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An 8.5-year prospective case-control study

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Bone mineral density and markers of bone turnover and inflammation in diabetes patients with or without a Charcot foot: An 8.5-year prospective case-control study

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Introduction

Charcot osteoarthropathy (Charcot foot, CF) is a rare but severe complication to diabetes mellitus and peripheral neuropathy, and is associated with increased morbidity and mortality (1). It manifests as an aseptic inflammation of bones and joints in the feet leading to progressive degeneration of the bone structures, which can cause deformity and ulceration (2–5). The treatment consists of long-term off-loading, often accompanied by assisted wound healing and surgery (6,7).

While the precise pathogenetic mechanism causing to the characteristic Charcot inflammation is unknown, some studies have shown an increased bone resorption in the affected foot, leading to a lowered BMD (8–11). This change has also been visualized on the micro-structural level (12,13). Additionally, several recent studies have reported changes in biomarkers of bone resorption and inflammation in individuals with Charcot foot, specifically within the RANK/RANK-L/OPG system (14–19).

As a member of the TNF cytokine family, RANK-L (receptor activator of nuclear factor κ-B ligand) is a key activator of osteoclast maturation and differentiation (20). It also plays a role in activating the immune system as RANK-L expression on T cells can modulate other T cells and dendritic cells to increase the local inflammatory environment (21), and it could very well be a central piece to the puzzle of osteolysis in diabetic neuropathy (22–24).

Increased levels of the measurable free soluble fraction of RANK-L (fsRANK-L) can be taken as a marker for increased osteoclastic and inflammatory activity. The decoy receptor to RANK-L, called osteoprotegerin (OPG), inhibits this response. Therefore, the ratio of fsRANK-L to OPG might be taken as a marker for an individual's current state of bone resorption and inflammation (21,25). OPG and fsRANK-L have been a topic of debate in connection with Charcot foot over the last 10-15 years (9,10,15,21,24,26). Soluble receptor for advanced glycation end products (sRAGE), a marker
for vascular calcification, is connected to RANK-L through the common NF-κB pathway\(^{23,27,28}\), and might thus also play a role in this interaction.

It is still unclear whether changes in the concentration of these substances during a Charcot foot activation have long-term effects on local and systemic bone metabolism. To investigate this issue we conducted a follow-up study of a previously well-described Charcot population\(^{(9,29,30)}\). Our main goal was to elucidate if a previously acute Charcot foot has any long-term effects on bone mineral density or local or systemic bone metabolism. Additionally, we wanted to measure the time course of fsRANK-L, OPG and sRAGE concentrations in our Charcot foot population.

Methods

Design and follow-up time:
Participants from a previous study\(^{(9,29)}\) were invited to participate in the follow-up study. The participants all had diabetes mellitus, half (n=24) had an acute or chronic Charcot foot at baseline in 2005-2007 (17 acute and 7 chronic Charcot feet). A total of 49 patients were included at the baseline visit (from 2005-2007). At baseline inclusion, participants were matched on age, sex, type and age of diabetes.

Data collected in the previous study were used as baseline data. Follow-up was performed at one more visit after about 8 years. The length of follow-up time was chosen as a compromise between giving the Charcot condition time to settle and any reactivation to occur, while not losing too many participants due to the high mortality associated with late stage diabetes and diabetic foot disorder.

Participants were not examined or treated in any way between baseline and follow-up as part of the study. The treatment they received was standard care performed by regular medical staff as part of the routine treatment of diabetes and Charcot foot.

All participants (n=49) were followed up using in-hospital medical records, the "Danish Register of Cause of Death" and the "Danish Civil Registration System (CPR)". Additionally they were invited to participate in the present follow-up study when possible.

The participants were also screened for any use of bisphosphonates or other drugs related to the treatment of osteoporosis, both prior to baseline inclusion, and between baseline and follow-up.

Measurements

All examinations at the baseline visit in 2005-2007 were done by author TMC\(^{(9,29)}\), and at follow-up visit they were done by author RBJ. Examinations were performed using the same equipment and the same procedures, including the same DXA scanner used at baseline measurements.

Participants were examined in a random order based on their availability over a 2½ months period.
at Bispebjerg Hospital, Denmark.

