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Submitted 2014-09-01. Accepted 2015-02-08.

Background and purpose — Charcot neuropathy is characterized by bone destruction in a foot leading to deformity, instability, and risk of amputation. Little is known about the pathogenic mechanisms. We hypothesized that the bone-regulating Wnt/ß-catenin and RANKL/OPG pathways have a role in Charcot arthropathy.

Patients and methods — 24 consecutive Charcot patients were treated by off-loading, and monitored for 2 years by repeated foot radiography, MRI, and circulating levels of sclerostin, dickkopf-1, Wnt inhibitory factor-1, Wnt ligand-1, OPG, and RANKL. 20 neuropathic diabetic controls and 20 healthy controls served as the reference.

Results — Levels of sclerostin, Dkk-1 and Wnt-1, but not of Wif-1, were significantly lower in Charcot patients than in the diabetic controls at inclusion. Dkk-1 and Wnt-1 levels responded to off-loading by increasing. Sclerostin levels were significantly higher in the diabetic controls than in the other groups whereas Wif-1 levels were significantly higher in the healthy controls than in the other groups. OPG and RANKL levels were significantly higher in the Charcot patients than in the other groups at inclusion, but decreased to the levels in healthy controls at 2 years. OPG/RANKL ratio was balanced in all groups at inclusion, and it remained balanced in Charcot patients on repeated measurement throughout the study.

Interpretation — High plasma RANKL and OPG levels at diagnosis of Charcot suggest that there is high bone remodeling activity before gradually normalizing after off-loading treatment. The consistently balanced OPG/RANKL ratio in Charcot patients suggests that there is low-key net bone building activity by this pathway following diagnosis and treatment. Inter-group differences at diagnosis and changes in Wnt signaling following off-loading treatment were sufficiently large to be reflected by systemic levels, indicating that this pathway has a role in bone remodeling and bone repair activity in Charcot patients. This is of particular clinical relevance considering the recent emergence of promising drugs that target this system.

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Charcot neuropathy mainly affects diabetic patients with bilateral peripheral neuropathy and is characterized by degenerative changes of the bone, joints, and soft tissues of the foot and ankle. However, the pathophysiological processes that lead to acute Charcot foot remain obscure. The identification of a cytokine system belonging to the tumor necrosis factor family—consisting of RANKL (receptor activator of nuclear factor-κB ligand), its receptor RANK (receptor activator of nuclear factor-κB), and its soluble decoy receptor, osteoprotegerin (OPG)—has substantially increased our understanding of bone regulatory mechanisms responsible for osteolytic activity by osteoclasts. The RANKL/OPG pathway has therefore been implicated in an increasing number of diseases affecting the bone, and several review articles have hypothesized about the possible role of this pathway in Charcot arthropathy (Jeffcoate 2008, Mascarenhas and Jude 2013), although only a few studies have been published. Although there has been much interest in RANKL/OPG signaling, recent research has significantly increased our understanding of the pivotal role played by other pathways in bone regulation. One such pathway is that of Wnt, which has been shown to be a key regulator of embryonic bone development, mechanical loading and unloading of the skeleton, bone growth, bone remodeling, and fracture repair by increasing osteoblast commitment from progenitor cells and by stimulating their proliferation and activation (Agholme and Aspenberg 2011, Kim et al. 2013). In addition, there is crosstalk between the bone anabolic Wnt pathway and the bone-catabolic RANKL/OPG pathway (Diarra et al.

2007), with recent studies suggesting that the Wnt pathway is in control of osteoclast differentiation and activation through its actions on the osteoblasts, and by participating in osteoclast recruitment and the initiation of bone remodeling (Bellido 2014). We are not aware of any study investigating the role of the Wnt system in the pathology of Charcot arthropathy.

In this 2-year longitudinal, observational study in Charcot patients, we repeatedly measured plasma levels of endogenous mediators forming part of the RANKL/OPG and Wnt/ß-catenin pathways with the aim of elucidating their role in the pathophysiology of Charcot arthropathy from the acute phase until reaching a cold/chronic state.

Material and methods

Patient selection and treatment

26 consecutive ambulatory men (n = 15) and women (n = 11) admitted to Sahlgrenska University Hospital, Mölndal with clinical signs of unilateral acute Charcot's foot were included in the study during the period 2009–2014. 2 patients died shortly after inclusion and were excluded. 1 patient interrupted participation after 18 months, after being informed that radiographs showed finalized bone healing, but this patient did not object to data being processed in the study. Diagnosis of Charcot foot was based on medical history, clinical examination, and radiological findings including the following criteria: (1) type-1 or type-2 diabetes with a duration of ≥ 1 year, (2) peripheral bilateral neuropathy as defined below, and (3) clinical signs of active Charcot arthropathy with hot/reddened/swollen foot and skin temperature in the affected foot ≥ 2oC higher than in the contralateral foot. Off-loading treatment of the diseased foot by a non-weight bearing total contact cast (TCC) was introduced in all Charcot patients within 1 day of inclusion in the study. The TCC was repeatedly replaced as required from changes in foot volume due to increased or attenuated swelling. The non-weight-bearing protocol was supplemented with crutches or wheel-chair and was continued until the difference in skin temperature between the feet was ≤ 1oC and there had been no signs of redness or swelling for the previous 30 days. The TCC was then replaced by orthosis for partial weight bearing. Evaluation of skin temperature and toe pressure was continued until 2 years after inclusion. When full weight bearing was allowed, the patient was given accommodative shoes. Bilateral foot radiographs and magnetic resonance imaging (MRI) at inclusion and repeatedly during the follow-up period were used to monitor fractures, dislocations, and soft-tissue- or bone marrow edema.

Exclusion criteria were plantar ulcerations, documented history of trauma or surgery involving bone tissue during the year before inclusion, current immunosuppressive therapy (steroids or cancer treatment), or medication known to affect bone metabolism (e.g. bisphosphonates, denosumab). A control group of 20 ambulatory diabetes type-1 or type-2 patients with neuropathy documented as described below was included at Frölunda Specialist Hospital during 2011 and another control group of 20 healthy subjects was included at Sahlgrenska University Hospital/Mölndal during 2010. Neither of the control groups had any history of joint/bone disease or bone trauma/surgery in the year before inclusion and they had not received any osteoporosis medication.

