherlands (European group 1, n=609, 351 cases, 258 controls, and European group 2, n=794, 412 cases, 382 controls) and two cohorts of Polynesian individuals (East Polynesian, n=901, 435 cases, 466 controls, and West Polynesian, n=538, 277 cases, 261 controls). In a Dutch gout patient subset of European group 2 ex vivo stimulation experiments were performed with MSU crystals and IL-1β and IL-6 responses were measured by ELISA.

Results

We identified by GWAS a deletion at chr5:658,692-728,796 that protected from gout. This region includes the gene for TPPP. In the validation cohorts, we could replicate this association only in the Western Polynesian cohort where increased copy-number associated with increased risk. A meta-analysis of the validation cohorts combined unfortunately showed no statistically significant association between TPPP copy number and gout risk. A logistic regression analysis of the interaction between TPPP CNV and presence of the ABCG2 141K risk allele (rs2231142) on gout risk showed a non-significant trend, with all four cohorts showing the same direction of increased risk. Functional data suggests that the effect of TPPP copy number on ex vivo IL-1β response is dependent on Q141K genotype, with higher TPPP copy number giving lower cytokine production in the presence of the 141K risk allele.

Conclusion

Increased copy number in the identified CNV region including the TPPP gene was associated with increased gout risk in 2 of 5 sample sets and provides an interesting candidate for genetic control of gout inflammatory mechanisms. The combined effects of TPPP CNV and ABCG2 rs2231142 on gout risk and ex vivo MSU-induced cytokine production suggest a functional interaction effect.
INTRODUCTION

Gout is a complex and debilitating disease that has afflicted humans for millennia (1). During a flare of acute gouty arthritis, endogenous monosodium urate (MSU) crystals elicit a local cellular inflammatory response (2), causing the joint to be red, swollen and severely painful. The fundamental cause of gout is urate. Hyperuricemia can lead to precipitation of MSU crystals in the joints, after which resident macrophages phagocytose these crystals and initiate an interleukin (IL)-1β-driven inflammatory response. That not all individuals with hyperuricemia develop gout (3) indicates that host and environmental factors are required for the progression from hyperuricemia to clinically evident gout.

Secretion of active IL-1β is a two-step process. Firstly, the MSU crystals induce the assembly of the NLRP3 inflammasome, a protein complex existing of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 (4). This assembly leads to the activation of caspase-1, an enzyme with the ability to process pro-IL-1β into its active form. Secondly, the transcription of inactive pro-IL-1β is initiated. This requires an additional signal, such as LPS or long-chain free fatty acids binding to Toll-like receptors (5). The inactive pro-IL-1β can subsequently be cleaved by caspase-1 and form a self-perpetuating loop of inflammatory cytokine production through the IL-1R.

Colchicine is a natural product that is extracted from the Colchicum (autumn crocus) plant. It is a widely used therapeutic drug, which resolves the acute inflammatory process in gout, and disrupts proper assembly of the NLRP3 inflammasome. NLRP3 activation requires microtubule-driven spatial co-localization of ASC located on the mitochondria and NLRP3 on the endoplasmic reticulum (6). This dynein-mediated transport is facilitated by the presence of acetylated α-tubulin in microtubules (6). Colchicine interferes with this transport by inhibiting microtubule polymerization. It binds to both α- and β-tubulin, forming a complex that is unable to polymerize into stable microtubules (7). However, colchicine can induce side effects such as gastrointestinal complaints and neutropenia, resulting in the need for more targeted therapeutics to inhibit NLRP3 inflammasome assembly to prevent acute gouty arthritis.

One approach to identifying such therapeutic targets is to identify genetic risk factors for gout. Genome-wide association studies have currently identified up to 30 genetic loci marked by SNPs that are associated with serum urate concentrations in studies of people with European ancestry (8-11), most of which are involved in urate transport. However, little is understood about the genetic influence on inflammatory responses to MSU crystals (12). One of the key variants influencing serum urate levels and gout risk is the Q141K variant of the ABCG2 protein, encoded by the rs2231142 genetic variant. ABCG2 is an important secretory transporter of urate in the gut, and its dysfunction leads to an extra-renal underexcretion, resulting in a renal overload type hyperuricemia (13, 14). The K allele has been shown to result in both decreased surface expression of the ABCG2 protein and impaired function (15, 16). Interestingly, acetylated α-tubulin does not only play a role in gout through facilitating NLRP3 activation, but may also play a role in regulating expression and function of the protein containing 141K (15). Colchicine and histone deacetylase inhibitors have been shown to disturb trafficking along microtubules, inhibiting the aggregation of the Q141K ABCG2 variant in the perinuclear region called the aggresome (15). A similar effect was found with small molecule corrector VRT-325 (16). The above-described
results highlight the importance of microtubule networks in ABCG2 membrane expression and NLRP3 inflammasome activation.

Structural variation, such as copy number variations (CNVs), will account for some of the missing heritability of complex diseases not explained by single nucleotide polymorphisms (17), including gout (18). Copy number variants are caused by deletion or duplication of DNA segments, varying from kilobases (kb) to large events of over 1 megabase (Mb) in size, and have already been associated with a range of complex diseases (19-21). Such CNVs can have an abundant effect on gene expression levels (22, 23). Identifying gout-associated CNVs could provide valuable knowledge on the genetic control on MSU-induced inflammatory responses. To our knowledge, such a CNV association study in gout has only been performed once in a small Chinese gout patient cohort (24). In this study, three gout-associated CNVs were identified containing exons for inflammation- and immunity-related genes (ABCF1, IL17REL, and FCGR3A). However these associations are yet to be replicated.

In the current study we utilized a hypothesis-free approach to detect CNVs that are associated with gout in individuals of European and Polynesian ancestry. We detected CNV in the TPPP gene associated with gout and test for genetic non-additive interaction (epistasis) with ABCG2 rs2231142. Moreover, we assessed the effects of the gout-associated CNV on functional gout-related inflammatory markers in an ex vivo system.

**MATERIALS AND METHODS**

**Study participants**

For identification of CNVs, a cohort of 468 gout patients of European descent from New Zealand and Australia was used. The control cohort consisted of 1000 randomly chosen individuals from the WTCCC 1958 Birth Cohort, previously genotyped on the Immunochip platform (25, 26). The New Zealand Multi-Region Ethics Committee (MEC/105/10/130) gave ethical approval for the New Zealand participants. The Research ethics committee of the University of New South Wales granted approval for the Australian participants. All participants gave written informed consent.

For the validation of the CNV region, we selected four independent cohorts: Two cohorts of individuals of European ancestry from New Zealand (NZ) and the Netherlands (European group 1, n=609, 351 cases, 258 controls, and European group 2, n=794, 412 cases, 382 controls) and two cohorts of Polynesian individuals (East Polynesian, n=901, 435 cases, 466 controls, and West Polynesian, n=538, 277 cases, 261 controls). West Polynesian (Samoa, Tonga, Niue, Tokelau) and East Polynesian (New Zealand and Cook Island Māori) individuals were stratified by clustering of genome-wide principal component vectors as described by Krishnan et al. (27). Both the European and Māori and Pacific (Polynesian) cohorts were recruited from 2006 to 2016 for studies on gout as part of the EuroGout / NZ consortium (28). Among the East Polynesian dataset are 168 participants recruited in collaboration with Ngāti Porou Hauora Charitable Trust, the healthcare provider located in the East Coast (Tairāwhiti) region of the North Island of Aotearoa New Zealand. The New Zealand Multi-Region Ethics Committee (MEC/105/10/130) gave ethical approval for the New Zealand participants. The ethics committee of Radboud University Nijmegen granted approval for the Dutch gout patient cohort (reference number...
TPPP copy number variation is associated with gout risk in Polynesian people

2012/482) and healthy participants (reference number 42561.091.12). All experiments were conducted in accordance with the Declaration of Helsinki.

Gout diagnosis in the New Zealand patients was determined using the preliminary criteria of the American Rheumatism Association (29). In the Dutch patient cohort all patients were diagnosed by confirming presence of MSU crystals in joint aspirate by light microscopy (30). In the Dutch gout patient and control samples from Nijmegen, which is part of the European group 2 cohort, additional ex vivo stimulation experiments and plasma measurements were performed to gain insight into the underlying immune functions.

Illumina Immunochip array

The Illumina Immunochip platform (Illumina, San Diego, CA, USA) was used to genotype participants for approximately 250,000 variants genome-wide using the Illumina Iscan system at AgResearch (Invermay, Dunedin, New Zealand). The SNP intensity data were loaded into Illumina GenomeStudio (v1.9.0) and clustering was performed using the Gentrain algorithm of the Genotyping module (v1.9.4). The log reference ratios (LRRs), B allele frequencies (BAFs), and genotype calls were extracted. Samples with a genotyping call-rate of > 98%, a LRR standard deviation > 0.28, a waviness factor > 0.05 or < -0.05, or CNV count > 100 were not utilized in downstream analyses. SNPs with a call-rate of less than 95% were excluded. A PCA adjustment of the LRR matrix was used to remove batch-effects from the SNP array dataset. To reduce the chance that CNV would be removed, a random 15% of the SNPs with 100% call-rate were selected for inclusion in calculation of the principal components. Each individual had their LRR intensities adjusted for none, 12, 24, and 36 principal components. Technical replicates were available for 9 participants with gout, with eight being run in duplicate, and one individual run 5 times. Consistency was determined using concordance. Specifically, for each CNV call in a sample the technical replicates were checked to determine whether a similar CNV (duplication or deletion) +/- 70kb was detected. The percentage of replicates confirming this CNV call was then calculated. These numbers were averaged within each sample, and then across all samples with technical replicates. For example, in an individual sample with only one CNV call, if this call was agreed upon by 4 out of 5 replicates, it would be assigned a concordance score of 80%. The R (v3.2.0) function prcomp was used to generate principal components from the sample-by-LRR (1,431 x 189,993) matrix that contained a subset of the SNPs (R Core Team, 2015). This was followed by linear regressions using the principal components. This set of operations was performed independently for each SNP on (Equation 2.1 of (26)). After running the regression, the residuals were extracted and added to the mean of the LRRs for that SNP (Equation 2.2 of (26)).