Bone mineral density (BMD) was assessed using a DXA-scanner from Lunar Prodigy (GE, Madison, WI, USA, with encore 2005 software, version 9.15.010), and the calculations were done by the scanner software. Weight and height measurement were done using a hospital-grade electronic weight, and a wall-fixed height scale. Measuring was done with participants wearing light indoor clothing and no shoes.

The BMD and T-scores were registered for total hip and lumbar spine (L2-L4). For total hip comparisons, the previous Charcot foot side in the Charcot foot group were compared to the left side of the control group, and the contralateral side (non-Charcot side) in the Charcot foot group were compared to the right side of the control group.

The BMD values were also compared to the 1994 WHO criteria for osteoporosis (31). The lowest T-score value of lumbar (L2-L4) and either hip was used for comparisons. The T-score was calculated by the scanner using normal reference material (provided by the scanner software) of age, sex and ethnicity.

Calcaneal BMD was measured by DXA-scanning the calcaneus using specialized software for "small animals". The feet were placed flat on the scanner bed with the lateral side pointing downwards and the ankle joint bent 90° so that the calcaneus could be assessed with a perpendicular beam direction. Both os. calcaneii of all participants were scanned. BMD was estimated based on a region of interest (ROI) drawn by hand to encompass the entire bone. For comparisons with the control group the previously affected Charcot foot has been compared to the left foot of the control group.

Blood samples were drawn from a vein in the antecubital fossa. The samples were centrifuged at 4 °C and frozen at -80 °C. All samples were analyzed together at the end of the study. Baseline blood samples from 2005-2007 had been stored at -80 °C continuously since sampling (up to 11 years prior to analyzing) with no intermittent thawing.

Measurement of fsRANK-L, OPG and sRAGE was performed at BioLab, Dept. of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Denmark. The remaining samples were analyzed at the same place as part of the daily test sample routines. The accepted intra-individual sample CV for the fsRANK-L, OPG and sRAGE assays was 20%. The accepted intra-individual sample CV for the remaining samples was at most 10%.

- RANK-L was measured as the free soluble form (fsRANKL) with a sandwich ELISA, high sensitivity kit at 450 nm (Biomedica Medizinprodukte GmbH & Co KG, A-1210 Wien). The assay had a limit of detection (LoD; std.0 blank+3SD blank) of 0.01 pmol/L with normal serum
median values of 0.14 pmol/L, and a standard range of 0-2 pmol/L. The fsRANK-L/OPG ratio was calculated as: \((\text{fsRANK-L} \times 100)/\text{[OPG]}\).

Pearson et al. have argued that human serum levels of RANK-L are difficult to measure due to the lower limits of the normal range being close to the lower limit of quantification for available assays (32). We worked around this by continuing the serial dilution row further by one step than what was described in the assay instructions, and calibrating the standard curves after this lower signal.

- Osteoprotegerin (OPG) was measured with a sandwich ELISA, high sensitivity kit at 450 nm (Biomedica Medizinprodukte GmbH & Co KG, A-1210 Wien). LoD 0.07 pmol/L with normal serum median values of 2.7 pmol/L, standard range of 0-20 pmol/L.
- sRAGE was measured by a quantitative sandwich ELISA with biotin-labelled antibodies at 450 nm (Biovendor – Laboratorní medicína a.s., 621 00 Brno, Czech Republic). LoD 19.2 pg/mL with normal serum median values of 692.0-832.1 pg/mL (males) and 769.8-827.5 pg/mL (females), upper assay limit 3200 pg/mL.
- Baseline measurement of CTX (Carboxy-Terminal collagen crosslinks) and osteocalcin:
  - CTX was measured with a Serum CrossLabs® ELISA (Nordis Bioscience Diagnostic A/S, Herlev, Denmark). Detection limit 0.020 ng/mL, intra-assay variation <6.0%.
  - Osteocalcin was measured as N-MID (N-MID® Osteocalcin ELISA (Nordic Bioscience Diagnostic A/S, Herlev, Denmark). Detection limit 0.5 ng/mL, intra-assay variation 2.2%.
  - At follow-up CTX, P1NP (Procollagen type 1 N-terminal Propeptide), osteocalcin and bone-specific alkaline phosphatase assays were all chemiluminescence immunoassays. They were carried out on a dedicated automated analyzer, iSYS (Immunodiagnostic Systems) according to the manufacturer’s instructions.
  - CTX-I was measured using the IDS-iSYS CTX (CrossLaps®) assay (Immunodiagnostic Systems, plc, Tyne and Wear, UK).
  - P1NP was measured using the IDS-iSYS intact P1NP assay (Immunodiagnostic Systems). Osteocalcin was measured using the N-Mid Osteocalcin assay (Immunodiagnostic Systems).
  - Bone-specific alkaline phosphatase (BAP) was measured using the IDS-iSYS Ostase® BAP assay (Immunodiagnostic Systems).