Skin sensitivity, skin temperature and toe pressure

Skin sensitivity (neuropathy) was measured in Charcot foot patients and diabetic control patients bilaterally at inclusion, at 4 different locations of the foot using the Semmes-Weinstein monofilament test. The monofilament (10 g) was pressed against the skin and the patient's ability or inability to feel the sensation upon buckling of the monofilament was registered. Neuropathy was present if 3 or more sites were insensitive to the monofilament. Skin temperature was measured bilaterally on the dorsal foot, 5 cm distal to the ankle and 2 cm proximal to the mid-toe. Toe blood pressure was measured bilaterally using a specially designed cuff (Samuelsson et al. 1996). Measurements of skin temperature and toe pressure were performed at inclusion, at 1 week, and 2, 4, 6, 8, 12, 18, and 24 months thereafter.

Blood samples

Peripheral venous blood was collected for routine analysis (hemoglobin, serum creatinine, CRP, SR, and HbA1c) and for cytokine analysis (pre-chilled EDTA tubes immediately centrifuged at 3,000× g for 10 min at 4°C before being stored at −85oC until analysis). Blood was collected at inclusion, at 1 week, and 2, 4, 6, 8, 12, 18, and 24 months after inclusion. Before analysis, samples were thawed on ice before being mounted on assay plates and analyzed for OPG, RANKL, sclerostin, dickkopf-1 (Dkk-1), Wnt ligand-1 (Wnt-1), and Wnt inhibitory factor-1 (Wif-1).

Determination of plasma biomarkers by ECL technology

The Sector Imager 2400 assay platform from MesoScaleDiagnostics (MSD; Gaithersburg, MD) was used for biomarker analysis (for details see www.meso-scale.com). Quantitative measurements were done using a matched pair of anti-human (ah) antibodies, i.e. capture antibody (CA) and biotinylated detection antibody (DA). Standard curves were created using the corresponding human recombinant proteins (hRPs). Samples were applied to uncoated standard plates from MSD (cat. no. L15XA). OPG CA: affinity-purified polyclonal rabbit ahOPG antibody (PeproTech cat. no.500-P149); OPG DA: biotinylated polyclonal rabbit ahOPG antibody (ReproTech cat no. 500-P149Bt), hRP (450-14). RANKL CA: monoclonal mouse IgGκ ahRANKL antibody (ReproTech cat. no. 500-M46); RANKL DA: biotinylated goat polyclonal ahRANKL antibody (ReproTech cat. no. 500-P133GBT), hRP (310-01). RANKL antibodies were directed against the soluble form of RANKL corresponding to the amino acid sequence 143–317

of the full-length RANKL molecule. Sclerostin CA: monoclonal mouse IgG2B ahsclerostin antibody (R&D Systems (Minneapolis, MN) cat. no. MAB1406); sclerostin DA: biotinylated monoclonal mouse IgG1 ahsclerostin antibody (R&D Systems cat no. BAM1406), hRP (1406-ST-025). Dkk-1 CA: monoclonal mouse IgG2B ahDkk-1 antibody (R&D Systems cat. no. MAB10962); Dkk-1 DA: biotinylated affinity-purified goat polyclonal IgG ahDkk-1 antibody (R&D Systems cat. no. BAF1096), hRP (PeproTech cat. no. 120-30). Wnt-1 CA: affinity-purified polyclonal rabbit ahWnt-1 antibody (Reprotech cat. no. 500-P250); Wnt-1 DA: biotinylated polyclonal rabbit ahWnt-1 antibody (Peprotech cat. no. 500-P250Bt), hRP (450-14). Wif-1 CA: monoclonal mouse IgG1 ahWnt-1 antibody (R&D Systems cat. no. MAB1342); Wif-1 DA: biotinylated monoclonal mouse IgG1 ahWnt-1 antibody (R&D Systems cat. no. BAM1341), hRP (1341-WF-50). Optimization of primary and secondary antibodies was done by checkerboard titration and subsequent checking of standard curves against plasma from healthy donors.

Radiography

Radiographs of both feet were performed in supine position with dorso-plantar, oblique, and lateral projections as well as weight bearing in frontal and lateral projections. MRI was performed on a 1.5-T Siemens Magnetom Symphony scanner with the patient supine and feet-first into the gantry. All examinations were performed with a head-neck surface coil and with the feet in flexed position. The feet were examined with 4 sequences each and without intravenous contrast medium: T1, T2, T2 3d, and STIR sequences in sagittal, transverse, and coronary positions. Following administration of intravenous contrast medium, transverse and sagittal projections were obtained by T-sequences. Radiographic and MRI examinations were performed according to a preset schedule within a week of being assigned to the study, and then 6, 12, 18, and 24 months after inclusion.

For anatomical classification of Charcot arthropathy, we used the system previously described (Papanas and Maltezos 2013) with 5 different patterns depending on the areas of the foot affected; i.e. pattern 1: metatarsophalangeal and interphalangeal joints; pattern 2: tarso-metatarsal joints; pattern 3: naviculocuneiform, talonavicular and calcaneocuboid joints; pattern 4: ankle and subtalar joints; and pattern 5: calcaneus. As the sensitivity differs substantially between plain radiographs and MRI regarding identification of the early stages of Charcot, we included both a modified Eichenhotz staging based on plain radiographs as described by Sella and Barrette (1999) and an MRI-based identification of early inflammation-induced signs of Charcot arthropathy (Chantelau and Grutzner 2014). The staging by Sella and Barrette comprises 5 stages: stage 0 (warm, reddened, swollen foot and normal radiographs); stage 1 (clinical findings and radiographic cysts, erosions, localized osteopenia, and occasionally diastases); stage 2 (joint subluxation); stage 3 (dislocation and arch collapse); and stage 4 (healed stage of bony process). The severity of inflammation in the Charcot foot on MRI was graded in 2 stages, inactive or active, depending on the presence or absence of skeletal inflammation/bone edema.