The PCA adjusted LRR files were processed with PennCNV (version May 2011) to generate raw CNV calls (31). This was performed using a trained hidden Markov model transition file that was provided for use with alternative arrays, such as the Immunochip; and a guanine-cytosine (GC) content file that was created for all the SNPs on the Immunochip. GNU parallel (v20150822) was used to reduce the run-time of PennCNV (32). Any CNV calls that were supported by less than 6 SNPs or were less than 50 basepairs in length were not analyzed further.
PLINK (v1.07) was used to perform an association analysis. Specifically, CNVs (frequency \( \geq 1\% \), PLINK flag = –cnv-freq-exclude-below 14) were summarized 70kb from each probe and the significance was determined in PLINK by calculating a two-sided empirical p-value using 1,000,000 null permutations (PLINK flags = –cnv-z-sided, –mperm 1000000, –cnv-test-window 70). This analysis was performed in the deletions and duplications separately. Successive probes with the same p-values were merged into a single CNV event, and any event with less than 6 probes was removed. As the duplications and deletions were processed separately, the multiple-testing correction provided by PLINK only accounted for the number of tests within each CNV grouping (i.e deletions only). P-values reported in the text as \( P_{\text{deletions}} \) are adjusted for all deletions in the dataset, and \( P_{\text{duplications}} \) for all duplications. To provide an overall p-value adjustment for CNVs with a group-corrected p-value of less than 0.05, a trimmed CNV list was created, whereby the copy-number calls opposing the associated copy-number were removed. For example, if a deleted region was associated with gout, duplications in the same region were removed. PLINK was then used to calculate the overall multiple-testing adjusted empirical p-value for that CNV. P-values reported in the text as \( P_{\text{corrected}} \) were adjusted in this way. The PCA-adjusted LRRs for all regions associated with gout were visualised with ggplot2. Manual copy-number calling for these associated regions was performed by two human operators who were blinded to the original PennCNV calls. When the operators disagreed on a CNV call, it was removed from the downstream comparisons. These manual calls were compared to the CNV calls from PennCNV using Spearman’s correlation, this calculation was also performed for the deletions and duplications separately.

Validation of CNV region by qPCR

Quantitative PCR (qPCR) primer and probe sequences were obtained for the CNV between chromosome 5 between 658,692 and 728,796 (hg19) from Handsaker et al. (23). The sequences were forward (GTTCCTGTTCGTTGTTACTGTT), reverse (GTGCAAACTCAAGGAGCTGG), and probe (ACATCAAGAGACAAACGACAAGCAGC). The qPCR was performed with the use of a Roche LightCycler 480 RT-PCR where a custom FAM probe was ordered using the above sequences, and the HEX probe used was the standard RNase P reference (Applied Biosystems catalogue no. 4403328). The protocol consisted of pipetting 16ng of genomic DNA in 2μl volumes in triplicate onto a 384 well plate, the DNA was then left to dry. The total volume of each reaction was 10μl, this consisted of 1x custom probe, 1x RNase P probe, and 1x TaqMan®R Genotyping Master mix. The plates were centrifuged then placed into the LightCycler 480 with the following cycling conditions: 10 minute incubation at 95 degrees, followed by 40 cycles that consisted of a 15 second incubation at 95 degrees and a 60 second incubation. The second derivative maximum method implemented in the LightCycler software was then used to calculate the cycling threshold (Ct), which is an estimate of the number of cycles until the beginning of the exponential phase of the PCR. Applied Biosystems CopyCaller software was used to process the raw qPCR data into integer copy calls for the locus. The raw data were analyzed without a calibrator sample, with the most frequent sample copy number set to 4, which is the most common number of copies identified among Europeans (23). Because each plate of qPCR has subtle differences in fluorescence which directly impacts the calculation of Ct, the results of each plate of PCR was run through the CopyCaller software individually, so as to avoid the data being artificially skewed by inter-plate differences.
**PBMC isolation and cell culture**

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn venous blood by means of Ficoll-Paque (GE Healthcare) density gradient centrifugation. Cells were resuspended in Dutch Modified RPMI 1640 medium (Life Technologies) supplemented with 50 μg/mL Gentamycin (Centrafarm), 2 mM GlutaMAX and 1 mM pyruvate (Life Technologies) and plated at 0.5x10⁶ cells per well (U-bottom 96-well plate). Cells were cultured for 24 hours either with medium alone, or with a combination of monosodium urate crystals (MSU crystals) and palmitic acid (C16:0).

**Reagents for ex vivo experiments**

MSU crystals were precipitated from urate (Sigma) and sodium hydroxide (Merck) according to methods described previously (33). Palmitic acid (Sigma) was dissolved in 100% ethanol at high concentrations and conjugated to human albumin (Albuman 200g/L, Sanquin, Amsterdam). Levels of IL-1β and IL-6 were measured in the supernatant according to the manufacturer’s protocol (R&D).

**RESULTS**

**Deletions in CNV region on chromosome 5 are associated with gout**

CNV regions were identified from Illumina array intensity data using 1000 controls from the WTCCC 1958 Birth Cohort and a cohort of 468 gout patients from New Zealand and Australia. After quality control, 989 of the 1000 controls, and 444 of the 468 gout patients remained (Supplementary figure 1). The detected CNVs range in size from 62 bp to 1,940,031 bp (1.94 Mb), with an average length of 33.8 kb, and a median length of 7.6 kb (Figure 1).

![Figure 1. Distribution of CNVs](image)

Size (in kb) compared to the number of CNVs. The CNVs were summarized in 1 kb windows. For the purpose of clear visualization, large CNVs (>100 kb) were artificially resized to 100 kb. The blue and red lines represent the mean and median CNV size, respectively.
All CNVs with a frequency >1% were analyzed between controls and gout patients using PLINK. The association results are provided in Table 1. This showed a significant association (p_{unadjusted} = 0.007, p_{deletions} = 0.02, p_{corrected} = 0.09) of a deletion on chromosome 5 at positions 658,692 to 728,796, and was found in 30 controls and 3 gout patients. Analyzing these counts with a Fisher’s exact test revealed that the deletion had a protective effect on gout risk (OR = 0.22, p = 0.0064). Duplications in this region on chromosome 5 were not associated with gout. Nor were any of the other identified CNVs in Table 1. Figure 2 shows the Immunochip results for the deletion at chr5:658,692-728,796. The only gene that lies entirely within this CNV region is TPPP, encoding for the tubulin polymerization-promoting protein.

### Table 1. Association results between CNVs and gout.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Start (bp)</th>
<th>End (bp)</th>
<th>Length (kb)</th>
<th>P-value (raw)</th>
<th>P-value (group-corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deletions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chr1</td>
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<td>161,542,727</td>
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<td>1.00</td>
</tr>
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<td>0.18</td>
<td>0.95</td>
</tr>
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<td>0.99</td>
</tr>
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<td>chr1</td>
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<td>1.00</td>
</tr>
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<td>chr3</td>
<td>18,708,341</td>
<td>22,708,536</td>
<td>42.11</td>
<td>0.27</td>
<td>1.00</td>
</tr>
<tr>
<td>chr5</td>
<td>658,692</td>
<td>728,796</td>
<td>70.10</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>chr5</td>
<td>150,157,360</td>
<td>150,224,378</td>
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<td>1.00</td>
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<tr>
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</tr>
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<td>1.00</td>
</tr>
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</tr>
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<td>28,651,733</td>
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<td>1.00</td>
</tr>
<tr>
<td>chr12</td>
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<td>1.00</td>
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<tr>
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<td>55,387,953</td>
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<td><strong>Duplications</strong></td>
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<td>17,631,811</td>
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</tr>
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</tr>
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<td>161,611,005</td>
<td>38.65</td>
<td>0.23</td>
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</tr>
<tr>
<td>chr1</td>
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<td>161,631,174</td>
<td>18.60</td>
<td>0.26</td>
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<tr>
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<td>161,643,644</td>
<td>5.11</td>
<td>0.30</td>
<td>0.99</td>
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<tr>
<td>chr5</td>
<td>634,660</td>
<td>728,796</td>
<td>94.15</td>
<td>0.52</td>
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</tr>
<tr>
<td>chr6</td>
<td>31,229,462</td>
<td>31,368,126</td>
<td>138.66</td>
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<td>1.00</td>
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<td>57,098,251</td>
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<td>0.31</td>
<td>0.99</td>
</tr>
<tr>
<td>chr12</td>
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<td>9,727,587</td>
<td>83.57</td>
<td>0.04</td>
<td>0.83</td>
</tr>
<tr>
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<td>35,615,461</td>
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<td>1.00</td>
</tr>
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<td>28,651,733</td>
<td>50.55</td>
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<td>chr17</td>
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<td>44,262,951</td>
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</tr>
<tr>
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<td>105.83</td>
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<td>1.00</td>
</tr>
<tr>
<td>chr20</td>
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<td>1,605,166</td>
<td>44.27</td>
<td>0.75</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Summary of association results to gout for deletions and duplications with a frequency >1% in 989 controls from the WTCCC 1958 Birth Cohort and 444 gout patients from New Zealand and Australia. P-values were calculated using PLINK (v1.07).
TPPP copy number variation is associated with gout risk in Polynesian people.

Figure 2. The deletion at chr5:658,692-728,796
UCSC genome browser (hg19) view of the gout-associated deletion identified at chromosome 5 between positions 658,692 and 728,796. The red and blue lines depict the deletions identified by PennCNV in individuals with gout and controls, respectively. Reprinted from (26).

