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# The role of genetic factors and monocyte-to-osteoclast differentiation in the pathogenesis of Charcot neuroarthropathy

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## ABSTRACT

Charcot neuroarthropathy is a chronic, progressive condition of the skeletal system that affects some patients with diabetic neuropathy. It results in progressive destruction of bones of the foot and disorganisation of pedal joints and ligaments. Effective prevention and treatment for Charcot neuroarthropathy remain a challenge. Currently, there are no reliable repeatable markers to identify patients with diabetes who are at higher risk of developing Charcot neuroarthropathy. The pathogenesis underlying the development of Charcot neuroarthropathy also remains unclear. In this review, we provide an overview of the history, prevalence, symptoms, risk factors, diagnostics and treatment of Charcot neuroarthropathy. We also discuss the potential for OPG and RANKL gene variants to act as predictive markers for the development of Charcot neuroarthropathy. Finally, we summarise the latest research on the role of monocyte-to-osteoclast differentiation in the development of acute Charcot neuroarthropathy.

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## 1. Introduction

Diabetes mellitus is one of the most common disorders in the modern world, and its prevalence has reached epidemic proportions [1]. Long-term and uncontrolled hyperglycaemia leads to a number of severe consequences. The most common complication of diabetes is neuropathy which, according to some sources, affects up to 60% of patients with diabetes [2,3]. Neuropathy most often manifests as diabetic foot syndrome with hard-healing ulcers. Some patients with symmetrical distal neuropathy develop a chronic, and progressive condition called Charcot neuroarthropathy (CN), also known as diabetic neuropathic osteoarthropathy (DNOAP), Charcot foot or Charcot joint disease. It results in the progressive destruction of bones of the foot and soft tissues at weightbearing joints; in its most severe form, it may cause significant disruption of the bone architecture [4,5]. The degree of bone destruction, subluxation, dislocation, and deformity varies in the clinical picture of Charcot neuroarthropathy; neuropathy, trauma, and disturbances in bone metabolism underlie this complication [4–7]. In Charcot neuroarthropathy, the acute (or active) and chronic (or inactive) phases are distinguished to describe the inflamed or the stable phase in the clinical course of this condition, respectively [8].

Although the number of reports on genetic studies on Charcot neuroarthropathy and treatment guidelines have increased in recent years, the exact molecular mechanism of the disease remains unclear. Moreover, there are no effective therapies to prevent or inhibit its development. Here, we provide a comprehensive overview of the history, prevalence, symptoms, risk factors, diagnostics and treatment of Charcot neuroarthropathy. We also discuss the association of OPG and RANKL variants with Charcot neuroarthropathy. Finally, we summarise the latest research on the role of monocyte-to-osteoclast differentiation in the development of acute Charcot neuroarthropathy.

## 2. History

In 1703, William Musgrave first described a neuropathic joint as an arthralgia caused by venereal disease [9]. The association between nerve injury of different origins and arthropathy was first mentioned in the nineteenth century by John Kearsley Mitchell [10], and the idea was further developed by the French neurologist Jean-Marie Charcot [11]. The condition was first described in association with venereal disease and tabes dorsalis. Syphilis was believed to be the most common cause of Charcot arthropathy until 1936, when William Jordan first noted its relationship with diabetes [12]. Nowadays, Charcot neuroarthropathy is predominantly observed in

patients with diabetes during the course of diabetic neuropathy [8].

## 3. Prevalence, symptoms and risk factors

The reported incidence of Charcot neuroarthropathy in the general population of patients with diabetes is around 0.16% [13], and may be even higher in subjects with diabetic neuropathy, where it may reach up to 29%. The prevalence is reported to vary from 0.08% in the general population of patients with diabetes to 13% in high-risk patients with diabetes and peripheral neuropathy [14,15]. It is usually unilateral, but according to some sources it can affect both feet in up to 75% of cases [16]. Mortality in patients with this complication is high, exceeding the mortality of some cancers such as prostate or breast cancer [17].