Statistics

Before analyzing the data by repeated measures ANOVA, we tested for a number of assumptions to be met. The variables tested (biomarkers) were measured at a continuous level over time, and a comparison was made between the 3 independent study groups for each individual biomarker at inclusion and 2 years after inclusion by means of 1-way repeated measures ANOVA followed by the Holm-Šidak test. Where applicable, this model was used for comparisons of blood pressure, Hb, CRP, SR, and S-kreatinin between the 3 study groups (Table 1). This statistical model ensures that the probability of incorrectly rejecting the null hypothesis for any of the pairwise comparisons in the family does not exceed alpha, thereby controlling the family-wise error rate (FWE) and setting the alpha value according to Šidak correction of Bonferroni inequality. This stepwise method is generally more powerful than the corresponding single-step procedures. The Holm-Šidak test applies an accept/reject criterion to a sorted set of null hypotheses, starting from the lower p-value and going up to the acceptance of the null hypothesis. For each comparison, the alpha value is set according to Šidak correction of Bonferroni inequality. The Šidak formula is: Šidak corrected alpha = $1 - (1 - \alpha)^{1/k}$ where k is the number of comparisons performed for each individual biomarker (3 in our study). The Šidak procedure has slightly more power than the Bonferroni procedure when alpha = 0.05. A multiplicative model was assumed to be more accurate for the current data, and the logarithms of measurements were therefore used for the comparisons. Comparisons between 2 datasets of individual biomarkers within the Charcot group (e.g. at inclusion vs. 4 months) were performed by Wilcoxon rank sum test. Comparison of HbA1c between the Charcot and diabetic control groups was done by Mann-Whitney U-test (Table 1)

The statistical program identified significant outliers in the related groups for RANKL when analysis was run using polyclonal detection antibody. In order to satisfy the condition of not having outliers (i.e. ≥10× the median value), we tested with a monoclonal ahRANKL capture antibody, which eliminated significant outliers and satisfied this requirement.

Ethics

The study was approved in 2007 by the Review Board of the Västra Götaland Region (EPN entry number 499-07), with additional approval in 2010 (T 762-10 ad 499-07). All the subjects provided informed consent before participating in the study. The study complied with the STROBE statement for observational studies (www.strobe-statement.org).

Table 1. Clinical and demographic data in Charcot patients, diabetes controls, and healthy subjects

	Charcot arthropathy at inclusion (n = 24)	Charcot arthropathy at end of study (n = 23)	Diabetic controls at inclusion (n = 20)	Diabetic controls at 2 years (n = 20)	Healthy subjects (n = 20)
Age, median (range), years	61 (42–87)	63 (42–87)	55 (20–94)	57 (20–94)	58 (24–78)
Gender, female/male, n	10/14	10/14	8/12	8/12	9/11
Diabetes type-1/type-2, n	10/14	10/14	6/14	6/14	-
Duration of diabetes, years	26 (4)	-	14 (6)	-	-
Debut foot symptoms, median (range), weeks	8.8 (1–24)	-	-	-	-
Systolic blood pressure, mmHg	151 (6)	150 (6)	127 (4)	-	138 (4)
Diastolic blood pressure, mmHg	80 (3)	78 (2)	76 (2)	-	81 (3)
Arterial toe pressure, mmHg					
Charcot foot	118 (12)	123 (8)	115 (4)	-	-
Contralateral foot	131 (8)	119 (7)	114 (5)	-	-
Skin temperature, °C					
Charcot foot	31.5 (0.7) [a]	29.6 (0.7)	30.0 (0.4)	-	-
Contralateral foot	29.3 (0.8)	29.1 (0.7)	30.2 (0.4)	-	-
Pain at rest, n	4	0	0	-	-
Pain on bearing weight, n	16	0	0	-	-
Foot distortion, n	3	3	0	-	0
Total contact cast, months	-	7.6 (1.0)	-	-	-
Charcot pattern 1/2/3/4/5, n	2/15/4/0/3	-	-	-	-
Nephropathy/ cardiac disease, n	7/4	7/4	2/1	2/1	0/0
Hb, g/L	127 (3)	128 (3)	136 (2)	139 (3)	143 (5)
S-CRP, mg/L	11.2 (3) [b,c]	9.9 (2) [d,e]	3.6 (0.8)	3.8 (0.8)	2.7 (0.7)
SR, mm	31.0 (6) [f,g]	24 (4)	11 (2)	10 (2)	6 (4)
S-creatinine, µmol/L	116 (19)	99 (12)	83 (8)	89 (7)	78 (5)
HbA1c, mmol/mol	59 (4)	67 (3)	58 (3)	56 (2)	-
HbA1c, %	7.5 (0.4)	8.3 (0.3)	7.5 (0.2)	7.3 (0.2)	-

Table data are mean (SEM) when not expressed differently.
[a] p = 0.04 for Charcot foot vs. contralateral foot at inclusion.
[b] p = 0.04 for Charcot foot patients at inclusion vs. diabetes controls at inclusion.
[c] p = 0.01 for Charcot foot patients at inclusion vs. healthy subjects.
[d] p = 0.04 for Charcot foot patients at end of study vs. diabetes controls at 2 years.
[e] p = 0.03 for Charcot foot patients at end of study vs. healthy subjects.
[f] p = 0.003 for Charcot foot patients at inclusion vs. diabetes controls at inclusion.
[g] p < 0.001 for Charcot foot patients at inclusion vs. healthy subjects. All other differences were not significant.
Charcot pattern 1–5 on radiographs (see Papanas and Maltezos 2013).

Results

Clinical and demographic data are presented in Table 1. In Figures 4, 5C, 6C, 7C, and 8C, the results are given as linear graphs representing repeated measurements of individual biomarkers ranging over the entire period of observation from inclusion until 2 years after inclusion. In the other figure panels, the box plots illustrate inter-group comparisons at inclusion and 2 years after inclusion with the median represented by the horizontal line within the box, the 25th and 75th percentiles represented by the box, the 5th and 95th percentiles represented by the whiskers, and outliers represented by dots.