**Statistical analysis**

Data are expressed as [means ±1SD] for normally distributed data, and as [median ±range] for data not normally distributed. Normal distribution in data was controlled by the Shapiro-Wilks test (no
transformations were used). T-tests and ANOVA was used for variance analysis between groups in normally distributed data sets, while in ranked groups and groups not normally distributed non-parametric tests in the form of the Mann-Whitney rank sum test and the Kruskal-Wallis one way analysis of variance on ranks were used. For matched groups (e.g. baseline versus follow-up) paired t-tests and Wilcoxon signed rank tests were used. Test results were adjusted using Bonferroni corrections in cases of multiple comparisons to avoid issues with mass significance.

Statistics and general data handling was done using IBM SPSS Statistics v. 23 by IBM Corporation, SIGMAPLOT v. 11.0.0.77 by Systat Software Inc., Microsoft Excel 2000 v. 9.0.2812 by Microsoft Corporation and Apache OpenOffice 4.0.1 by The Apache Software Foundation.

Results
A total of 21 participants from the baseline visit in 2005-2007 were included in the follow-up examinations. All participants had diabetes mellitus type 1 or 2 at baseline. At baseline, 10 of the follow-up participants also had an acute Charcot foot (DM+CF group), while 11 participants did not (DM-CF group). None of the participants had an active Charcot foot at follow-up.

Of the 17 participants with acute Charcot foot at baseline, 3 had passed away, 2 declined participation, 1 was excluded due to lower leg amputation since baseline, and 1 we were unable to contact. Antropomorphic data on the participants at the follow-up visit are listed in Table 1.

Due to the high loss to follow-up we have tested for differences between those baseline participants lost to follow-up, and those who were able to participate in both examinations. We dividing the baseline population into follow-up participants (n=21) and non follow-up participants (n=28) and testing the two groups against each other for variations regarding age, diabetes age, BMI, HbA1c or BMD in total hip and L2-L4 without any significant finds.

Of the 21 participants included in both baseline and follow-up, none had received treatment with bisphosphonates or similar osteoporosis-related treatment. It was not possible to confirm whether some, or all, had taken calcium and/or vitamin D supplements, as these are not necessarily registered in the prescription database. However, none of the participants reported using any of these supplements in the intervening period.

DXA-scanning
There were no differences in the change in total hip BMD ($\Delta(BMD)_{\text{followup}} - \text{baseline}$) from baseline to follow-up in either the DM+CF group (previous Charcot foot side versus non-Charcot foot side) or the DM-CF group (left side versus right side)(Table 2). In addition, there were no difference in total hip $\Delta(BMD)_{\text{followup}} - \text{baseline}$ between the DM+CF and DM-CF groups for either the Charcot foot
leg/left leg (p=0.294) or non-Charcot foot leg/right leg (p=0.717).

Lumbar L2-L4 DXA scans showed an increase in BMD in both groups from baseline to follow-up of +0.036 g/cm$^2$ (+2.9%) in the DM+CF group, and +0.125 g/cm$^2$ (+10.1%) in the DM-CF group.

The change in calcaneal BMD difference ($\Delta$(BMD)$_{\text{followup}}$–baseline) was not significant between the previously affected Charcot foot (DM+CF group) and the control group left foot (DM-CF group)(p=0.232), or between the non-Charcot foot (DM+CF group) and the control group right foot (DM-CF group)(p=0.549)(Table 3).

At baseline, 14 out of the 21 participants had a normal T-score, 4 had osteopenia and 3 had osteoporosis. There were no difference in the distribution among the two groups. At follow-up, these values had not changed significantly with 15, 2 and 4 participants having normal, osteopenic or osteoporotic T-scores respectively.

Additionally, we estimated a calcaneal T-score using reference data collected by Zerahn et al. (33). This data was collected on a population similar to the participants in this study regarding ethnicity and sex. Using this dataset, most participants had a normal T-score, and none had a calcaneal T-score below -2.5, i.e. osteoporosis.