The acute Charcot neuroarthropathy manifests as a triad of symptoms: unilateral oedema, redness, and a local increase in temperature. In some cases, slight pain is present. The clinical picture resembles other aetiologies of swelling of the foot, such as cellulitis, sprains or deep vein thrombosis; therefore, the diagnosis is often difficult and delayed [4,7,8]. Chronic Charcot neuroarthropathy is easily recognizable with the rocker-bottom appearance of the foot, resulting from irreversible deformation due to metatarsal collapse or joint destruction [4,7,8]. These deformities predispose to skin ulceration, an established risk factor for amputation [4].

The risk factors for the development of Charcot neuroarthropathy are not well understood. According to some authors, the highest incidence of this complication is observed in patients between 60 and 70 years of age with a diabetes duration of more than 10 years [18]. However, Petrova and colleagues observed a higher incidence of Charcot neuroarthropathy in patients with diabetes who were aged in their 40 s and 50 s [19]. Type 1 and type 2 diabetes differ with regard to diabetes duration and Charcot neuroarthropathy risk. When compared to patients with type 2 diabetes, patients with type 1 diabetes and Charcot neuroarthropathy tend to be younger, and these patients have generally had diabetes for a longer time [19]. The risk factor that is most strongly correlated with Charcot neuroarthropathy is social isolation. Nobrega and colleagues suggest that acute Charcot neuroarthropathy primarily affects patients aged under 55 years who live alone, are literate, and have a prior history of ulcers [20]. Peripheral arterial disease is a protective factor [20]. Contradictory results have been obtained regarding body mass index (BMI); some studies report that obesity is associated with an increased incidence of Charcot neuroarthropathy

[21,22], while according to others, no association has been observed [20,23]. It is not clear whether low bone mineral density (BMD) is a risk factor for the development of Charcot neuroarthropathy; the calcaneal BMD of patients with diabetes and Charcot neuroarthropathy is lower compared to healthy controls, but is similar between patients with Charcot neuroarthropathy and patients with diabetes alone [24]. A higher incidence and poorer outcome of this complication is observed in patients with diabetes and concurrent renal disease [25]. Impaired renal function, haemodialysis, peritoneal dialysis, and kidney or simultaneous kidney/pancreas transplantation correlate with the incidence and progression of Charcot neuroarthropathy [25–27].

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#### 4. Diagnostics

In the acute stage, Charcot neuroarthropathy is diagnosed mainly on clinical grounds, supported by diagnostic imaging. Plain foot radiographs are recommended as the initial diagnostic method [3]. Radiographs allow for an overall assessment of anatomy and the detection of bone destruction, dislocations and deformity of the foot, all findings that are typical of advanced Charcot neuroarthropathy [28]. Unfortunately, plain X-ray films have low sensitivity for diagnosing subtle, initial abnormalities within the skeletal system, as X-rays in the very early stages of Charcot neuroarthropathy may appear normal. In the earliest stage of Charcot neuroarthropathy, magnetic resonance imaging (MRI) or nuclear medicine studies following initial X-rays are recommended to confirm the acute bone pathology. MRI is a highly sensitive imaging method that, in cases of acute Charcot neuroarthropathy, allows the detection of signs of bone marrow and soft tissue oedema, stress fractures without cortical disruption and joint effusion [28]. Radionuclide imaging with labelled white cells may help to differentiate Charcot neuroarthropathy from osteomyelitis with a specificity of 80% and sensitivity of about 80% [29]. Positron emission tomography-computed tomography (PET-CT) may prove to be a valuable method for diagnosing Charcot neuroarthropathy; however, due to its high cost and limited availability, it is not widely applied. In the chronic stage of Charcot neuroarthropathy, plain radiographs show dislocation or subluxation, deformity, destruction, disorganisation, debris and increased subchondral bone mineral density, known as the rule of “six Ds” [30]. At this stage, an intervention that would prevent gross deformity of the foot is not usually possible.