Replication and validation of the deletion at chr5:658,692-728,796
The gout-associated CNV at chr5:658,692-728,796 was validated by qPCR in four different cohorts consisting of both healthy volunteers and gout patients (table 2). By means of logistic regression analyses, we studied the effect of TPPP copy number on gout risk. Figure 3 shows the results for the four different cohorts. In the Polynesian cohorts, there was evidence for an increased gout risk per unit increase in TPPP copy number in the West Polynesian group and significant evidence when meta-analyzed with the East Polynesian group (OR=1.171, CI 1.002-1.369) (Figure 3B). No evidence for association between TPPP copy number variation and gout risk was found in either of the European cohorts or by meta-analysis (Figure 3A). There was no statistically significant association between gout risk and TPPP copy number in a meta-analysis of all four cohorts (Figure 3C).
### Table 2. Cohort characteristics for CNV validation by qPCR

<table>
<thead>
<tr>
<th></th>
<th>European group 1</th>
<th>European group 2</th>
<th>East Polynesian</th>
<th>West Polynesian</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean ± SD)</strong></td>
<td>62.2±14.6</td>
<td>48.9±21.3</td>
<td>51.7±14.7</td>
<td>42.4±13.2</td>
</tr>
<tr>
<td><strong>Disease status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, n (%)</td>
<td>258 (42)</td>
<td>382 (48)</td>
<td>466 (52)</td>
<td>261 (49)</td>
</tr>
<tr>
<td>Gout, n(%)</td>
<td>351 (58)</td>
<td>412 (52)</td>
<td>435 (48)</td>
<td>277 (51)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n(%)</td>
<td>119 (20)</td>
<td>269 (34)</td>
<td>381 (42)</td>
<td>140 (26)</td>
</tr>
<tr>
<td>Female, n(%)</td>
<td>490 (80)</td>
<td>525 (66)</td>
<td>520 (58)</td>
<td>398 (74)</td>
</tr>
<tr>
<td><strong>rs2231142 frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG, n(%)</td>
<td>421 (69.1)</td>
<td>541 (68.2)</td>
<td>755 (83.8)</td>
<td>223 (41.4)</td>
</tr>
<tr>
<td>GT, n(%)</td>
<td>161 (46.5)</td>
<td>221 (27.8)</td>
<td>144 (16.0)</td>
<td>228 (42.4)</td>
</tr>
<tr>
<td>TT, n(%)</td>
<td>27 (4.4)</td>
<td>32 (4.0)</td>
<td>2 (0.2)</td>
<td>87 (16.2)</td>
</tr>
<tr>
<td>2. n(%)</td>
<td>4 (0.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>3. n(%)</td>
<td>70 (11.5)</td>
<td>36 (4.5)</td>
<td>137 (15.2)</td>
<td>131 (24.3)</td>
</tr>
<tr>
<td><strong>TPPP integer copy number</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. n(%)</td>
<td>333 (54.7)</td>
<td>445 (56.0)</td>
<td>451 (50.1)</td>
<td>279 (51.9)</td>
</tr>
<tr>
<td>5. n(%)</td>
<td>154 (25.2)</td>
<td>271 (34.1)</td>
<td>225 (25.0)</td>
<td>95 (17.7)</td>
</tr>
<tr>
<td>6. n(%)</td>
<td>37 (6.1)</td>
<td>38 (4.7)</td>
<td>74 (8.2)</td>
<td>26 (4.8)</td>
</tr>
<tr>
<td>7. n(%)</td>
<td>9 (1.5)</td>
<td>4 (0.5)</td>
<td>11 (1.2)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>8. n(%)</td>
<td>2 (0.3)</td>
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<td>2 (0.2)</td>
<td>1 (0.2)</td>
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<tr>
<td>9. n(%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (0.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
TPPP copy number variation is associated with gout risk in Polynesian people

**Figure 3. Association between TPPP CNV and gout risk**

Logistic regression analysis was performed in R corrected for age, gender, and PCR plate number: Gout status ~ integer TPPP copy number + age*gender + PCR plate number. A. Logistic regression analysis of European groups 1 and 2. B. Logistic regression analysis of West and East Polynesian cohorts. C. Meta-analysis of all four cohorts.

**Interaction analysis of TPPP copy number and rs2231142 genotype on gout risk**

The presence of the rs2231142 risk allele (141K ABCG2 variant) is one of the key genetic risk factors for developing hyperuricemia and gout (11). In addition, Basseville and colleagues showed that small molecules, such as colchicine or HDAC inhibitors, are able to restore Q141K ABCG2 expression on the cell surface by facilitating trafficking via the tubulin network (15). A non-additive interaction could explain inconsistent results in main effect analysis of association of TPPP copy number with gout.

We therefore studied the effects of group clustering based on TPPP copy number and presence of the rs2231142 risk allele on gout risk. Table 3 shows the odds ratios for gout risk in the four cohorts stratified into four groups for the TPPP CNV and presence of the ABCG2 risk allele. Overall, in all cohorts the presence of the rs2231142 risk allele increases gout risk (as is already well-known), but specifically in combination with increased copy numbers of TPPP the risk is amplified. For the stratifications within the non-risk allele groups, higher TPPP copy numbers does not appear to have a significant effect in increasing gout risk. In the meta-analysis of all cohorts (table 3) the already increased odds ratio with the presence of the rs2231142 risk allele is increased from 2.59 (CI 2.01-3.33) to 3.68 (CI 2.63-5.20) when TPPP copy number is above 4.
Graphical representations of the stratified odds ratios per cohort are shown in Supplementary figure 2. However this observation was not supported by a formal test for genetic interaction that was done by including an interaction term in the logistic regression analysis. In figure 4, the interaction between TPPO CNV and the rs2231142 genotype shows a trend towards increased gout risk in all four cohorts, although none are statistically significant, including a non-significant finding by meta-analysis.

### Figure 4. Association between TPPO CNV’rs2231142 interaction and gout risk

Logistic regression analysis was performed in R corrected for age, gender, and PCR plate number: Gout status ~ integer TPPO copy number * rs2231142 + age*gender + PCR plate number. A. Logistic regression analysis of European groups 1 and 2. B. Logistic regression analysis of West and East Polynesian cohorts. C. Meta-analysis of all four cohorts combined.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>OR</th>
<th>[95% CI]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>European Group 1, n=609</td>
<td>1.332</td>
<td>[0.806; 2.203]</td>
<td>0.263</td>
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<td>European Group 2, n=794</td>
<td>1.144</td>
<td>[0.536; 2.440]</td>
<td>0.728</td>
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</tbody>
</table>

**Overall Effect**

- Heterogeneity: $\chi^2 = 0.11 (p = 7.4e-01)$
- Test for overall effect: $z = 1.12 (p = 2.6e-01)$

<table>
<thead>
<tr>
<th>Cohort</th>
<th>OR</th>
<th>[95% CI]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>East Polynesian, n=901</td>
<td>1.253</td>
<td>[0.807; 1.948]</td>
<td>0.315</td>
</tr>
<tr>
<td>West Polynesian, n=548</td>
<td>1.272</td>
<td>[0.867; 2.391]</td>
<td>0.456</td>
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</tbody>
</table>

**Overall Effect**

- Heterogeneity: $\chi^2 = 0.00 (p = 9.7e-01)$
- Test for overall effect: $z = 1.25 (p = 2.1e-01)$

<table>
<thead>
<tr>
<th>Cohort</th>
<th>OR</th>
<th>[95% CI]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>European Group 1, n=609</td>
<td>1.332</td>
<td>[0.806; 2.203]</td>
<td>0.263</td>
</tr>
<tr>
<td>European Group 2, n=794</td>
<td>1.144</td>
<td>[0.536; 2.440]</td>
<td>0.728</td>
</tr>
<tr>
<td>East Polynesian, n=901</td>
<td>1.253</td>
<td>[0.807; 1.948]</td>
<td>0.315</td>
</tr>
<tr>
<td>West Polynesian, n=548</td>
<td>1.272</td>
<td>[0.867; 2.391]</td>
<td>0.456</td>
</tr>
</tbody>
</table>

**Overall Effect**

- Heterogeneity: $\chi^2 = 0.11 (p = 9.9e-01)$
- Test for overall effect: $z = 1.68 (p = 9.3e-02)$
Table 3. Logistic regression analysis per TPPP CNV and rs2231142 (Q141K) cluster group