OPG

OPG (ng/mL) levels were significantly higher in Charcot patients and diabetic controls than in healthy subjects, whereas OPG levels in Charcot patients and diabetic controls were similar (Figure 1A). OPG at 2 years was significantly higher in diabetic controls than in healthy subjects and Charcot patients, but they were similar in Charcot patients and healthy subjects (Figure 1B). Plasma OPG was significantly higher in Charcot patients at inclusion than at 2 years (p < 0.001).

As circulating OPG has been linked to cardiovascular and renal disease (Bjerre 2013, Klejna et al. 2009), we identified Charcot patients with such diseases (Table 1) and compared their plasma OPG levels to those of the diabetic control group as a whole (excluding nephropathy and cardiopathy patients). There was no significant difference (p = 0.3) between Charcot patients with nephropathy (n = 7) and those without (n = 17) (mean 2.44 (SEM 0.15) ng/mL vs. 2.15 (SEM 0.28) ng/mL). Similarly, mean OPG levels in Charcot patients with cardiopathy (n = 4) and those without (n = 20) were not significantly different (p = 0.5) (2.40 (SEM 0.29) ng/mL vs. 2.0 (SEM 0.24) ng/mL). The numbers of patients with renal/cardiac affection in the diabetes control group were too small for statistical analysis (Table 1).
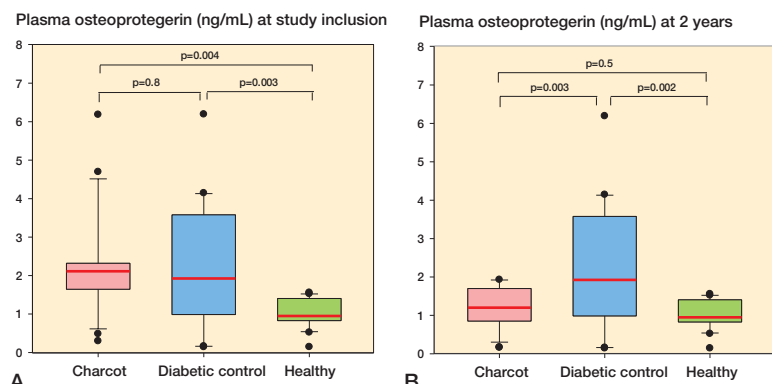
Figure 1. A and B. Box plots of plasma osteoprotegerin values (OPG, ng/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B). Inter-group comparison performed by 1-way repeated measures ANOVA and post-hoc Holm-Šidak test.
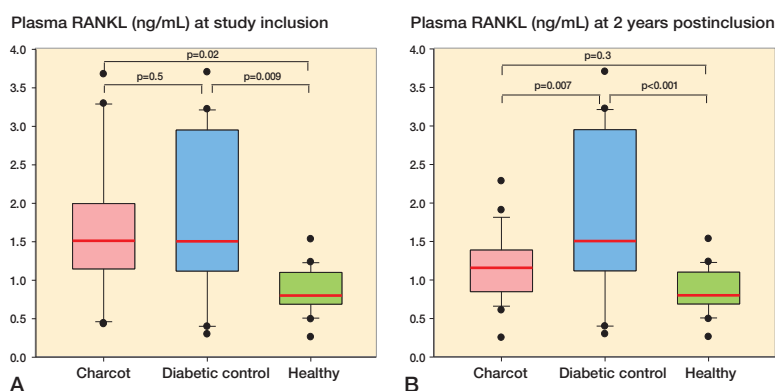

Figure 2. A and B. Box plots of plasma receptor activator of nuclear factor-κB ligand (RANKL, ng/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B).
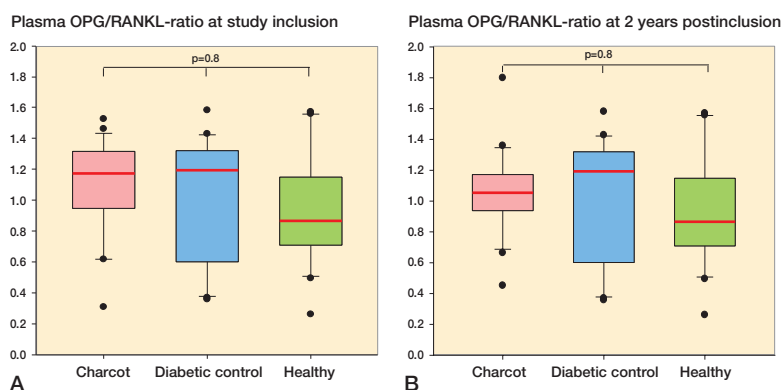

Figure 3. A and B. Box plots of plasma OPG-RANKL ratio in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B).

RANKL

At inclusion, plasma RANKL levels (in ng/mL) were significantly higher in Charcot patients than in healthy subjects and significantly higher in diabetic controls than in healthy subjects, but they were similar in Charcot patients and in diabetic controls (Figure 2A). At 2 years, plasma RANKL levels were significantly higher in diabetes controls than in healthy subjects and Charcot patients, whereas they were not significantly different in Charcot patients and healthy subjects (Figure 2B). Plasma RANKL levels were significantly higher in Charcot patients at inclusion than at 2 years (p = 0.04).

OPG-RANKL ratio

OPG-RANKL ratio was similar between Charcot patients, diabetes controls, and healthy subjects at inclusion (Figure 3A) and at 2 years after inclusion (Figure 3B). Repeated measurements of plasma OPG, plasma RANKL, and OPG-RANKL ratio from diagnosis until 2 years showed a sharp decline in OPG and RANKL at 8 months after inclusion, whereas OPG-RANKL ratio remained in balance throughout the study (Figure 4, see Supplementary data).

Sclerostin

Sclerostin levels at inclusion were significantly higher in diabetes controls than in Charcot patients and healthy subjects, but they were similar in Charcot patients and healthy subjects (Figure 5A). At 2 years, sclerostin levels were significantly higher in diabetes controls than in healthy subjects and Charcot patients, but they were similar in Charcot patients and in healthy subjects (Figure 5B). The trajectory of sclerostin in Charcot patients throughout the observation period of 2 years—based on repeated sampling over time and relative to diabetes controls and healthy subjects—was similar at inclusion and 2 years after inclusion (Figure 5C, see Supplementary data).