**Calcium and bone turnover parameters**

Measurements of calcium metabolic parameters at follow-up are listed in Table 4. There were no differences in any marker between the DM+CF and DM-CF groups at follow-up.

CTX and osteocalcin were also measured at baseline, and these values were compared to the follow-up values. CTX showed a significant decrease from baseline to follow-up (387 ±136 versus 95 ±83 ng/L)(p<0.001) in the DM+CF group, and (305 ±141 versus 90 ±40 ng/L)(p<0.001) in the DM-CF group. Osteocalcin did not show any difference from baseline to follow-up in either the DM+CF group (16.9 ±5.7 versus 14.7 ±11.5 µg/L)(p=0.153) or in the DM-CF group (11.1 ±4.0 versus 10.4 ±3.6 µg/L)(p=0.695).

**fsRANK-L, OPG and sRAGE**

At baseline, there were no difference in the level of fsRANK-L between the DM+CF and DM-CF groups. There was a significant decrease in fsRANK-L from baseline to follow-up in the DM+CF group (p=0.002), but no difference in the DM-CF group (p=0.232)(Table 5). The OPG values showed no differences between the groups.

The fsRANK-L/OPG ratio at baseline was 3.4 in the DM+CF group versus 1.3 in the DM-CF group (p=0.156)(Figure 1). However, the fsRANK-L/OPG ratio was significantly decreased from
baseline to follow-up in the DM+CF group (3.4 versus 0.5)(p=0.009), but not in the DM-CF group (1.3 versus 1.1)(p=0.302). Furthermore, the Δ fsRANK-L/OPG ratio follow-up-baseline between DM+CF and DM-CF from baseline to follow-up was significantly larger for the DM+CF group (-2.9 versus -0.1) (p=0.046).

Regarding sRAGE, there was no difference between the two groups at baseline (Figure 2). Both the DM+CF and the DM-CF groups had a significant increase from baseline to follow-up (p=0.005 in both cases). However, there was no difference in the change from baseline to follow-up between the two groups (p=0.470).

Discussion

We have re-investigated a population with diabetes mellitus, half of whom had an acute Charcot foot 8.5 years prior. We have focused on calcaneal and total hip BMD measured by DXA as well as markers of bone remodelling and inflammation. These long-term follow-up data are unique, as this has not been reported before.

DXA-scanning

We did not find a decreased BMD in our population using DXA. Actually, we found higher-than-expected total hip BMD and T-scores in our participants at follow-up regardless of a previous Charcot foot, as well as a lower-than-expected annual BMD loss in both total hip and calcaneus in both groups. The increase in BMD in lumbar L2-L4 was attributed to decreased height of the lumbar vertebrae (e.g. compression fractures) or the generation of osteophytes, and have thus not been attributed any importance.

Several studies have suggested lower BMD in a Charcot foot but, to our knowledge, none have reported on the changes with time. Some authors have reported decreased calcaneal BMD in Charcot feet when using ultrasound evaluation. Using DXA, Young et al. reported similar findings in lower limb BMD, which however, could not be confirmed by Witzke et al. across both hip and foot measurements. Our results match the findings of Clasen, who also found unaltered tibial BMD in Charcot patients. The present data now support these findings with DXA measurements of the os. calcaneii of previously acute Charcot feet.

Data indicate that the development of a Charcot foot is not due to a generally increased rate of spontaneous bone demineralization as suggested by others. Furthermore, we have previously reported that a chronic Charcot foot may result in a lower BMD in the affected foot. Thus, parts of the reported lower BMD in Charcot patients found by other groups might be a secondary effect due to immobilisation.
Additionally, the lack of local demineralization in an acute Charcot foot supports the neuro-traumatic over the neuro-vascular model for the development of a Charcot foot \(^{(36-39)}\), as the neuro-vascular theory states that local neuro-degeneration results in pedal bone dissolution and instability in the Charcot foot.

The estimated calcaneal T-scores reported are based on material which as been collected using a slightly different method for aligning the ROI and were obtained using a different scanner \(^{(33)}\). They should thus be interpreted with caution. However, they do point in the same direction as the total hip measurements, which is that the participants in this study had BMD above average. This is in line with current evidence suggesting that type 2 diabetics have increased BMD \(^{(40)}\).