<table>
<thead>
<tr>
<th>Cohort and cluster group</th>
<th>Cases (n)</th>
<th>Controls (n)</th>
<th>OR</th>
<th>2.5% CI</th>
<th>97.5% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>European group 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 non-risk</td>
<td>147</td>
<td>132</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 non-risk</td>
<td>79</td>
<td>63</td>
<td>1.30</td>
<td>0.83</td>
<td>2.05</td>
<td>0.255</td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 risk</td>
<td>85</td>
<td>47</td>
<td>2.05</td>
<td>1.28</td>
<td>3.32</td>
<td>0.003</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 risk</td>
<td>40</td>
<td>16</td>
<td>3.38</td>
<td>1.71</td>
<td>7.04</td>
<td>6.78x10^{-4}</td>
</tr>
<tr>
<td><strong>European group 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 non-risk</td>
<td>155</td>
<td>188</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 non-risk</td>
<td>80</td>
<td>118</td>
<td>0.75</td>
<td>0.43</td>
<td>1.29</td>
<td>0.301</td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 risk</td>
<td>115</td>
<td>45</td>
<td>2.89</td>
<td>1.60</td>
<td>5.37</td>
<td>5.57x10^{-4}</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 risk</td>
<td>62</td>
<td>31</td>
<td>3.65</td>
<td>1.70</td>
<td>8.21</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>West Polynesian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 non-risk</td>
<td>59</td>
<td>129</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 non-risk</td>
<td>12</td>
<td>23</td>
<td>1.42</td>
<td>0.54</td>
<td>3.62</td>
<td>0.468</td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 risk</td>
<td>141</td>
<td>87</td>
<td>4.92</td>
<td>2.91</td>
<td>8.49</td>
<td>4.79x10^{-9}</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 risk</td>
<td>65</td>
<td>22</td>
<td>8.49</td>
<td>4.18</td>
<td>17.94</td>
<td>8.11x10^{-9}</td>
</tr>
<tr>
<td><strong>East Polynesian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 non-risk</td>
<td>223</td>
<td>281</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 non-risk</td>
<td>123</td>
<td>128</td>
<td>1.07</td>
<td>0.73</td>
<td>1.57</td>
<td>0.734</td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 risk</td>
<td>55</td>
<td>35</td>
<td>1.85</td>
<td>1.06</td>
<td>3.28</td>
<td>0.032</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 risk</td>
<td>34</td>
<td>22</td>
<td>2.39</td>
<td>1.20</td>
<td>4.92</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>Meta-analysis (all cohorts)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 non-risk</td>
<td>584</td>
<td>730</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 non-risk</td>
<td>294</td>
<td>332</td>
<td>1.06</td>
<td>0.84</td>
<td>1.35</td>
<td>0.614</td>
</tr>
<tr>
<td>TPPP CNV≤4, ABCG2 risk</td>
<td>396</td>
<td>214</td>
<td>2.59</td>
<td>2.01</td>
<td>3.33</td>
<td>1.55x10^{-13}</td>
</tr>
<tr>
<td>TPPP CNV&gt;4, ABCG2 risk</td>
<td>201</td>
<td>91</td>
<td>3.68</td>
<td>2.63</td>
<td>5.20</td>
<td>8.00x10^{-14}</td>
</tr>
</tbody>
</table>

In these logistic regression analyses, the TPPP CNV≤4, ABCG2 non-risk allele (141Q) group is used as reference group.
Effects of TPPP CNV and rs2231142 genotype on functional immunological parameters

In a Dutch gout patient subset of the European group 2, PBMC IL-1β and IL-6 production was measured in response to a combined stimulation with MSU crystals and palmitate. By means of clustered analyses, we aimed to study whether TPPP copy number or rs2231142 genotype affect proinflammatory cytokine production. Table 4 lists the characteristics of this immunological phenotype cohort. A Chi-square test could not provide evidence for an association between TPPP CNV and rs2231142 genotype in this cohort (table 5).

Table 4. Cohort characteristics for immunological phenotyping in the Dutch gout cohort

<table>
<thead>
<tr>
<th>Gout patient cohort</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>142</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>65 ± 13</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>121 (85)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>21 (15)</td>
</tr>
<tr>
<td>rs2231142 frequency</td>
<td></td>
</tr>
<tr>
<td>GG, n (%)</td>
<td>83 (58.5)</td>
</tr>
<tr>
<td>GT, n (%)</td>
<td>48 (33.8)</td>
</tr>
<tr>
<td>TT, n (%)</td>
<td>11 (7.7)</td>
</tr>
</tbody>
</table>

Table 5. Crosstabs of rs2231142 genotype and TPPP CNV in Dutch gout cohort

<table>
<thead>
<tr>
<th>ABCG2 rs2231142 genotype</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GT&amp;TT</td>
<td></td>
</tr>
<tr>
<td>TPPP CNV</td>
<td>≤4</td>
<td>&gt;4</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>55 (66.3%)</td>
<td>32 (54.2%)</td>
<td>87</td>
</tr>
<tr>
<td>&gt;4</td>
<td>28 (33.7%)</td>
<td>27 (45.8%)</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>59</td>
<td>142</td>
</tr>
</tbody>
</table>

We aimed to analyze the effects of TPPP CNV and rs2231142 genotype on functional immunological parameters. Due to the highly skewed distribution of in vitro cytokine production, an ANOVA analysis would not be appropriate to assess the effects of TPPP CNV and rs2231142 genotype. Therefore, we chose to analyze these data by clustering the groups based on the TPPP CNV and rs2231142 genotype. Interestingly, it appears that the effect of increasing TPPP copies on ex vivo production of IL-1β and IL-6 depends on whether or not the rs2231142 risk allele is present (figure 5) with production decreasing in the presence of the 141K allele. Despite the appearance of an interaction effect in figure 5, a Kruskall-Wallis test showed no evidence for differences between the four clusters.
TPPP copy number variation is associated with gout risk in Polynesian people

Figure 5. In vivo and ex vivo cytokine production in Dutch gout patient cohort
Freshly isolated PBMCs were cultured for 24 hours in the presence of a combination of MSU crystals (300 µg/mL) and palmitic acid (C16.0, 50 µM). IL-1β (A) and IL-6 (B) levels were measured in the supernatant after culture. C and D. Line graph representations of the data in A and B, respectively.
**DISCUSSION**

Despite our knowledge on the pathogenesis of gout, we still know very little about the genetic control of gout-related inflammatory pathways. The SNPs that have so far been associated to serum urate levels and gout have relatively small effect sizes and are mostly involved in urate transport and glycolysis (11). In this study, we identify a copy number variable region (CNVR) on chromosome 5 that is associated with gout risk from a genome-wide study in Europeans. More specifically, deletions in chr5:658,692-728,796 gave a protective effect on gout risk, whereas duplications did not increase the risk. This CNVR on chromosome 5 overlaps with the **TPPP** gene, which provides us with a highly interesting candidate gene for inflammatory mechanisms in acute gout.

Microtubules are of crucial important for the cytoskeleton of a cell and serve important functions in the transport of cytoplasmic structures. Microtubules are dynamic, as they have to respond to the cell’s need to execute various functions, such as cell division, differentiation, activation and migration. Microtubule architecture is controlled by various post-translational modifications of tubulin. Both HDAC6 and SIRT2 are known $\alpha$-tubulin deacetylases (34, 35) and inhibition of SIRT2 has been shown to increase acetylated $\alpha$-tubulin and facilitate transport required for NLRP3 inflammasome assembly (6). TPPP is a protein that promotes the dynamic stabilization of microtubules by counteracting SIRT2 and HDAC6 effects (36, 37). In this way, we envisaged that a TPPP copy number variation might affect gout risk via its effects on microtubule stabilization and NLRP3 inflammasome activation. In the TPPP qPCR replication cohorts we observe that the copy number is associated with slightly increased gout risk, but this effect was significant only in the cohort of Western Polynesian ancestry.

A previous study showed that colchicine and HDAC inhibitors can restore cell surface expression and function of the Q141K ABCG2 by disturbing transport via microtubules of the Q141K variant into aggresomes (15). We hypothesized that there might be an interaction between the TPPP CNV and the gout-associated rs2231142 SNP in ABCG2: Separately, both genetic variations may increase gout risk, but in the presence of the rs2231142 risk allele, a higher TPPP copy number may rescue ABCG2 surface expression and decrease gout risk. By stratified analysis our data suggested an amplified effect of increased TPPP CN and ABCG2 141K on the risk of gout in all four cohorts. However a formal test for non-additive interaction was not statistically significant.

For a Dutch gout subset of the European cohorts, functional immunological data were available. We used this data to assess any functional effects of the TPPP copy number variation. For the ex vivo production of IL-1$\beta$ and IL-6 in response to C16.0+MSU co-stimulation the effect of TPPP copy number was dependent on the presence or absence of the Q141K ABCG2 protective allele such that IL-1$\beta$ response decreased in combination with 141K and increased TPPP copy number. At face value this contradicts the epidemiological data suggesting increased risk of gout with the combination of increased TPPP copy number and Q141K. It would be interesting to study this further. The combination of the 141K risk allele and high TPPP copy number may increase gout risk for example by altering cellular urate processing and MSU crystal formation, but have an opposite effect on subsequent MSU-induced cytokine production in the ex vivo model.

In conclusion, we show that copy number variation of the TPPP gene is associated with gout risk in 2 of 5 cohorts. On a functional level, however, the effect of TPPP copy number is dependent
on the presence of particular Q141K alleles, i.e. there may be an interaction effect of these two
genetic variations on ex vivo cytokine production. As reviewed previously (13), the Q141K ABCG2
variant not only affects urate transport, but may also affect several inflammation-related cellular
pathways, such as autophagy and cellular responses to stress. These results provide insight
into the possible contribution of structural genetic variation in *TPPP* on inflammatory pathways
in gout.
REFERENCES

TPPP copy number variation is associated with gout risk in Polynesian people

Supplementary figure 1. Flow-chart of sample quality controls
A. Flow-chart of each step in the quality control on the gout participant cohort from New Zealand and Australia (n=468) and the dataset with controls from the WTCCC 1958 Birth Cohort (n=1000). B. The remaining numbers of participants in each cohort after quality control.
TPPP copy number variation is associated with gout risk in Polynesian people.
9

Summary and general discussion
SUMMARY AND GENERAL DISCUSSION

Gout is an ancient complex disease with records dating back to Ancient Egypt (1). Due to its association with life in luxury it was often referred to as the ‘disease of kings’. Today, in a time of Western dietary habits and a sedentary lifestyle, gout is no longer preserved only for the rich. It currently affects 1-4% of the adult population in Western countries (2-5) and its incidence and prevalence has been rising over the past decades (3,4). Despite our current elaborate knowledge on the pathogenesis of gout, treatment of gout is still suboptimal even in developed countries. Kuo et al. reported that only 48% of prevalent gout patients in the UK in 2012 were seen by a medical professional for their gout or treated with urate-lowering therapy (ULT) (4). A study conducted in Australian general practitioner care in 2008-2013 showed that only 57% were given allopurinol, 55% had their serum urate levels checked in these five years (6). Overall, of all chronic diseases, gout has some of the poorest adherence rates (4, 7). A meta-analysis of therapy adherence rates in gout patients showed an overall adherence rate of 47% (8). Part of these poor adherence rates may be caused by poor disease perception. Historically, gout is viewed as a disease self-inflicted by excessive alcohol consumption and overeating. Gout has been (and still is) often portrayed comically, further stigmatizing this disease (9). Furthermore, annual primary hospitalizations rates for gout in the US have increased from 4.4 (1993) to 8.8 (2011) per 100,000 US adults, whereas hospitalization rates for rheumatoid arthritis decreased (10).