At present, there are no reliable markers that could confirm the diagnosis of Charcot neuroarthropathy in its acute phase. In addition, it is not possible to determine which patients affected by neuropathy are at greater risk of developing this complication. It also remains unclear why this process is unilateral and self-limiting in the majority of cases. Due to the lack of typical laboratory and radiological characteristics, acute Charcot neuroarthropathy is misdiagnosed and mistreated in up to 25% of cases [31]. Diagnostic errors resulting in improper or delayed treatment can lead to profound deformity, an increased risk of ulceration or lower-limb amputation. Acute Charcot neuroarthropathy is com-

monly misdiagnosed as bacterial inflammation and phlegmon of the foot, which is referred for surgical intervention [4]. Advanced cases with bone destruction evident on X-rays are difficult to distinguish from osteomyelitis, especially if a foot ulcer is present [32,33]. A careful patient history and examination may aid in the diagnosis of acute Charcot neuroarthropathy [32]. A history of minor trauma, lack of previous (or present) ulcer, and a reduction of erythema upon elevation of the leg are all characteristic features of Charcot neuroarthropathy. Patients also display very dense neuropathy and, in most cases, there is no foot ischemia [32]. Measurement of uric acid levels and a negative venous Doppler exam may help to exclude gout and deep vein thrombosis. Levels of procalcitonin (PCT) and C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and leukocyte count are usually normal [34].

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#### 5. Treatment

In the very early stage, Charcot neuroarthropathy is usually treated with custom footwear, a cast shoe containing a custom-made insole, or orthoses to protect the foot and ankle [32]. Using this footwear effectively reduces swelling and protects the bones of the foot at risk from developing Charcot neuroarthropathy. The gold standard of treatment of the acute Charcot neuroarthropathy is non-weight bearing or a reduction in weight-bearing and immobilisation of the affected foot, preferably in a total contact cast [35]. Application of this treatment modality requires expertise. Protective padding should be applied between the toes, with additional padding over bony prominences. The cast should be removed regularly to assess possible wounds caused by friction while the oedema resolves. The length of necessary immobilisation varies between individuals, with one study reporting a mean duration of casting as long as  $18.5 \pm 10.6$  weeks [36]. A prolonged period of casting may result in overloading of the contralateral foot, muscle wasting, a further reduction in bone mineral density and increased body mass index. Offloading should continue until the patient has entered the healing phase, clinically defined as a reduction in oedema and a temperature difference between the affected and contralateral foot of less than  $2^\circ\text{C}$  [33,35]. The transition to weight-bearing and regular activity of the patient must be gradual, preferably supported by a physiotherapist; beginning strenuous exercise too soon after removal of the total contact cast may result in the recurrence of acute Charcot neuroarthropathy. For long-term management of patients after weaning out of the total contact cast, custom footwear or total contact insoles are recommended to ensure that the foot is accommodated and protected [32,37].

Surgical treatment of Charcot neuroarthropathy is recommended for those patients who have severe ankle and foot deformities that are unstable and at a high risk of developing a foot ulcer [7]. If the deformity makes braces and orthotics difficult to use, surgery may also be indicated. The clinical outcome of a patient after surgical correction often results in improved quality of life [38], but it may also be associated with a high complication rate [39].

Pharmacological treatments for Charcot neuroarthropathy are under study as an additional option to classical treatments. These treatments include the administration of antiresorptive agents (bisphosphonates and calcitonin), anabolic agents (recombinant parathyroid human hormone), or anti-RANKL monoclonal antibody (denosumab). However, the clinical efficacy of these agents is still inconclusive (reviewed in [37,40]).

## 6. Pathogenesis of Charcot neuroarthropathy

The underlying pathogenesis of Charcot neuroarthropathy is still not completely understood. Two main theories to explain the pathogenesis of the condition have been described in the literature: the neurovascular theory, and the neurotraumatic theory. Michael E. Edmonds, in his study, observed that the peripheral neuropathy was frequently accompanied by hyperaemia, which could result in osteopenia and, consequently, bone fractures caused by even minor trauma (neurovascular theory) [31]. Other authors associated the development of Charcot neuroarthropathy with repetitive trauma acting on a foot with sensory neuropathy (neurotraumatic theory) [41]. It seems that the interaction of certain factors, such as presence of diabetes, peripheral neuropathy (both sensory and motor) and metabolic abnormalities affecting bone (osteopenia), are key factors in the pathogenesis of the disease. A reduction in BMD is often reported in the affected foot compared to the contralateral foot [42–45]. How immobilization and off-loading therapy affects BMD is still inconclusive. According to some authors, BMD falls over time in the affected foot as casting therapy is introduced [45], while other authors have shown no long-term effects of this therapy on further BMD loss [46,47].