**Calcium and bone turnover parameters**

CTX showed no difference at baseline, but a significant decrease in both groups from baseline to follow-up, without a difference in the change between the DM+CF and DM-CF groups from baseline to follow-up \((p=0.290)\). Thus, the decrease could be an age-related phenomenon, or due to the change of assays. It is worth noting that our follow-up CTX values are at the very low end of the newly suggested normal range for a Danish population \(^{(41)}\).

Osteocalcin (N-MID) showed a significant difference between the groups in the baseline study \(^{(9)}\), however this was not present when comparing the smaller groups at follow-up. There was no difference in the change in osteocalcin from baseline to follow-up between the DM+CF and DM-CF groups \((p=0.664)\).

Thus, overall, there were no notable differences in bone turnover markers from baseline to follow-up between the DM+CF and DM-CF groups.

**fsRANK-L, OPG and sRAGE**

We found a significant decrease in fsRANK-L in the DM+CF group from baseline to follow-up, as well as a significant difference in the change in fsRANK-L from baseline to follow-up between the DM+CF and DM-CF groups. This is also seen in the fsRANK-L/OPG ratios and could suggest that the fsRANK-L/OPG ratio is only elevated in the acute phase of the Charcot foot, and thus might not reflect a permanently higher-than-average ratio in individuals who develop a Charcot foot. An increased level of RANK-L and an elevated RANK-L/OPG ratio in individuals with Charcot foot in general has also been suggested by Ndip et al. \(^{(25)}\).

However, RANK-L can also be elevated for other reasons, such as trauma or physical stress. While the level of RANK-L might not rise immediately after short physical activity \(^{(42)}\), it does increase
shortly after extended physical stress\(^{(43)}\). Additionally, post surgery measurements show an increase which seems to peak after 2 months\(^{(44)}\). As shown by Folestad et al.\(^{(19)}\), the RANK-L level for an acute Charcot foot is initially elevated, and continues to hold this level for 4 months.

Regarding the OPG levels, we did not find these altered between the groups at baseline or at follow-up, which does not correspond to the findings of Folestad et al.\(^{(19)}\). It is possible that this is due to our low sample number. Furthermore, it is worth noting that the difference found by Folestad et al. was in comparison to a control group consisting of healthy non-diabetics. The control in the present study consists of comparable diabetes patients without a Charcot foot, which is a strength of our study.

Other authors have reported that OPG in diabetics is related to neuropathy\(^{(45,46)}\). As such, it is possible that any elevation in OPG in our population is masked by the fact that both groups had neuropathy at follow-up.

Regarding sRAGE, there was an increase for both groups from baseline to follow-up. It is possible that this increase is purely an age-related phenomenon or related to the progression of the participants' advancing diabetes. There was no difference in the change from baseline to follow-up between the two groups of participants with diabetes, with or without previous Charcot.

There is currently no consensus on the exact relationship between sRAGE levels, diabetes and neuropathy\(^{(23,28,47)}\). Witzke et al. have reported that sRAGE is lowered in individuals with chronic Charcot foot compared to controls both with and without diabetes mellitus\(^{(23)}\). It should be noted that our participants had an acute Charcot foot at baseline (as opposed to non-acute for Witzke et al.) which could play a role in this difference. The control group does not match either, as we have no healthy controls. Furthermore, our Charcot group has much higher levels of sRAGE in general than what was reported by Witzke et al.

**Limitations**

There are several shortcomings in this study. First and foremost, the number of participants available for recruitment has been limited to the population from the baseline study. As there was a high mortality from baseline to follow-up, we ended up with relatively few participants in the follow-up study, thus lowering the statistical power of this study. As the original study was never powered to look for differences in BMD, negative results in this area should be interpreted with caution.

Regarding BMD measurements of the calcaneus DXA, the precision of repeated measurements might be affected by the manual fitting of the ROI, as well as the precise alignment of the foot in the scan window.
In relation to the blood tests, very little sample volume was left from the baseline visit, which has limited the range of analyzes we could perform. The baseline samples had been stored frozen for up to 10 years, but Stern et al. (48) have shown that both RANK-L and OPG are stable while stored for up to 19 years.

Conclusion

In conclusion, we have documented an elevated level of fsRANK-L, as well as an increased fsRANK-L/OPG ratio, in patients with diabetes and acute Charcot foot compared to follow-up values for the same patients after 8.5 years. We also found a significant drop in fsRANK-L/OPG ratio from baseline to follow-up in patients with acute Charcot foot compared to diabetes patients without Charcot foot over 8.5 years.