Recent guidelines from the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) on gout management highlight the importance of treating to a serum urate target <0.36 mmol/l (11,12). Next to ULT, there are several recommended options to treat and prophylactically prevent acute gout flares. Despite their efficacy, the most commonly used anti-inflammatory treatment options, colchicine, NSAIDs and corticosteroids, may be poorly tolerated or contra-indicated due to the presence of comorbidities (13,14). In patients with limited treatment options, an IL-1 blocking agent, such as canakinumab (15,16) may be used. However, IL-1 blocking agents are extremely costly. As an example, one dose of Canakinumab of 150 mg costs around € 12,000 in the Netherlands (17). This poses the need for the development of new well-tolerated, targeted and cost-effective treatment options.

The current thesis is an endeavor to further elucidate the inflammatory mechanisms that are at play during acute gouty arthritis, exploring the potential of using histone deacetylase inhibitors to inhibit monosodium urate crystal-induced inflammation, and touching upon the genetic control of gout-related inflammatory pathways. This chapter summarizes the work presented in this thesis and provides future perspectives for follow-up research in the field.

Advances in our knowledge of acute gout pathogenesis

Chapter 2 reviews the current knowledge and recent advances in the field of gout pathogenesis. The role of the gut microbiome in gouty arthritis is discussed. A recent study from China found an altered gut microbiome profile in gout patients compared to healthy controls (18). Interestingly, they found that the gut microbiome of gout patients was characterized by impaired butyrate synthesis from dietary fiber. The potential role of butyrate in gout pathogenesis has been corroborated by several murine studies (19, 20). Short-chain fatty acids, and butyrate in particular, are known HDAC inhibitors (21) and the therapeutic mechanism of HDAC inhibitors
in MSU-induced inflammation will be discussed more elaborately in the next section of this chapter.

Furthermore, recent findings on intracellular sensing of monosodium urate crystals and the different cellular pathways leading up to the damaging and self-perpetuating production of interleukin (IL-1β) were summarized. We have known for over a decade now that monosodium urate crystals trigger the activation of the NOD-like receptor pyrin-containing 3 (NLRP3) inflammasome (22). However, there is still discussion as to how this is mediated. Many studies have implicated mitochondrial stress and the production of reactive oxygen species (ROS) as a crucial step in this process (23, 24). Others have shown that defective autophagy induced NLRP3-dependent IL-1β production (25-27). Finally, one group recently reported that MSU-crystals inhibit AMP-activated protein kinase (AMPK) activation, leading to a significantly increased inflammatory response in a murine air pouch model (28).

Next to intracellular sensors, there are several important neutrophilic processes at play during acute gouty arthritis. Neutrophils serve in the first place to promote MSU-induced cytokine production. Around a decade ago, dying neutrophils were shown to release several serine proteases, capable of activating IL-1β in a caspase-1-independent manner (29, 30). More recently, several research groups showed roles for neutrophils in the resolution of acute gouty arthritis through release of microvesicles which induce suppressor of cytokine signaling 3 (SOCS3) expression and release of tumor growth factor (TGF)-β (31), and the formation of neutrophil extracellular traps (NETs) (32).

New insights into the effects of soluble uric acid

Taking a step back from MSU-induced inflammation, we also elaborated on the role of uric acid in human evolution, metabolic dysfunction and autoimmune diseases in chapter 2. Humans and ape species are especially prone to develop hyperuricemia due to the step-wise loss of the uricase gene during the course of evolution (33). Uricase mediates the breakdown of uric acid in to the soluble allantoin. Recently, an interesting theory on the evolutionary advantage of increased serum urate levels was proposed by Johnson et al. Apes were likely to consume fruits during warmer periods. Fruit-derived fructose has been shown to stimulate accumulation of triglycerides and uric acid. They further propose that uric acid protected our them in times of famine, when fruit was unavailable, by stimulating fat storage and gluconeogenesis. Finally, they suggest that this fructose-induced fat and glucose accumulation may underlie the current metabolic syndrome pandemic.

Indeed, many groups have studied and found associations between uric acid and metabolic syndrome (34-36). Additionally, several trials with allopurinol have reported beneficial effects on renal function (37), insulin resistance and CRP levels (38), and blood pressure (39). Therefore, in chapter 3, we aimed to study in vitro the effects of soluble uric acid on the inflammatory potential of human peripheral blood mononuclear cells. MSU crystals do not induce any cytokine production in PBMCs on their own. They activate the NLRP3 inflammasome, but as inactive pro-IL-1β is not constitutively present in the cells, we need an additional stimulus, such as Toll-like receptor (TLR) ligands to induce the transcription of pro-IL-1β (40). For the in vitro experiments described in this chapter, we used a combination of MSU crystals and palmitic acid (C16:0), Pam3CSK4 (P3C), or lipopolysaccharide (LPS) to induce a synergistic production of active IL-1β.
We observed that gout patients produce higher amounts of IL-1β and IL-6 compared to healthy volunteers. Moreover, this effect was positively correlated with serum urate levels.

To mimic the in vivo exposure of circulating monocytes to high levels of uric acid, we pre-cultured cells with uric acid for 24h, after which the supernatant was washed away and the cells were restimulated for another 24h with various TLR-ligands with or without MSU crystals. This resulted in an intriguing cytokine response. Very consistently, we were able to show that IL-1β, IL-6 and TNFα increased with uric acid priming in a dose-dependent manner, whereas the anti-inflammatory IL-1Ra decreased. This was a very interesting finding, as the anti-inflammatory IL-1Ra mostly increases and decreases simultaneously with IL-1β. The dose-dependent uric acid priming effect was also reflected in IL-1β and IL-1Ra mRNA levels. Comparison of ex vivo uric acid priming in cells from gout patients and healthy controls revealed that cells from gout patients show less capacity to upregulate IL-1β production upon uric acid priming compared to controls. Additionally, we observed higher basal IL-1β mRNA levels in gout patients compared to healthy controls. Both findings are indicative of long-term in vivo hyperuricemia-induced priming effects. Finally, we observed that the histone methyltransferase inhibitor MTA reversed the uric acid priming effect, indicating that uric acid may induce non-specific immunological memory via epigenetic modifications.

One thing to consider from our experimental setup are the high concentrations of uric acid required to induce priming effects. A concentration of 50 mg/dl is considerably higher than physiological concentrations of serum urate. However, as shown in the supplementary data from chapter 5, similar trends in IL-1β/IL-1Ra ratios were found with lower concentrations of uric acid, when it is still fully soluble. For our in vitro experiments, high concentrations of uric acid were required to show effects over 48 hours, in an attempt to mimic the in vivo situation of exposure to elevated urate over (sometimes many) years. Furthermore, although we have checked for the presence of MSU crystals in our in vitro experimental setting, we cannot fully exclude the possibility that there are (micro)crystals remaining in the supernatant. However, the specific pattern of IL-1β upregulation and IL-1Ra downregulation re-assures us that this is not caused by MSU crystals, as stimulation experiments with MSU crystals and TLR ligands show a significant increase of IL1β and IL-1Ra production.

It has been shown previously that non-specific immunological memory (or trained immunity) can convey protection against subsequent infection by a pathogen unrelated to the initial training stimulus (41, 42). This new layer of regulation of inflammatory responses may be beneficial in case of infection or vaccination strategies, it also may have detrimental consequences for the many chronic inflammatory diseases we currently face. If uric acid is able to induce long-term epigenetic reprogramming, this may explain in part the chronic low-grade inflammation observed in gout, diabetes, cardiovascular diseases and other related metabolic inflammatory diseases.

Considering histone deacetylase inhibition as anti-inflammatory treatment

Chapters 4-6 explore the anti-inflammatory properties of histone deacetylase inhibitors, their potential to inhibit MSU-induced cytokine production, and the cellular pathways involved in this. Chapter 4 starts out to study the naturally occurring broad HDAC inhibitor butyrate. Broad-spectrum HDAC inhibition has already been shown to have beneficial effects in murine models.
of arthritis and even provided clinical improvement in a trial with juvenile idiopathic arthritis patients (43, 44).

We show that 1 mM butyrate significantly inhibited the production of IL-1β, IL-6, and IL-8 induced by in vitro stimulation of PBMCs by a combination of MSU crystals and C16:0. This inhibition is accompanied by a significant decrease in IL1β mRNA. Intriguingly, the dose-dependent effects of butyrate are very different with LPS-stimulation. In this case, butyrate actually increased IL-1β production, indicating that the effects of butyrate on cytokine production are both dose- and stimulus-dependent. We reported that butyrate most strongly inhibits HDAC8 activity, but is in general a class I HDAC inhibitor, with IC50s for blocking HDAC1, HDAC2, HDAC3, and HDAC8 of around 100-700 M.

Comparing the effects of butyrate with a highly specific HDAC8-inhibitor revealed that HDAC8 is not involved in the suppression of MSU-induced cytokine production. Moreover, HDAC inhibitors panobinostat and ITF-B, which both have specifically low IC50 values for inhibiting class I and class IV HDACs, show dose-response effects with MSU+C16:0 and LPS stimulation very similar to butyrate. As butyrate itself has only low inhibitory capacity for class IV HDACs at 1 mM, these findings led us to conclude that the inhibition of MSU+C16:0-induced cytokine production is mediated through one or a combination of HDAC1, HDAC2 and/or HDAC3.