At the beginning of the twenty-first century, inflammation was identified as a key component in the pathogenesis of Charcot neuroarthropathy. The “inflammatory theory” stresses the pathogenic importance of local ongoing inflammation and the increased expression of proinflammatory cytokines, including IL-1 $\beta$ , IL-6, TNF- $\alpha$  and other factors, in the pathogenesis of Charcot neuroarthropathy [48,49]. The proinflammatory state is linked to the bone turnover observed in Charcot neuroarthropathy. However, it is still unclear whether it is the cause or the consequence of the ongoing bone destruction process occurring in the affected foot. The venous-arterial flux of IL-6 is increased in the affected foot of patients with Charcot neuroarthropathy compared to the healthy foot, suggesting that IL-6 is locally produced in the affected foot [50]. Serum levels of TNF- $\alpha$ , IL-6 or C-reactive protein are higher in patients with acute Charcot neuroarthropathy prior to treatment, with a decrease observed following casting therapy, a standard treatment method for Charcot neuroarthropathy [51]. Serum TNF- $\alpha$  or IL-6 have also been reported to be positively correlated with C-terminal telopeptide, a bone turnover marker, and serum osteoprotegerin (OPG) levels at presentation but not after casting therapy [51]. Other studies suggest that activation of the proinflammatory cytokines IL-6, IL-17 and TNF- $\alpha$  in patients with Charcot neuroarthropathy occurs after they begin

offloading therapy, representing a key step in bone repair and remodelling during recovery [52,53].

### 6.1. Role of the OPG-RANKL-RANK axis in bone resorption and formation

The physiological balance between pro- and anti-inflammatory cytokines plays an important role in the course of Charcot neuroarthropathy. The release of proinflammatory cytokines leads to increased activity of the metabolic pathway consisting of OPG, receptor activator of nuclear factor  $\kappa$ B (RANK) and its ligand, receptor activator of nuclear factor  $\kappa$ B ligand (RANKL). RANKL is the main factor in the bone matrix responsible for the differentiation of osteoclast precursor cells and their activation to mature osteoclasts [54]. It exists as a membrane-bound protein produced by osteoblast lineage cells and activated T cells, as well as a soluble protein which can be detected in blood serum [55]. As there are at least three isoforms of RANKL mRNA, it is plausible that different forms of RANKL are produced from different mRNAs [56]. RANKL exerts its biological activity through the RANK receptor, which is present on the surface of preosteoclasts, mature osteoclasts and several other cell types. Binding of RANKL to RANK initiates a signal cascade within the cell, which involves the recruitment of TRAF factors (TRAF2, TRAF5 and TRAF6) to the cytoplasmic domain of RANK and results in the activation of nuclear transcription factors NF- $\kappa$ B and JNK. This process leads to the differentiation of preosteoclasts into mature osteoclasts. Importantly, the inhibition of osteoclast differentiation is counterbalanced by the action of OPG, which serves as the soluble decoy receptor for RANKL, preventing its association with RANK [57,58].

Imbalance of the OPG/RANKL/RANK axis may lead to uncontrolled bone loss and various pathological conditions [59], as observed in many diseases such as Paget's disease [60], rheumatoid arthritis [61], osteopenia [62] and Charcot neuroarthropathy [63]. Ndip and colleagues found that serum RANKL and OPG levels, as well as the RANKL/OPG ratio, are increased in patients with Charcot neuroarthropathy when compared to patients with diabetes or a healthy control group [63]. The local inflammation and related increased expression of the RANKL gene are associated with fractures and bone destruction in Charcot neuroarthropathy [64]. In addition, there is a correlation between increased bone resorption and vascular calcification that may contribute to the development of the disease [64–66].