In addition, we found that at 8.5 years follow-up diabetes patients with a previous acute Charcot foot had similar calcaneal and total hip BMD and bone turnover markers as patients with diabetes without previous Charcot foot. In fact, very few of the participants in this study had osteoporosis, and the annual decrease in BMD was less than expected for healthy non-diabetic elderly. Thus, bone mineral in general does not seem to be affected by a previous Charcot foot.

References


Table 1: Antropomorphic data at follow-up visit in 2015 of participants with diabetes and previous Charcot foot (DM+CF), and participants with diabetes without a previous Charcot foot (DM-CF).

<table>
<thead>
<tr>
<th></th>
<th>Diabetes and previous CF (n=10)</th>
<th>Diabetes without previous CF (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67.7 ±8.6</td>
<td>70.4 ±3.8</td>
</tr>
<tr>
<td>Sex (m/f)</td>
<td>7 / 3</td>
<td>10 / 1</td>
</tr>
<tr>
<td>Diabetes type (I/II)</td>
<td>5 / 5</td>
<td>2 / 9</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>28.9 ±15.9</td>
<td>26.7 ±12.8</td>
</tr>
<tr>
<td>Affected foot (right/left)</td>
<td>6 / 4</td>
<td>--</td>
</tr>
<tr>
<td>BMI</td>
<td>27.0 ±3.3</td>
<td>30.0 ±4.8</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>61.3 ±10.1</td>
<td>58.3 ±12.8</td>
</tr>
</tbody>
</table>

Data listed as #n or mean ±1SD.
None of the parameters differ significantly between the groups.
Table 2: Total hip BMD at baseline and changes in BMD (from baseline to follow-up 8.5 years later) in participants with diabetes with (DM+CF) or without (DM-CF) previous Charcot foot.

<table>
<thead>
<tr>
<th></th>
<th>BMD, total hip</th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DM+CF (n=10)</td>
<td>DM-CF (n=11)</td>
<td>P-value</td>
<td>Left side</td>
<td>Right side</td>
<td>P-value</td>
</tr>
<tr>
<td>Previous Charcot</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>foot side</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Contralateral</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>foot side</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Baseline (g/cm²)</strong></td>
<td>1.010 ±0.175</td>
<td>0.995 ±0.186</td>
<td>0.850</td>
<td>1.092 ±0.176</td>
<td>1.086 ±0.177</td>
<td>0.936</td>
</tr>
<tr>
<td><strong>Δ follow-up (g/cm²)</strong></td>
<td>0.003 ±0.120</td>
<td>-0.033 ±0.061</td>
<td>0.402</td>
<td>-0.041 ±0.059</td>
<td>-0.023 ±0.065</td>
<td>0.517</td>
</tr>
<tr>
<td><strong>%-change during</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>follow-up period</td>
<td>0.9%</td>
<td>-3.4%</td>
<td>--</td>
<td>-3.9%</td>
<td>-2.3%</td>
<td>--</td>
</tr>
<tr>
<td><strong>%-change per year</strong></td>
<td>0.1%</td>
<td>-0.4%</td>
<td>--</td>
<td>-0.5%</td>
<td>-0.3%</td>
<td>--</td>
</tr>
</tbody>
</table>

Data listed as mean ±1SD. Compared with t-tests.
Table 3: Calcaneal BMD at baseline and changes in BMD (from baseline to follow-up 8.5 years later) in participants with diabetes with (DM+CF) or without (DM-CF) previous Charcot foot.

<table>
<thead>
<tr>
<th>Calcaneal BMD</th>
<th>DM+CF (n=10)</th>
<th>DM-CF(n=10)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Previous Charcot foot</td>
<td>Contralateral foot</td>
</tr>
<tr>
<td>Baseline (g/cm²)</td>
<td>0.715 ±0.099</td>
<td>0.728 ±0.101</td>
</tr>
<tr>
<td>Δfollow-up (g/cm²)</td>
<td>-0.023 ±0.069</td>
<td>-0.023 ±0.082</td>
</tr>
<tr>
<td>% change during follow-up period</td>
<td>-3.3%</td>
<td>-3.3%</td>
</tr>
<tr>
<td>% change per year</td>
<td>-0.4%</td>
<td>-0.4%</td>
</tr>
</tbody>
</table>