If butyrate is an effective HDAC inhibitor capable of reducing inflammatory responses in circulating PBMCs, this would be an interesting, safe, and cheap option to limit systemic low-grade inflammation. In chapter 5, we therefore studied the effects of 4-week oral butyrate supplementation in healthy and obese males on ex vivo cytokine production profiles. For this, we envisaged that circulating monocytes encounter high butyrate concentrations in the gut, inducing epigenetic changes in their inflammatory potential. Unfortunately, we were unable to detect consistent effects of butyrate supplementation on ex vivo cytokine production upon direct stimulation. We did find reduced trained immunity effects, specifically in the group of obese males trained with oxidized low-density lipoprotein (oxLDL).

Next to the small numbers of participants in this study, a limitation was our inability to detect increased butyrate levels in feces or plasma. It is known that butyrate is a primary energy source for colonocytes, is largely metabolized by the liver, and only a small fraction is excreted via feces (45,46). Moreover, in a recent human trial, butyrate concentrations were already undetectable 2 hours after colonic infusion (47). All in all, this study provided no indication that oral butyrate supplementation may be beneficial for gout patients. Considering the high concentrations of butyrate required for class I HDAC inhibition, it is very unlikely that we will achieve such concentrations in the plasma for extended periods of time to induce acetylation changes in circulating PBMCs. Although butyrate treatment may be an effective treatment option in patients with inflammatory bowel disease, where butyrate can exert its effects locally in the gut, it most likely is not the answer to limit systemic inflammation.

As shown in chapter 4, synthetic HDAC inhibitors are effective at much lower concentrations and even achieve cytokine suppression in the nM range. With the current efforts in pharmaceutical companies to develop highly specific HDAC inhibitors, this may be a more valuable treatment option, possibly with large effectiveness while reducing side effects. In chapter 6, we aim to narrow down on the specific HDAC(s) involved in MSU+C16:0-induced cytokine production. We
show that specific inhibition of HDAC2, HDAC3, or HDAC6 did not reduce MSU+C16.0-induced IL-1β production. Entinostat, a specific HDAC1/3 inhibitor, significantly decreased IL-1β production by 30-40%, whereas 50 nM of the HDAC1/2 inhibitor romidepsin even decreases IL-1β production by 75%. Possibly due to redundancy and compensatory mechanisms, dual HDAC inhibition is more effective than single specific HDAC blocking. Even at 25nM, romidepsin significantly decreased IL-1β, IL-1Ra, IL-6, and IL-8. In addition, we observed that romidepsin upregulated transcription of suppressor of cytokine signaling 1 (SOCS1) and decreased activation of signal transducer and activator of transcription (STAT)1 and STAT3. Interestingly, to our knowledge, this is an unexplored signaling pathway in the context of gout. Next to suppression of STAT1, SOCS1 has been shown to negatively regulate TLR signaling via proteasomal degradation of various signaling molecules (48-50). Indeed, we showed that the cytokine-suppressive effects of romidepsin were reversed by the addition of proteasome inhibitor bortezomib.

The results described in chapters 4-6 provide a rationale for the use of specific HDAC inhibitors as a potential treatment strategy in acute gouty arthritis. There are, however, still many issues that have to be addressed. The largest concern is the differential response of butyrate and romidepsin when added to different TLR ligands. In unpublished results from in vitro experiments, we observe an adverse response with both HDAC inhibitors when we subsequently stimulate cells with LPS or a combination of MSU-LPS. After 24 hours of culture, there was a consistent increase in extracellular IL-1β, but a decrease in both intracellular IL-1β and IL-1β mRNA with 1 mM butyrate or 50 nM romidepsin. In contrast, in stimulation experiments with TLR2 ligand Pam3CSK4 and C16.0 (with or without MSU) both butyrate and romidepsin significantly decrease extracellular, intracellular, and mRNA IL-1β. This could be a matter of dosing, as we observed in chapter 4 that extracellular IL-1β decreases with very low concentrations of panobinostat and ITF-B. Alternatively, it is possible that the combination of LPS and HDAC inhibitors provide a particular inflammatory condition in which caspase-1 dependent activation of pro-IL1β is surpassed and the inactive form is secreted into the supernatant. Our findings are in line with other studies showing differential effects of HDAC inhibitors with LPS stimulation (51,52). The differential effects with LPS stimulation compared to Pam3CSK4 or C16.0 may be caused by differences in metabolic responses to these stimuli. As shown by Lachmandas et al., both stimuli induce glycolysis to provide a quick energy supply for the inflammatory response. However, only LPS stimulation led to decreased oxidative phosphorylation, whereas Pam3CSK4 stimulation was shown to increase oxygen consumption and mitochondrial enzyme activity (53).

Stimulus-dependent differential effects of HDAC inhibitors may pose a challenge for their therapeutic use as anti-inflammatory agents. In gout patients, it is often observed that acute gout develops in the night after consuming a large meal. This makes the free fatty acid C16.0 a likely candidate to trigger the MSU-induced inflammatory responses. However, we may never be sure what triggers this response in patients and it may vary between individuals (or even between attacks within an individual). It is therefore very important to be cautious with the use of HDAC inhibitors as anti-inflammatory agents. An alternative would be to target the inflammatory pathway that is regulated by HDAC1/2, such as our finding with the SOCS/JAK/STAT pathway in chapter 6.
Summary and general discussion

Gene control of urate transport and inflammatory pathways in gout

In chapters 7 and 8, we explore the genetic control of gout inflammatory pathways. In particular, we focus on the effects of ABCG2 polymorphisms and effects of copy number variable regions associated with gout. We have reviewed the current knowledge of ABCG2 polymorphisms in gout in chapter 7. The Q141K ABCG2 variant, caused by the rs2231142 polymorphism, is the most widely studied common variant in ABCG2 associated with both hyperuricemia and gout risk. This missense mutation has been shown to reduce ATPase activity, uric acid transport activity and its total and surface membrane expression levels (54-57). Previous studies have provided evidence that the Q141K variant is targeted for proteasomal degradation and sequestered in aggresomes (57). Next to its role as a urate transporter, we also discussed a role of ABCG2 in autophagy. Ding et al. reported that autophagy was increased in cell lines overexpressing ABCG2 (Ding 26983466). In this way, functional ABCG2 expression may limit MSU-induced inflammatory responses by mediating autophagy of damaged mitochondria, thereby limiting NLRP3 inflammasome activation. Interestingly, two groups have reported that HDAC inhibitors were able to restore surface membrane expression of the Q141K ABCG2 variant (56, 57). Basseville et al. show this is mediated via disturbing microtubule-mediated transport of the Q141K variant to the aggresome (57).

In chapter 8, a hypothesis-free approach was utilized to detect copy number variable regions (CNVs) that are associated with gout risk. CNVs are a form of structural genetic variation leading to deletions or duplications of genetic loci. Up until today, only a small part of the hereditability of gout has been explained by common single-nucleotide polymorphisms and CNVs may provide additional genetic factors that play a role in urate control or gout-related inflammatory pathways. We showed that a CNV at chromosome 5, position 658,692-728,796, is significantly associated with increased gout risk. We were able to validate this finding of TPPP copy number variation by PCR in a Polynesian cohort, but not in a cohort of European descent. Further exploring this particular locus, we found the TPPP gene to be located within this region. TPPP encodes the tubulin polymerization-promoting protein. Based on the findings by Basseville et al. described above, we hypothesized that this CNV may interact with the ABCG2 rs2231142 polymorphism (Q141K variant), as changes in tubulin-mediated transport were shown to affect Q141K ABCG2 expression (57). When stratifying the cohorts for TPPP copy number and presence of the rs2231142 risk allele, we observed that increased TPPP copy numbers did not increase gout risk when the rs2231142 risk allele was not present. However, in the presence of the rs2231142 risk allele, increasing TPPP copy numbers even further increased gout risk. Unfortunately, functional data in a Dutch subset of the European cohort did not reflect this finding. None of the immunological functional parameters measured showed significant changes with increased TPPP copy number in the rs2231142 risk allele group. Observing the direction of the effects of TPPP copy number and comparing it between presence and absence of the rs2231142 risk allele does indicate that there may be an interaction effect. However, if this is true, the direction of the effect is opposite to what we would expect, as ex vivo IL-1 and IL-6 production seemingly decrease with higher TPPP copy numbers in the presence of the rs2231142 risk allele.
FUTURE PERSPECTIVES

With this thesis, an attempt was made to elucidate the MSU-crystal independent effects of soluble uric acid, to explore the anti-inflammatory mechanism and therapeutic potential of HDAC inhibitors, and assessed the genetic control of copy number variable regions on gout risk. And although we have provided several scientific advancements in the field of gout pathogenesis, still many questions remain.

Although we have shown priming effects of soluble uric acid in vitro which are indicative of a pro-inflammatory role independent of MSU crystal formation, its direct causative role in gout, cardiovascular disease, diabetes, and other chronic metabolic diseases remains elusive. Several trials have reported beneficial effects of allopurinol besides lowering urate levels (37-39), but it remains to be determined whether these effects are caused by a decrease in uric acid or inhibition of xanthine oxidase induced ROS. To study the direct inflammatory effects of soluble uric acid, and to provide possible rationale for treating asymptomatic hyperuricemic patients (with for example metabolic syndrome and at risk for cardiovascular events), it would be valuable to compare a uricosuric agent such as probenecid to the xanthine oxidase inhibitor allopurinol and a placebo control in a large prospective trial.

Additionally, as the epigenetic landscape in gout is still largely unexplored, mapping the epigenome of participants with low and high uric acid levels may provide us with specific profiles of regulatory regions that mediate a chronic low-grade inflammatory state. If indeed we would be able to map such specific uric acid-induced pro-inflammatory epigenetic profile, we might track its reversal during urate-lowering therapy in the future, giving us insight into the longevity of the in vivo uric acid priming. Furthermore, there is still the question which cells would be primed in vivo by uric acid. Is it the continuous exposure to serum urate that maintains the primed state in circulating cells, or are also progenitor cells affected by persistent epigenetic reprogramming?