Dkk-1

Dkk-1 levels at inclusion were significantly higher in diabetes controls than in Charcot patients and healthy subjects, but they were similar in Charcot patients and healthy subjects (Figure 6A). At 2 years, Dkk-1 levels were significantly higher in diabetes controls than in healthy subjects, but not significantly higher than in Charcot patients. Again, they were not significantly different in Charcot patients and healthy subjects (Figure 6B). Figure 6C illustrates the trajectory of plasma Dkk-1 (in pg/mL) in Charcot patients throughout the observation period of 2 years, based on repeated sampling over time and relative to diabetes controls and healthy subjects. Plasma Dkk-1 levels in Charcot patients

Plasma sclerostin (ng/mL) at inclusion

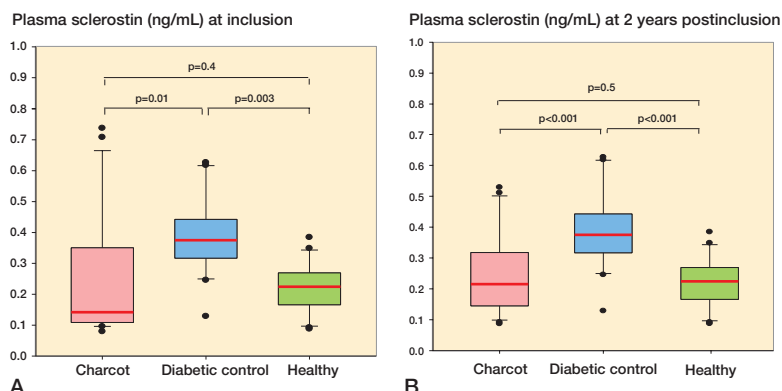Plasma sclerostin (ng/mL) at 2 years postinclusion

Figure 5. A and B. Box plots of plasma sclerostin (ng/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B).

Wnt-1

Wnt-1 levels at inclusion were significantly higher in diabetes controls than in healthy subjects and Charcot patients, but they were similar in Charcot patients and in healthy subjects (Figure 7A). At 2 years, Wnt-1 levels were significantly higher in diabetes controls than in healthy subjects and Charcot patients, but they were similar in Charcot patients and healthy subjects (Figure 7B). Figure 7C illustrates the trajectory of Wnt-1 (in pg/mL) in Charcot patients throughout the observation period of 2 years, based on repeated sampling over time and relative to diabetes controls and healthy subjects. Plasma Wnt-1 levels in Charcot patients were significantly higher at 4 months than at inclusion, but at 2 years they were similar to the levels at inclusion.

were significantly higher at 4 months and 2 years after inclusion than at inclusion.
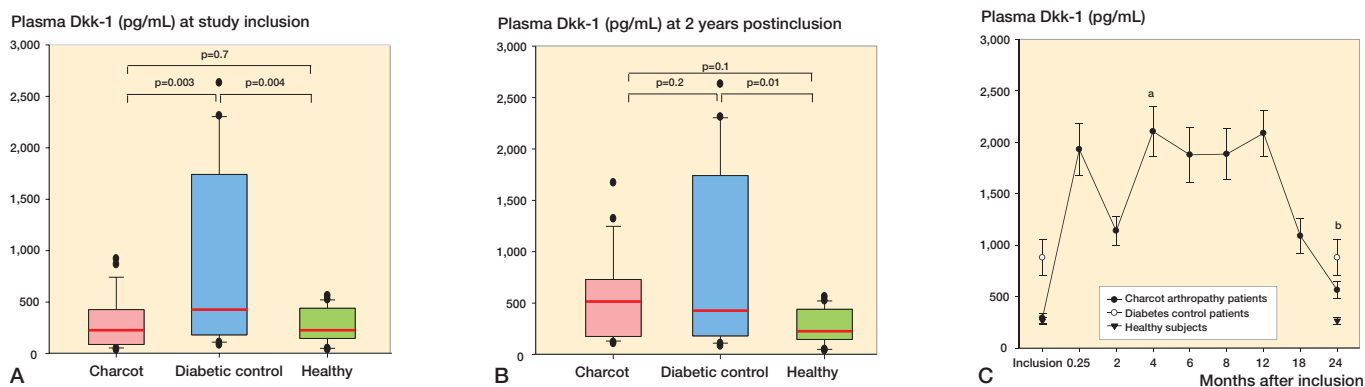

Figure 6. A and B. Box plots of plasma Dickkopf-1 (Dkk-1, pg/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B). C. Trajectory of plasma Dkk-1 in Charcot patients throughout the observation period of 2 years, based on repeated sampling over time and relative to diabetic controls and healthy subjects. Mean (SEM). [a] p = 0.009 for Charcot patients at 4 months vs. Charcot patients at inclusion; [b] p = 0.01 for Charcot patients at 2 years vs. Charcot patients at inclusion, as analyzed by Mann-Whitney rank sum test.
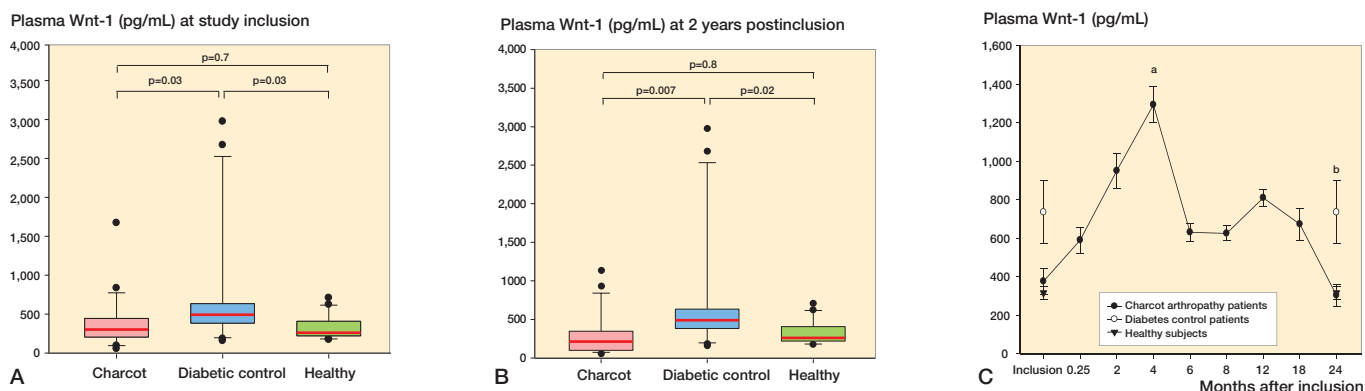