Data listed as mean ±1SD. Compared with t-tests.
+ = One participant was unable to assume the required position for calcaneal DXA-scanning due to severe ankle arthrosis.
Table 4: Biochemical parameters related to bone health at follow-up in participants with diabetes with (DM+CF) or without (DM-CF) previous Charcot foot.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Follow-up samples</th>
<th></th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DM+CF (n=10)</td>
<td>DM-CF (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-OH-vitamin D (nmol/L) (50 – 160 nmol/L)</td>
<td>75.8 ±46.7</td>
<td>85.2 ±37.9</td>
<td></td>
<td>0.620</td>
</tr>
<tr>
<td>PTH (pmol/L) (1.6 – 6.9 pmol/L)</td>
<td>4.6 ±2.4</td>
<td>5.9 ±2.4</td>
<td></td>
<td>0.228</td>
</tr>
<tr>
<td>Ca²⁺(free, ionized) (mmol/L) (1.18 – 1.32 mmol/L)</td>
<td>1.21 ±0.03</td>
<td>1.24 ±0.04</td>
<td></td>
<td>0.072</td>
</tr>
<tr>
<td>Alkaline phosphatase (bone specific) (µg/L) (&lt; 20 µg/L)</td>
<td>17.1 ±7.6</td>
<td>16.1 ±4.8</td>
<td></td>
<td>0.725†</td>
</tr>
<tr>
<td>PINP (µg/L) (19 – 82 µg/L)</td>
<td>33.0 ±16.2</td>
<td>27.0 ±7.5</td>
<td></td>
<td>0.277</td>
</tr>
<tr>
<td>CTX (ng/L) (90 – 1086 ng/L)</td>
<td>95 ±83</td>
<td>90 ±40</td>
<td></td>
<td>0.620†</td>
</tr>
<tr>
<td>Osteocalcin (µg/L) (9 – 42 µg/L)</td>
<td>14.7± ±11.5</td>
<td>10.4 ±3.6</td>
<td></td>
<td>0.287†</td>
</tr>
</tbody>
</table>

Data listed as mean ±1SD. Compared with t-tests unless otherwise noted.
Values listed in the first column are the normal reference ranges for the assays used.
+ = Compared with a Mann-Whitney rank sum test
† = One value excluded as outlier due to being 102.1 above max value of the remaining sample.
$ = Reference range listed for 50 y.o. male where ranges differ with age and/or sex.
Table 5: Blood test results for the 21 participants with diabetes and with (DM+CF) or without (DM-CF) previous Charcot foot (CF), who participated in the follow-up after 8.5 years.

<table>
<thead>
<tr>
<th></th>
<th>fsRANK-L (pmol/L)</th>
<th>OPG (pmol/L)</th>
<th>sRAGE (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DM+CF (n=10)</td>
<td>DM-CF (n=11)</td>
<td>P-value</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.13 ; 0.68</td>
<td>0.06 ; 0.25</td>
<td>0.148</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.03 ; 0.04</td>
<td>0.07 ; 0.10</td>
<td>--</td>
</tr>
<tr>
<td>P-value</td>
<td>0.002*</td>
<td>0.232</td>
<td>--</td>
</tr>
</tbody>
</table>

Data listed as median ; range. Compared with the Mann-Whitney rank sum test or Wilcoxon signed rank test (for paired data).

fsRANKL = free soluble receptor activator of nuclear factor κ-B.
OPG = Osteoprotegerin.
sRAGE = soluble receptor for advanced glycation end products.

* = Significant at chosen α-level of 0.05.
Figure 1: The fsRANK-L/OPG ratio at baseline and follow-up for participants with diabetes and with (DM+CF)(n=10) or without (DM-CF)(n=11) Charcot foot.

In the DM+CF group the fsRANK-L/OPG-ratio is significantly different from baseline to follow-up (3.4 versus 0.5)(p=0.009), which is not the case in the DM-CF group (1.3 versus 1.1)(p=0.302).

Error bars shown as SEM.

Figure 2: The level of sRAGE at baseline and follow-up after 8.5 years in the two groups with diabetes with (DM+CF)(n=10) or without (DM-CF)(n=11) Charcot foot.

There was a significant increase in sRAGE in both groups from baseline to follow-up, but no difference in the change from baseline to follow-up between the two groups (p=0.470).

Error bars shown as SEM.