The interplay between RANKL and OPG proteins controls bone resorption and formation. However, other factors, such as advanced glycation end products (AGE), reactive oxygen species (ROS) and cytokines, influence this process and may contribute to the development of Charcot neuroarthropathy (as reviewed in [67]). Hyperglycaemia stimulates the formation of AGEs, and interplay between impaired AGE defence, bone turnover and bone quality has been observed in Charcot neuroarthropathy [68]. Patients with Charcot neuroarthropathy display reduced levels of circulating soluble receptor of advanced glycation end products (sRAGE), increased serum osteocalcin, a marker of bone formation, and reduced calcaneus bone stiffness. It is hypothesised that low levels of

sRAGE fail to protect against the production of AGE due to hyperglycaemia. Excess AGE may accumulate in tissues such as tendons, bone or cartilage, where it may increase the production of RAGE, which may in turn enhance RANKL activation leading to increased osteoclastogenesis, predisposing the bone to fracture. Bone resorption by osteoclasts is also stimulated by either TNF- $\alpha$  or RANKL, which upregulate the expression of RANK and promote osteoclast differentiation *in vitro* [69].

### 6.2. Association of OPG and RANKL variants with Charcot neuroarthropathy

Different studies suggest the involvement of genetic factors associated with OPG and RANKL variants in the development of various skeletal system diseases with underlying osteopenia [70–72]. There are reports of an association of Charcot neuroarthropathy with variants of OPG, RANKL and RANK genes.

The OPG gene variants 245T>G, 1181G>C and 1217C>T have been reported to be associated with Charcot neuroarthropathy, but the allele frequencies at specified loci differ between studies. Pitocco and colleagues found a positive association with G alleles for both the OPG 1181G>C and 245T>G variants in Italian patients with Charcot neuroarthropathy when compared to patients with diabetic neuropathy and healthy controls [73]. Subjects with diabetes and neuropathy with CC/TT genotypes had an approximately six-fold lower risk of Charcot neuroarthropathy. In a Polish population, for both 1217C>T and 245T>G OPG gene variants, a positive correlation between TT genotypes and Charcot neuroarthropathy was found [74]. The TT genotype at these residues increased the risk of developing Charcot neuroarthropathy by more than three times. Polish patients with Charcot neuroarthropathy had the CC genotype with the OPG 1181C>G variant almost two times more often than patients with diabetic neuropathy or diabetes. Studies involving larger groups of patients seem to confirm these observations. In particular, a positive correlation between the 245T>G OPG gene variant and TT genotype with the development of Charcot neuroarthropathy was observed [75]. It appears that the 1181G>C variant of the OPG gene, which might be a potential marker of Charcot neuroarthropathy, is a marker specific to this complication alone.

The allele and genotype frequencies of RANKL 290C>T, 643C>T and 693G>C variants also differ between patients with Charcot neuroarthropathy, neuropathy and diabetes [74]. The T alleles in RANKL 290C>T and 643C>T and the C allele in 693G>C have been found to occur more frequently in patients with neuropathy and Charcot neuroarthropathy. This was correlated with an increased frequency of the corresponding homozygotes in groups with neuropathy and Charcot neuroarthropathy (TT for 290C>T and 643C>T, and CC for 693G>C), and these genotype frequencies differed significantly from those observed in the diabetes group. No significant changes in genotype and allele frequencies were observed between groups for two variants of the RANK gene (421C>T and 575C>T) [74].

An association between OPG 245T>G and OPG 1217C>T, as well as between RANKL 290C>T and 693G>C variants, was found in patients with Charcot neuroarthropathy, and the

linkage disequilibrium was the highest between these variants [74]. Based on similarities in their gene variant profiles, the analysed group of patients was divided into three clusters that differ in regard to the proportion of cases with diabetes, neuropathy and Charcot neuroarthropathy: cluster 1 contained mostly patients with diabetes, cluster 2 contained similar proportions of patients with neuropathy and Charcot neuroarthropathy, and cluster 3 consisted of about 50% of patients with Charcot neuroarthropathy [74].