Figure 7. A and B. Box plots of plasma Wnt ligand-1 (Wnt-1, pg/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B). C. Trajectory of plasma Wnt-1 in Charcot patients throughout the observation period of 2 years, based on repeated sampling over time and relative to diabetic controls and healthy subjects. Mean (SEM). [a] p = 0.003 for Charcot patients at 4 months vs. Charcot patients at inclusion; [b] p = 0.2 for Charcot patients at 2 years vs. Charcot patients at inclusion, as analyzed by Mann-Whitney rank sum test.
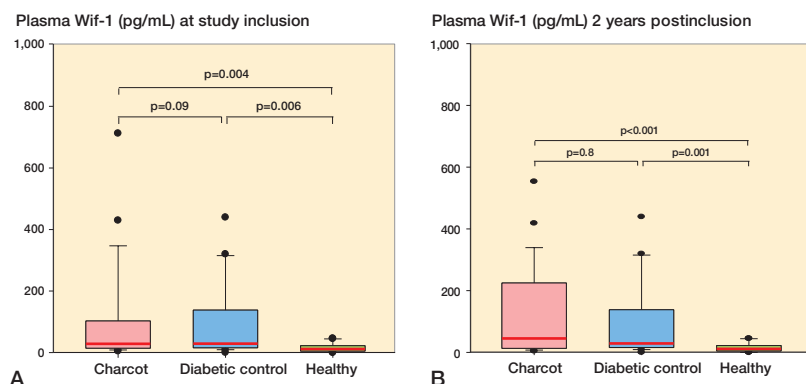
Figure 8. A and B. Box plots of plasma Wnt inhibitory factor-1 (Wif-1, pg/mL) in Charcot patients (n = 24), diabetic controls (n = 20), and healthy subjects (n = 20) at inclusion (A) and at termination of the study after 2 years (B).

Wif-1

Wif-1 levels at inclusion were significantly higher in diabetes controls than in healthy subjects and significantly higher in Charcot patients than in healthy subjects, but they were similar in Charcot patients and diabetes controls (Figure 8A). At 2 years, Dkk-1 levels were significantly higher in diabetes controls than in healthy subjects and significantly higher in Charcot patients than in healthy subjects but they were similar in Charcot patients and diabetes controls (Figure 8B). Wif-1 levels in Charcot patients at 4 months and at 2 years were similar to the levels in these patients at inclusion (Figure 8C, see Supplementary data).

Radiographic data

1 patient lacked data after 18 months due to interrupted participation. All the study patients had reached complete mineralization on radiographs and there were no signs of inflammation on MRI at 2 years after inclusion (Table 2).

Table 2. Staging of radiographic changes in the Charcot arthropathy foot as described by Sella and Barrette (1999) based on weight-bearing radiographic examinations of the diseased foot; also, inflammation/bone edema on MRI based on activity (see Chantelau and Grutzner 2014) . Numbers in the table are numbers of patients.

	At inclusion	1 week	6 months	12 months	18 months	24 months
Radiography						
Stage 0	4	3	0	0	0	0
Stage 1	3	3	2	0	0	0
Stage 2	9	9	9	6	4	0
Stage 3	8	8	8	5	5	0
Stage 4	0	1	5	13	15	23
MRI						
Inactive	7	9	12	15	18	23
Activite	17	15	12	9	6	0

Discussion

Patient selection and confounding factors

We did not do repeated blood sampling in healthy and diabetic controls, as we did not expect their clinical and laboratory variables to undergo substantial changes during the short duration of the study. Biomarker data from these groups were thus used as a reference for the Charcot group, both at inclusion and at the end of the study. Frequent routine monitoring in diabetic control patients enabled us to compare laboratory data at inclusion and 2 years later, confirming that no substantial changes had occurred during the course of the study.

OPG is involved in cardiovascular disease (Bjerre 2013) and chronic renal disease (Klejna et al. 2009), conditions that often affect diabetic patients. However, comparison of plasma OPG levels in Charcot patients with or without nephropathy and/or heart disease (Table 1) did not reveal any substantial differences, which is in line with previous reports mainly linking high OPG to HbA1c and the hyperglycemic state rather than to diabetes-related vascular complications (Bjerre 2013).

The RANKL/OPG pathway

Only 2 previous studies have investigated OPG and RANKL in Charcot patients. Mabilleau et al. (2008) compared peripheral blood monocytes from diabetic Charcot patients, diabetic controls, and healthy subjects and found higher numbers of osteoclasts and increased osteolytic activity in Charcot patients. Osteolysis was augmented further by RANKL but only partially inhibited by OPG in Charcot patients, suggesting that other mechanisms or pathways participate in Charcot-induced bone pathology. Ndip et al. (2011) investigated the link between arterial calcification and RANKL/OPG in Charcot patients, but also measured serum levels of OPG and RANKL. They found higher OPG levels in Charcot patients and diabetic controls than in healthy subjects, and higher RANKL levels in Charcot patients than in diabetic controls and healthy subjects. In contrast, our results showed that both OPG and RANKL were higher in Charcot patients and diabetic controls at diagnosis than in healthy controls. In contrast to Ndip et al. (2011), who found that RANKL-OPG ratio was higher in Charcot patients and diabetic controls than in healthy subjects, we found similar ratios between all groups at inclusion. There are several possible explanations for these differences. One was the small number of subjects in the study by Ndip et al. (12 Charcot patients, 10 diabetic controls, and 5 healthy subjects). Another was the fact that they analyzed differences between groups on a single occasion, whereas we made repeated measurements of OPG, RANKL, and OPG-RANKL ratio in Charcot patients over a period of 2 years after inclusion. We were thus able to show that the OPG-