Similarly, assessing chromatin accessibility and expression data would be an important advancement in our knowledge of the anti-inflammatory mechanisms of HDAC inhibitors. The difficulty remains that HDAC inhibitors not only affect histones but many other cellular proteins as well, such as signaling molecules or transcription factors. With this in mind, we may also more specifically target the inflammatory pathways affected by HDAC inhibitors, such as the SOCS/JAK/STAT pathway and further explore its role in MSU-induced inflammatory responses. Secondly, we discussed that the therapeutic use of HDAC inhibitors urges for caution as their effects appear to be dose- and stimulus-dependent.

Finally, associations between the genetic control of gout-related inflammatory pathways and gout susceptibility remain very limited. We observed an association between a copy number variable region in chromosome 5 and gout risk, but it is still unclear whether this CNV directly affects TPPP expression levels. CNVs may emerge as important genetic factors controlling gout susceptibility, but genetic data, expression data, and preferably even immunological functional data of large cohorts would be required to advance our understanding of their role in gout.
REFERENCES


10

Nederlandse samenvatting
Jicht

De mens is al sinds de Egyptische Oudheid bekend met de pijn die jicht kan veroorzaken. Voor lange tijd werd jicht gezien als een aandoening voor de elite, veroorzaakt door een overdaad aan eten en drinken. In onze huidige Westerse samenleving, waarin voedsel gemakkelijk verkrijgbaar is en we steeds minder fysiek actief zijn, is jicht echter uitgegroeid tot een ziekte die voorkomt bij 1-4% van de volwassen populatie.

Opvallend is dat we eigenlijk precies weten hoe jicht ontstaat. Verschillende factoren, zoals onze genetische code (DNA) en eetgewoonten, kunnen de concentratie van urinezuur in het bloed verhogen. Urinezuur is op zich geen boosdoener. Het is een natuurlijk afbraakproduct uit onze voeding en ontstaat wanneer cellen in ons lichaam worden gerecycled. Echter, wanneer de concentratie urinezuur in ons bloed te hoog wordt, lost het niet meer op (zoals te veel zout niet meer oplost in water). Op dat moment worden er (vooral in gewrichten) urinezuurkristallen gevormd, welke er onder de microscoop uitzien als kleine puntige naaldjes. Onder bepaalde omstandigheden reageren de cellen van ons afweersysteem op deze kristallen. Ze zien deze dan als indringers in ons lichaam. Hierbij ontstaat een ontsteking, waarbij het gewricht dik, rood, warm en pijnlijk wordt. Dit heet jichtartritis.

Behandeling van jicht bestaat enerzijds uit therapie om de concentratie urinezuur in het bloed te verlagen en anderzijds uit therapie om ontsteking bij jichtartritis te remmen of te voorkomen. Ondanks dat deze behandelingen over het algemeen goed werken, blijft jicht een probleem voor veel mensen. De meest voorgeschreven ontstekingsremmende therapieën, zoals colchicine, NSAIDs, en corticosteroïden, worden in sommige gevallen afgeraden door de aanwezigheid van andere ziekten, of kunnen voor bijwerkingen zorgen. Andere nieuwere therapieën, zoals de biologicals canakinumab en anakinra, zijn vooralsnog ontzettend duur. Er is daarom behoefte aan de ontwikkeling van nieuwe veilige en kosten-efficiënte medicatie om de ontsteking bij een acute jichtartritis te kunnen remmen.

In dit proefschrift heb ik geprobeerd te achterhalen welke mechanismen actief zijn in de afweercellen bij jichtartritis; heb ik onderzocht of een bepaalde groep bioactieve stoffen (histon deacetylase remmers) de ontsteking bij jicht kunnen remmen; en heb ik gekeken naar de rol van het DNA in het ontstaan van jichtartritis. In dit hoofdstuk vat ik de belangrijkste bevindingen van mijn proefschrift samen.

Het ontstaan van jicht en de rol van urinezuur

In hoofdstuk 2 vat ik de huidige kennis en recente ontwikkelingen met betrekking tot de ontwikkeling van acute jichtartritis samen. Ook gaat dit hoofdstuk dieper in op de vraag waarom mensen en apen, in tegenstelling tot andere zoogdieren, bijzonder gevoelig zijn voor verhoging van urinezuurconcentraties in het bloed. Voorheen werd gedacht dat urinezuur zelf geen rol speelde in het ontstaan van jichtartritis en dat alleen uraatkristallen de veroorzakers van jicht waren.
In hoofdstuk 3 hebben we daarom onderzocht of er een rol is voor urinezuur in oplossing in het ontstaan van jichtartritis. Om dit te testen isoleerden we mononucleaire perifere bloedcellen (PBMCs) uit bloed van gezonde donoren en patiënten met jicht. De PBMC-fractie uit het bloed bestaat uit cellen die het grootste deel van ons afweersysteem vormen, zoals T-cellen, B-cellen en monocyten. In onze experimenten stelden we PBMCs bloot aan urinezuur voor 24 uur, wassen vervolgens het urinezuur weg van de cellen, en stimuleerden de cellen daarna met bacteriedeeltjes en uraatkristallen om een ontstekingsreactie zoals in jichtartritis uit te lokken.

We observeerden dat PBMCs die waren blootgesteld aan urinezuur veel meer ontstekingsstoffen (cytokines) produceerden dan PBMCs die niet waren blootgesteld aan urinezuur. Zeer interessant hierbij was de disbalans in de vorm van een verlaagde productie van de ontstekingsremmende cytokine IL-1Ra (interleukine-1 receptor antagonist) en een verhoogde productie van de ontstekingsstimulerende cytokine IL-1β (interleukine-1beta). Verder zagen we dat deze disbalans in cytokines werd veroorzaakt door veranderingen in de manier waarop DNA is opgerold. DNA zit gewikkeld om bepaalde eiwitten, genaamd histonen. Of het DNA strak of losjes om de histonen is gewikkeld, bepaalt welke delen van het DNA ’aan’ en ’uit’ staan. Op die manier weet de cel welke stoffen moeten worden aangemaakt. We denken dat urinezuur de DNA structuur op zo’n manier aanpast dat de cel vervolgens hyperactief reageert op uraatkristallen. Op deze manier kan verhoogd urinezuur dus een rol spelen in de ontwikkeling van jichtartritis.

Gebruik van histon deacetylase remmers om ontsteking bij jicht te remmen

In hoofdstuk 4 hebben we de effecten van butyraat op afweercellen onderzocht. Butyraat is natuurlijk aanwezig in ons lichaam en wordt geproduceerd door bacteriën in ons darmen. Butyraat werkt echter ook als een HDAC remmer. In experimenten met PBMCs van gezonde vrijwilligers en patiënten met jicht konden we laten zien dat butyraat heel effectief is in het verlagen van de productie van ontstekingsstoffen in reactie op uraatkristallen. Dit zou daarom een veilige en goedkope optie voor therapie kunnen zijn voor patiënten met jicht. Echter zijn er hoge concentraties butyraat nodig om deze effecten bewerkstelligen erg hoog.

Om die reden onderzochten we in hoofdstuk 5 de effecten van butyraatpillen op de reactie van de afweercellen in gezonde mannen en mannen met obesitas. Op deze manier hoopten we de afweercellen te resetten, zodat ze minder sterk reageren met de productie van ontstekingsstoffen. Dit zou erg goed kunnen zijn om jichtartritis te voorkomen. We vonden in deze studie echter maar weinig effecten van butyraat op de afweercellen. Dit zou kunnen komen doordat er niet genoeg butyraat uit de pillen bij de afweercellen in het bloed kunnen komen. Cellen in de darmen gebruiken namelijk ook butyraat op als energiebron.
In hoofdstuk 6 probeerden we specifiek te achterhalen welke HDACs een rol spelen in acute jichtartritis. Er zijn 11 verschillende vormen HDACs bekend in ons lichaam. Sommige remmers, zoals butyraat, remmen een hoop van deze HDACs tegelijkertijd. Dit kan soms averechts werken. Daarom testten we verschillende specifieke HDAC remmers. Vooral de HDAC1 en HDAC2 remmer romidepsine was erg effectief in het blokkeren van ontstekingsstoffen bij blootstelling van PBMCs aan uraatkristallen. We toonden aan dat dit werd veroorzaakt door verhoogde transcriptie van het SOCS1 (suppressor of cytokine signalling) gen in het DNA. We denken daarom dat romidepsine ervoor zorgt dat het DNA rond dit gen meer openstaat, zodat er meer SOCS1 door de cel wordt gemaakt. SOCS1 remt ontstekingsmechanismen in de cel op verschillende manieren.

Rol van het DNA in controle van urinezuurconcentraties en jicht

Hoofdstuk 7 beschrijft onze huidige kennis over genetische veranderingen in het stukje DNA dat staat voor het eiwit ABCG2 (ATP-binding cassette G2) en het effect hiervan op het ontwikkelen van een hoge urinezuurconcentratie en jicht. In hoofdstuk 8 kijken we naar het effect van CNVs (copy number variaties) op het risico op het ontwikkelen van jicht. CNVs zijn delen van het DNA die verwijderd (deletie) of gedupliceerd (duplicatie) zijn. We vonden een specifieke deleterie in een stuk van het DNA dat codeert voor het eiwit TPPP (tubulin polymerization-promoting protein) welke geassocieerd was met een verhoogd risico op jicht. Deze bevinding vonden we ook terug in een Polynesische cohort van gezonde controles en patiënten met jicht, maar niet in een Europese cohort.