### 6.3. Serum OPG and RANKL levels in Charcot neuroarthropathy

Serum levels of the RANKL and OPG proteins are increased in patients with Charcot neuroarthropathy [63,74] and neuropathy [74] and the RANKL/OPG ratio is higher in patients with Charcot neuroarthropathy [46,63,74] than in patients with diabetes or healthy controls. Jansen and colleagues observed that the RANKL/OPG ratio was about three-fold higher in the Charcot group when compared to patients with diabetes without this condition and after few years of standard treatment. As the acute Charcot neuroarthropathy settled, the RANKL/OPG ratio decreased by approximately seven times when compared to the baseline ratio [46]. These authors suggested that the RANKL/OPG ratio is only elevated in the acute phase of Charcot neuroarthropathy. However, a more detailed study on a larger group of patients by Bruhn-Olszewska and colleagues revealed that the RANKL/OPG serum ratio is much higher in patients with neuropathy than patients with Charcot neuroarthropathy [74]. These authors suggested that an increased level of RANKL is specific for neuropathy, and is probably the key factor involved in bone loss. Interestingly, studies performed by two independent groups demonstrated that the OPG level is higher in patients with Charcot neuroarthropathy when compared to patients with diabetes, and postulated the hypothesis that OPG production is stimulated in response to elevated levels of RANKL, likely to counterbalance its effect on bone loss [63,74]. Increased serum levels of RANKL in patients with Charcot neuroarthropathy have also been demonstrated to promote osteogenic differentiation and the mineralisation of vascular smooth muscle cells (VSMCs) *in vitro* [63]. These studies support the hypothesis that RANKL is not only involved in osteolysis, but also in the vascular calcification associated with Charcot neuroarthropathy.

Despite the associations between the studied genetic variants, the available data indicate that the pathogenesis of Charcot neuroarthropathy is multifactorial. Petrova and colleagues have recently shown that osteoclasts from subjects with Charcot neuroarthropathy are characterised by an increased reaction to RANKL when compared to osteoclasts obtained from patients with diabetes (but not Charcot neuroarthropathy) and healthy controls [76]. RANKL-mediated osteoclastic resorption *in vitro* is modulated by TNF- $\alpha$  [69,77]. Expression of this proinflammatory cytokine on monocytes of patients with Charcot neuroarthropathy was found to be increased when compared to patients with diabetes who did not develop this complication [78]. The same authors also observed that serum concentrations of TNF- $\alpha$  and IL-6 were significantly higher in people with Charcot neuroarthropathy than in people with and without diabetes. On follow up, there

**Table 1 – Summary of the role of genetic factors and monocyte-to-osteoclast differentiation in Charcot neuroarthropathy pathogenesis.**

Factor or process	Characteristics and possible role in the pathogenesis of Charcot neuroarthropathy	Reference
Genetic factors	RANKL/OPG variation <ul style="list-style-type: none"> <li>■ Association of OPG 245T&gt;G, 1181G&gt;C and 1217C&gt;T variants with Charcot neuroarthropathy</li> <li>■ Association of RANKL 290C&gt;T, 643C&gt;T and 693G&gt;C variants with Charcot neuroarthropathy</li> <li>■ Increased serum RANKL and OPG levels in both patients with diabetic neuropathy and Charcot neuroarthropathy</li> <li>■ Bone loss</li> <li>■ Vascular calcification</li> </ul>	[73–757,463,74]
Monocyte-to-osteoclast differentiation	Microparticles <ul style="list-style-type: none"> <li>■ Increased level of microparticles from monocytes of patients with Charcot neuroarthropathy</li> <li>■ High content of inflammatory cytokines, i.e. G-CSF, GM-CSF, IL-1-ra, IL-2 and IL-16</li> <li>■ Increased osteoclast differentiation in vitro</li> </ul>	[81]
	miRNAs <ul style="list-style-type: none"> <li>■ Differential expression of circulating miRNAs in patients with Charcot neuroarthropathy: miR19a-3p, miR101-3p, miR144-3p, miR16-2-3p, miR16-5p, miR362-3p, miR30e-5p</li> <li>■ Differentiation of monocytes to osteoclasts</li> </ul>	[83]
	Gene methylation <ul style="list-style-type: none"> <li>■ Differential methylation of genes in circulating monocytes of patients with Charcot neuroarthropathy: e.g. HMGA1 and MAPK11</li> <li>■ Differential gene expression in circulating monocytes due to methylation changes, e.g. PPP2R5D, POC1A and FOSB</li> <li>■ Migration during monocyte-to-osteoclast differentiation</li> <li>■ Regulation of inflammatory pathways</li> </ul>	[79]

was a significant reduction in the concentration of these cytokines at resolution, suggesting that these markers may be useful in the assessment of disease activity [51].