RANKL ratio in Charcot patients was in balance—not only at inclusion and termination of the study, but throughout the 2-year follow-up—as shown by a stable trajectory over time. In addition to the above, the study by Ndip et al. also lacked documentation of the criteria used to verify Charcot; it had no radiographic examinations of the foot to confirm changes typical of Charcot and no information as to whether the diabetic controls were neuropathic or not, as neuropathy is a prerequisite for Charcot and circulating OPG has been shown to be significantly higher in diabetic patients with neuropathy than in those without (Terekeci et al. 2009). Our results showing higher OPG and RANKL levels in Charcot patients and diabetic controls at inclusion than in healthy subjects, despite the fact that only Charcot patients had bone pathology, could suggest that they are primarily related to the diabetic disease per se rather than to the arthropathy. However, despite the fact that previous studies have shown higher systemic OPG in diabetic patients than in healthy subjects (Bjerre 2013) and a correlation between plasma OPG and peripheral neuropathy in diabetic patients (Nybo et al. 2010), any link to the diabetic disease as the sole explanation was contradicted by results in Charcot patients showing that levels of both cytokines at 2 years had decreased to levels below those in diabetic controls. A possible reason for the similarly high levels of OPG and RANKL in Charcot patients and diabetic controls despite the fact that only Charcot patients have suffered fractures could be that they share another comorbidity apart from diabetes—i.e. peripheral neuropathy, which is characterized by insensate feet, therefore making victims prone to develop "silent" bone stress injuries (Chantelau et al. 2007). This would most probably trigger increased bone remodeling and repair activity, reflected by higher systemic levels of RANKL and OPG, and would suggest that both cytokines are already at a maximum in neuropathic diabetic patients with full weight bearing on their feet, with little ability to increase further in response to additional bone trauma. This could also explain our results showing that OPG and RANKL levels were significantly lower at 2 years in Charcot patients than in diabetic controls, as Charcot patients have had a long period of immobilization without weight bearing, allowing normalization of RANKL and OPG levels, although we can only speculate about the duration of these changes.

The integrity of the skeleton requires a balance between bone formation by osteoblasts and bone degradation by osteoclasts (Kohli and Kohli 2011). The OPG-RANKL ratio, which determines the net effect of osteoclast activity, is considered to be more relevant than plasma levels of the individual markers when describing the activity of these cytokines in the bone microenvironment (Rogers and Eastell 2005). Our results showing OPG-RANKL ratio to be close to 1 in all 3 groups at inclusion and in the Charcot group throughout the follow-up period of 2 years suggest that despite a higher bone turnover rate in both diabetic groups as suggested by higher RANKL and OPG, the ratio is in balance and does not contribute to net loss of bone or increased bone formation in Charcot patients. This does not, however, rule out the OPG/RANKL pathway playing a critical role at the very early and clinically undetected phase of the disorder, when OPG-RANKL ratio may have been shifted towards net bone loss before being counterbalanced by a compensatory increase in OPG—the soluble endogenous blocker of RANKL.

The Wnt/ß-catenin pathway

There have not been any previous studies investigating the role of Wnt in Charcot, despite accumulating evidence that this is an important bone anabolic pathway (Kim et al. 2013), which—through its actions on osteoblasts—can indirectly control osteoclast differentiation and activation, participate in osteoclast recruitment, and initiate bone remodeling (Bellido 2014). As there have been studies investigating Wnt in diabetes, we were able to compare the results with our findings. We found higher plasma sclerostin and Dkk-1 levels in diabetic controls at inclusion relative to healthy subjects, which is in line with studies showing higher systemic sclerostin and Dkk-1 levels and reduced ß-catenin levels in diabetes type-2 patients than in healthy subjects (Gaudio et al. 2014). These findings link Wnt to augmented fracture risk in diabetic patients, as suggested by a significant correlation between fractures and high systemic sclerostin levels in postmenopausal women (Arasu et al. 2012). In contrast, sclerostin and Dkk-1 levels in Charcot patients at inclusion were comparable to the levels in healthy subjects and significantly lower than those in diabetic controls. This inhibition of the antagonists reflecting increased bone anabolic activity in the Wnt/ß-catenin pathway is most likely a natural response to the destructive processes taking place in the Charcot foot, and is supported by experimental studies showing that inhibition of Dkk-1 and sclerostin improve healing of fractured bone (Agholme et al. 2010, 2011, 2014)

As sclerostin is mainly produced by osteocytes, it has been suggested to play a critical role in the bone anabolic responses to mechanical loading (Sapir-Koren and Livshits 2014). Thus, mechanical loading suppresses sclerostin, leading to increased bone mass and strength whereas unloading increases sclerostin and induces bone loss as part of a natural adaptation by the body to skeletal demands. One would therefore expect sclerostin to increase in response to unloading by TCC treatment in the Charcot group—in contrast to our results, which showed a stable and uninterrupted trajectory. However, previous studies showing an association between sclerostin and immobilization in humans did not involve a concomitant fracture or other bone pathology (Gaudio et al. 2010), factors that may have changed the priorities of this key Wnt regulator in favor of promoting bone repair activity in Charcot arthropathy patients. This is supported by a recent experimental study showing that unloading of rat hind limb induced a paradoxical downregulation of sclerostin gene expression in cancellous and cortical metaphyseal bone as opposed to diaphy-

seal cortical bone (Macias et al. 2013), suggesting that the role of sclerostin in unloading is more complex than previously assumed. We were also surprised to find that sclerostin levels at the end of the study remained suppressed in Charcot patients relative to the the levels in the diabetic controls, despite the fact that radiographs showed mineralized bone and no active inflammation on MRI. One plausible explanation to such ongoing Wnt stimulation would be that bone deformities in the wake of Charcot often cause unnatural high pressure on areas of the foot during weight bearing, increasing the need for continuous bone repair and remodeling (Gouveri and Papanas 2011). In contrast to sclerostin, off-loading treatment triggered an elevation of Dkk-1 levels in the present study, which persisted until 1 year after inclusion before retreating towards inclusion levels. This is in line with results suggesting a paradoxical upregulation of Dkk-1 in cancellous bone of rat hind leg in response to mechanical unloading (Agholme et al. 2011), and adds to the current results supporting a role for Dkk-1 in mechano-transduction of the skeleton.