Toekomstperspectief

Waar we graag naartoe willen werken is het voorkomen van aanvallen van gewrichtsontsteking bij patiënten met jicht. Urinezuur kan, zelfs voordat het uraatkristallen vormt, ervoor zorgen dat onze afweercellen als het ware hyperactief worden. Daarentegen hebben we aangetoond dat HDAC remmers de afweerreactie in de vorm van ontstekingsstoffen juist heel goed kunnen remmen. In de toekomst zou het heel mooi zijn te kunnen onderzoeken hoe urinezuur nu precies de manier veranderd waarop DNA om histonen opgerold zit. Welke delen van het DNA worden hierdoor aan- of uitgeschakeld? En kunnen we dit effect tegengaan met HDAC remmers om zo de afweercellen minder actief te maken? Hierbij is een subtiele balans noodzakelijk. We hebben onze afweercellen immers ook nodig om ons te beschermen tegen indringers, zoals bacteriën en virussen.
Appendices
Dankwoord

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Leef Carlo, Marleen, Geert en Dominique, waat ’n gelök heb ik met zó’n schoeënemfamilie! Ik veul mich gans thoes beej óg en ik kin altied met alles beej óg terecht. Bedank veur al óg hölp!

Leef Pap en Mam, zónder óg had ik dit gewoëen noëëts kinne doon. Geej heb mich gelierd um hard te werke en um neet op te geave, maar ouk um alles thoes gewoeën los te laote en van ’t laeve te genete. Geej heb mich zó’n ontzettend fijn en werm thoes gegaève en óndanks det ’t hoes neet vuuel langer mier van óg zal zien, zal ’t beej óg altied as thoes veule. Bedank veur alles!
Leef Gijs, Emmy en Tommy, ik bewonder óg alle dreej enorm um öggen drive um te doon waat geej ech leuk vindt. Det mak det geej sóms erg drök ziet, maar ’t is zoëë fijn det weej ós óndanks det dök zeen. Emmy, dich extra bedank veur de inspiratie veur de vormgaeving van de cover!

Leef Bart, dich hebs toch waal alles meigemak van dit werk. De hoeëgtepunte hebbe weej same gevierd en beej de deeptepunte waas dich d’r altied um mich alles weer efkes in perspectief te laote zeen. Dich bis (óndanks wie vuuel minse dich kinne) ech de rös in hoes en det haet mich d’r op vuuel momente doorhaer geholpe. Ik hald van dich! Twieë jaor geleje mochte weej ós ouk nog ens gruuetse elders neume.

Leef Thuur, op daag ein had ik ’t neet veur meugeli gehalde, maar idderen daag hald ik gewoeën nog mier van dich. ’t Is altied heerlik thoeskôme beej óg.
CURRICULUM VITAE


Van 2007 tot 2011 studeerde zij Voeding en Gezondheid aan de Wageningen universiteit. Tijdens deze bachelor verrichtte zij een literatuurstudie naar de rol van resistine in de ontwikkeling obesitas en diabetes onder begeleiding van dr. Sander Kersten. Ook was zij in 2010 4 maanden full-time actief als voorzitter van de almanakredactie van de Wageningse Studentenvereniging Ceres.

In 2011 behaalde ze haar bachelor diploma en startte ze met haar master Biomedical Sciences in Nijmegen. Voor een korte masterstage van 4 maanden werkte zij aan het Institute for Immunology and Infection Research van de University of Edinburgh in Schotland. Onder begeleiding van professor David Gray onderzocht ze de effecten van TLR7 en TLR9 liganden op de levensduur van plasmacellen. Dit is van belang voor de werking van het medicijn Rituximab bij patiënten met systemische lupus erythematous. Maartje rondde deze stage af met een 9.

Voor haar grote masterstage van 9 maanden werkte ze in het Laboratorium Experimentele Interne Geneeskunde in het Radboudumc in Nijmegen, onder begeleiding van professor Leo Joosten. In deze stage bestudeerde ze de effecten van korte-keten vetzuren op ontsteking veroorzaakt door uraatkristallen. Deze stage werd ook beloond met een 9.

In 2013 studeerde Maartje cum laude af voor haar master. Hierna is ze blijven plakken bij het Laboratorium Experimentele Interne Geneeskunde en startte ze haar promotieonderzoek onder begeleiding van haar promotoren professor Leo Joosten en professor Mihai Netea. In dit onderzoek bestudeerde ze de ontstekingsremmende effecten van histon deacetylaseremmers bij ontsteking veroorzaakt door uraatkristallen. Daarnaast onderzocht ze andere factoren die een rol kunnen spelen bij de ontwikkeling van jicht, zoals urinezuur en genetica.

LIST OF PUBLICATIONS


# RIMLS PHD PORTFOLIO

M.C.P. Jacobs-Cleophas  
Department: Internal Medicine  
Graduate School: RIMLS  
PhD period: 01-09-2013 – 30-11-2017  
Promotor(s):  
Prof. L.A.B. Joosten and Prof. M.G. Netea

## TRAINING ACTIVITIES

### a) Courses & Workshops

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year(s)</th>
<th>ECTS</th>
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<tbody>
<tr>
<td>Introduction day Radboudumc</td>
<td>2013</td>
<td>0.5</td>
</tr>
<tr>
<td>SNP Course 10th Edition: SNPs and Human Diseases</td>
<td>2014</td>
<td>0.75</td>
</tr>
<tr>
<td>How to conquer the scientific journals (General Technical Workshop, RIMLS)</td>
<td>2014</td>
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<tr>
<td>DCN PhD Council, PhD Workshop: ‘How to conquer the scientific journals?’</td>
<td>2014</td>
<td>0.1</td>
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<tr>
<td>Academic Writing course, Radboud University</td>
<td>2015</td>
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<tr>
<td>R programming course, VU Amsterdam</td>
<td>2015</td>
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<tr>
<td>Biostatistics Introduction, VU Amsterdam</td>
<td>2015</td>
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<tr>
<td>ENII Summer School Advanced Immunology (poster presentation)</td>
<td>2015</td>
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<tr>
<td>InDesign Workshop (PON)</td>
<td>2016</td>
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<tr>
<td>Scientific integrity course</td>
<td>2017</td>
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<tr>
<td>NFU eBROK</td>
<td>2018</td>
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### b) Seminars & lectures

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year(s)</th>
<th>ECTS</th>
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<tbody>
<tr>
<td>Radboud Research Round 23 March 2017: Inflammation in the pathogenesis of atherosclerosis and its risk factors (oral presentation)</td>
<td>2017</td>
<td>0.25</td>
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<tr>
<td>Gout seminar in Cluj-Napoca, Romania (oral presentation)</td>
<td>2017</td>
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### c) Symposia & congresses

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year(s)</th>
<th>ECTS</th>
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<tbody>
<tr>
<td>N4i Science Day (laptop presentation)</td>
<td>2013</td>
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<tr>
<td>CPC PhD Retreat (poster presentation)</td>
<td>2014</td>
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<tr>
<td>European Crystal Meeting, Paris (oral presentation)</td>
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<tr>
<td>AIG Department Science Day (laptop presentation)</td>
<td>2014</td>
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<tr>
<td>European Crystal Meeting, Paris (oral presentation)</td>
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<tr>
<td>NVVI Lunteren Symposium (poster presentation)</td>
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<tr>
<td>AIG Department Science Day (oral presentation)</td>
<td>2015</td>
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<tr>
<td>PhD Retreat IRB Barcelona (poster presentation)</td>
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<td>AMC-Radboudumc Retreat</td>
<td>2015</td>
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<tr>
<td>Heuvellanddagen Janssen-Janssen (oral presentation, “thought-lab”)</td>
<td>2016</td>
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<tr>
<td>European Crystal Meeting, Paris (oral presentation)</td>
<td>2016</td>
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### d) Symposia & congresses

<table>
<thead>
<tr>
<th>Event</th>
<th>Year</th>
<th>Hours</th>
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</thead>
<tbody>
<tr>
<td>Keystone Symposium Santa Fe, “Epigenetic and Metabolic Regulation of Aging and Aging-Related Diseases” (poster presentation)</td>
<td>2016</td>
<td>1.5</td>
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<tr>
<td>AIG Department Science Day (oral presentation)</td>
<td>2016</td>
<td>0.5</td>
</tr>
<tr>
<td>G-CAN 2016 annual meeting Washington DC (poster presentation)</td>
<td>2016</td>
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<tr>
<td>American College of Rheumatology ACR/ARP annual meeting Washington DC (poster presentation)</td>
<td>2016</td>
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<tr>
<td>BSI-NVVI joint meeting Liverpool (poster presentation)</td>
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### e) Other

<table>
<thead>
<tr>
<th>Event</th>
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<th>Hours</th>
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<tbody>
<tr>
<td>Weekly department oral presentations/Journal Club (presented 19 Feb and 25 Jun)</td>
<td>2014</td>
<td>2</td>
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<tr>
<td>Weekly department oral presentations/Journal Club (presented 1 Apr, 5 Aug, and 2 Dec)</td>
<td>2015</td>
<td>3</td>
</tr>
<tr>
<td>Weekly department oral presentations/Journal Club (presented 12 Jan and 5 Oct)</td>
<td>2016</td>
<td>2</td>
</tr>
<tr>
<td>Weekly department oral presentations/Journal Club (presented 31 Jan)</td>
<td>2017</td>
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### TEACHING ACTIVITIES

#### f) Lecturing

- Attend and assess Capita selecta presentations on gout from 3rd year medical students | 2013 | 0.4 |

#### g) Supervision of internships / other

- Supervision of bachelor student Biology from the UK (William Jones-Warner) for 3 months | 2014 | 1   |
- Coached starting PhD candidate (Viola Klück) | 2017 | 0.5 |

### TOTAL

<p>| | |</p>
<table>
<thead>
<tr>
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<tr>
<td>TOTAL</td>
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