#### 6.4. Monocyte-to-osteoclast differentiation in acute Charcot neuroarthropathy

The osteoclasts responsible for bone resorption differentiate from monocytes. Several studies have demonstrated that gene methylation in monocytes, monocyte-derived microparticles or microRNAs (miRNAs) can affect monocyte-to-

osteoclast differentiation, and may therefore play an important role in the development of Charcot neuroarthropathy. Recent whole-methylome studies have identified over a hundred genes that are differentially methylated in circulating monocytes isolated from patients with Charcot neuroarthropathy compared to patients with diabetes [79]. In patients with Charcot neuroarthropathy, most of these genes were found to be hypermethylated. More importantly, about 15% of the differentially methylated genes include genes that could be directly or indirectly involved in the differentiation of monocytes into osteoclasts, with *HMGA1* and *MAPK11* genes as the strongest candidates. The same study also demonstrated an association between DNA methylation and gene expression in patients with Charcot neuroarthropathy. The *PPP2R5D* gene was both hypermethylated and overexpressed in monocytes from patients with Charcot neuroarthropathy. Several differentially methylated genes were found to have an effect on other expression-associated genes including *POC1A* and *FOSB* genes, which are known to be involved in bone-related disorders and in bone formation.

Microparticles (also known as microvesicles) are small, membrane vesicles that are released from cells under different physiological and pathological conditions. Different patterns of circulating microparticles have been detected in patients with type 1 or type 2 diabetes and in patients with diabetic complications [80]. In patients with Charcot neuroarthropathy, microparticles from monocytes, but not platelets, are present at higher amounts when compared to patients with diabetic neuropathy or healthy controls [81]. Increased levels of cytokines such as G-CSF, GM-CSF, IL-1- $\alpha$ , IL-2 and IL-16 were detected only in microparticles from patients with acute Charcot neuroarthropathy, but levels in the serum were not elevated. Those cytokines are linked to pathways that are involved in osteoclast formation. Moreover, the incubation of monocytes from healthy controls with microparticles derived from patients with Charcot neuroarthropathy increased their differentiation into osteoclasts *in vitro* [81].

Circulating miRNAs may also play a role in the development of Charcot neuroarthropathy, presumably by the regulation of monocyte-to-osteoclast differentiation. MiRNAs are small, non-coding RNAs present in cells that act as post-transcriptional regulators of gene expression. MiRNAs are also detected in serum and extracellular fluids, which are termed circulating miRNAs and extracellular miRNAs, respectively [82]. Comparison of the serum levels of mature human miRNAs revealed differential expression of several miRNAs between patients with acute Charcot neuroarthropathy, patients with diabetes and neuropathy and those without neuropathy [83]. Deregulation of seven miRNAs (miR19a-3p, miR101-3p, miR144-3p, miR16-2-3p, miR16-5p, miR362-3p and miR30e-5p) was found to be strongly correlated with Charcot neuroarthropathy. The authors also identified that a number of circulating miRNAs that were differentially expressed between Charcot neuroarthropathy and neuropathy groups could be involved in the differentiation of monocytes into osteoclasts.

Effective prevention of the serious consequences of diabetes is still a challenge. Identifying reliable and repeatable genetic markers of Charcot neuroarthropathy would help to identify patients who are at higher risk of developing this condition soon after they are diagnosed with diabetes. Genetic factors play an important role in the development of Charcot neuroarthropathy. Recent observations suggest the involvement of *OPG* and *RANKL* gene variants in the development of osteopenia and vascular calcification that are characteristic of Charcot neuroarthropathy (Table 1). Multiple variant sites within these two genes could potentially be used as predictive markers of Charcot neuroarthropathy. Along with genetic markers, profiles of inflammatory cytokines in circulating microparticles or serum levels of specific miRNAs could be a simple and convenient screening tool to diagnose or predict the risk of Charcot neuroarthropathy development; however, studies on larger cohorts are necessary to confirm their usefulness as a diagnostic tool. As monocytes appear to be directly involved in the pathogenesis of Charcot neuroarthropathy (Table 1), further studies of the monocyte-to-osteoclast differentiation process may lead to a better understanding and perhaps early prevention of this condition.

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#### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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#### 7. Concluding remarks

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