A recent study showing lack of correlation between Dkk-1 and ß-catenin—as opposed to a strong correlation between sclerostin and ß-catenin (Gaudio et al. 2012)—could suggest that the seemingly paradoxical increase in the Wnt antagonist Dkk-1 during the bone repair phase of acute Charcot is the result of sclerostin being the main modulator of canonical Wnt with Dkk-1 playing a fine-tuning role. 2 recent studies in patients with primary hyperparathyroidism (PHTP) (Viapiana et al. 2013) and predialysis chronic kidney disease (Thambiah et al. 2012) have shown a similar discrepancy between serum sclerostin and Dkk-1 levels, with sclerostin being lower and Dkk-1 higher than in healthy subjects. The authors suggested that the bone anabolic effect of lower sclerostin may be counterbalanced in PHPT patients by higher Dkk-1 as part of a regulatory feedback mechanism. In a bone fracture study in rodents (Li et al. 2011), the authors were able to show that Dkk-1 was only able to influence bone formation in connection with inflammation and bone trauma, but had no effect on non-fractured bone in adult animals. These findings may shed some light on our results showing that Dkk-1 levels had risen during the most active inflammatory phase of bone repair before retreating, which could suggest that Dkk-1 had outplayed its role once bone healing had begun to abate. An interesting finding made by the authors (Li et al. 2011), albeit surprising, was that overexpression of Dkk-1 after fracture in adult mice increased serum OPG levels and reduced RANKL levels at the early stages of fracture healing with no inhibition of osteoclastogenesis. These results indicate that canonical Wnt, and particularly Dkk-1, may have a role in the bone remodeling phase of fracture healing and may, apart from being a fine-tuning tool of Wnt, also have a role in regulation of the RANKL/OPG pathway. As proposed by Diarra et al. (2007), Dkk-1 may be a master regulator of joint (and bone) remodeling, which shifts its balance from bone resorption when increased to formation of new bone when inhibited,

although the nature of its effects seem to vary depending on the cell type and timing of activation in the aftermath of a fracture (Komatsu et al. 2010).

Another possible partner in the complex regulation of canonical Wnt is the agonistic Wnt ligand Wnt-1. Its importance in bone and joint homeostasis was only recently highlighted by publications proposing that it acts as a key regulator of human bone mass despite its relatively low expression in bone (Laine et al. 2013, Wilson 2013). The suppression of the Wnt agonist Wnt-1 in Charcot patients relative to diabetic controls at inclusion, despite pronounced bone pathology in the Charcot foot, may appear to be paradoxical when considered independently. However, when viewed in conjunction with other changes in Wnt regulatory proteins, our results reveal that Wnt-1 inhibition is counterbalanced by a concomitant inhibition of the Wnt antagonists sclerostin and Dkk-1. Although we are presently unable to understand the inherent interplay between the regulators of Wnt at the time of Charcot diagnosis, it is reasonable to assume that the balance between the agonists and antagonists is aimed at preserving the damaged foot skeleton. Similar to Dkk-1, off-bearing treatment triggered a 3-fold increase in plasma Wnt-1, peaking at 4 months before retreating. This concomitant increase in Wnt-1 and Dkk-1 levels shortly after introducing TCC might suggest that the 2 opposing mediators of Wnt are part of the Wnt-controlled bone remodeling activity, as shown for the RANKL/OPG pathway. In support of this, Dkk-1 has been implicated in the osteolytic phase of osteoarthritis (Daoussis and Andonopoulos 2011), an action similar to that of RANKL, whereas Wnt-1 has been implicated in the bone anabolic activity of Wnt (Laine et al. 2013, Wilson 2013), thus exerting an action similar to that of OPG.

Wif-1 is a new type of secreted antagonist of Wnt signaling, which is structurally unrelated to Dkk-1 or sclerostin and is expressed in the cartilage, bone, and tendons. The antagonistic effect of Wif-1 is exerted by binding to several Wnt ligands, both canonical and non-canonical, thereby interfering with their induction of Wnt signaling—although not all of the regulatory mechanisms of Wif-1 are understood. Wif-1 has been shown to be a negative regulator of osteoblast differentiation and maturation and a promoter of bone erosion by increasing RANKL expression in osteoblasts, and thus the bone-resorbing activity of osteoclasts (Stock et al. 2013). Our results show for the first time that plasma Wif-1 levels were higher in Charcot patients and in diabetic controls at inclusion than in healthy subjects and that this paradoxical inhibition of Wnt in Charcot patients remained in place throughout the study. Interestingly, this continuous inhibition of Wnt by elevated Wif-1 was paralleled by equally persistent stimulation of Wnt through inhibition of sclerostin, suggesting that the 2 mediators exert a counterbalancing control of Wnt signaling during the entire course of the disease until recovery.

In summary, our results have shown a high degree of remodeling activity in Charcot patients and diabetic controls at inclusion, as reflected by high plasma OPG and RANKL levels.

A decrease in both cytokines in the Charcot group to levels similar to those in healthy subjects at 2 years, and below those in diabetic controls, could be secondary to a protracted off-loading and fracture-stabilizing treatment. The OPG-RANKL ratio being in balance from inclusion and onward suggest that this pathway did not lean in the direction of net bone anabolism or catabolism after diagnosis and treatment, but could have had an important role in bone catabolism during the extended period between trauma and diagnosis. We also provide evidence in support of the Wnt/ß-catenin pathway being involved in bone remodeling following off-loading treatment, and also being a regulator of bone healing in Charcot patients—as suggested by the actions of its endogenous antagonists and agonists. It is tempting to speculate about the possibility of Wnt signaling playing the role previously proposed for RANKL/OPG, although additional studies will be required to corroborate our findings.

Supplementary data

Figures 4, 5C, and 8C are available at Acta's website (www. actaorthop.org) identification number 7967.

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