

Collagen subtype mRNA over-expression in diabetic charcot neuroarthropathy: potential as pathogenic contributors and molecular signature

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Abstract:

Charcot neuroarthropathy (CN) remains a diagnostic and therapeutic enigma. Our hypothesis was that analysis of collagen gene expression would serve to further define, stage, and characterize this disease process. In a primary and secondary care university hospital, 10 patients with verified CN were studied in a prospective, assessor-blinded trial. They were compared with 10 age/sex matched controls without CN. Intraoperative capsule and bone samples were analyzed for differences in mRNA expression using DNA-microarray analysis. Inclusion criteria for the CN group included: reconstruction surgery of the foot because of instability, pseudo-exostoses not treatable with total contact casts, or orthoses due to polyneuropathy, and CN of the foot. Patients with osteomyelitis or peripheral vascular disease were excluded. Inclusion criteria for the control group was surgery for other indications without polyneuropathy or signs of CN. Five collagen subtypes were specifically noted to be significantly over-expressed, notably Types II, IV, IX, XI, and XVII. In pooled bone RNA samples collagen XVII A1 was up-regulated by 52.5x, collagen VI A6 by 6.3x, and collagen VI A5 by 5.9x from patients with CN compared to controls. In joint capsule, collagen II A1 was up-regulated in CN by 122.4x, collagen XI A2 by 9.5x, collagen XIX A1 by 6.7x, and collagen XXIIA1 by 5.4x. A distinct pattern of over-expression of collagen mRNA subtypes was observed in CN. These observations provide insight into the molecular pathogenesis of CN and may serve as a molecular and cellular biomarker signature with potential use for early diagnosis of CN.

Key words: Charcot Foot, Charcot Neuroarthropathy, Collagens II, VI, IX, XI, XVII, Diabetes Mellitus, Inflammation, mRNA Expression

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INTRODUCTION

Charcot neuroarthropathy (CN) is a severe inflammatory complication in patients with neuropathy resulting from diabetes mellitus or other diseases.¹ It may cause destructive lesions leading to subluxation, dislocation, deformity, and ulceration of the foot and ankle joints. This poses a high risk for developing ulceration, subsequent infection, and potential amputation. CN is estimated to affect 0.8-8% of the diabetic population, with an incidence that is on the rise (**Figure 1**).² In his early description, Charcot described not only the neuropathic changes of joints but also recognized joint inflammation and abnormal blood flow, likely due to denervation as

associated pathologic changes. It is now known that these changes may precede radiologic findings.³ Trauma, often in the form of repetitive minor trauma, has been reported as an inciting driver in 22-53% of CN cases and is a likely trigger of bone inflammation.^{4,5} This relationship to trauma may explain why CN is mostly unilateral in spite of bilateral neuropathy. Further, capillary leak, with subsequent edema formation, is an additional pathophysiological response to blunt trauma, leading to additional abnormalities in vasomotor regulation.⁶ Interestingly, acute CN may be brought into quiescence by immobilization, seemingly turning the clinical

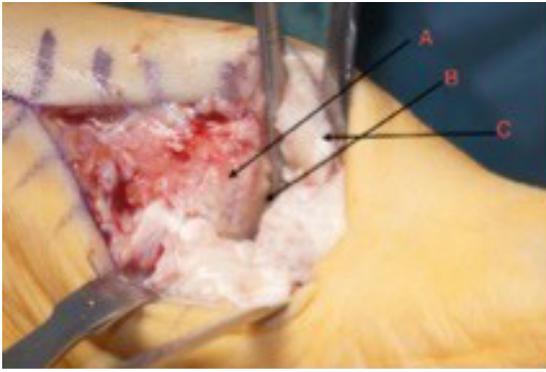


Figure 1. Intra-operative site of CN of the foot.

There was a gap of nearly 1 cm² where the bone had dissolved (B). The resisting bone appeared hard and rough on its surface (A). There were no remnants of cartilage visible on both sides of the former tarso-metatarsal-I-joint (B). The vanished bone had not been visible that way in the radiographs.

On examination the mid-foot was swollen, but not red or overheated. The patient suffered from severe polyneuropathy with subtotal loss of superficial sensibility. The skin was closed, and no ulcer was reported in the patient's history (Patient A).

course from destruction to condensation. The mechanisms responsible for this shift from bone destruction to restitution and regaining stability remain unclear. This phenomenon differs completely from other bone and joint diseases which typically are irreversible (eg, osteoporosis).

Jeffcoate and colleagues advanced the hypothesis that disruption of the RANKL/OPG pathway is responsible for both the vascular smooth muscle calcification as well as the osteopenia observed in CN.⁶ This hypothesis was 2000 and 2008, supported by blood analyses in patients with active CN and by the detection of impaired inhibition of RANKL expression. This further lead to impaired inhibition of osteoclast motility, recruitment, and differentiation by OPG and calcitonin gene-related peptide (CGRP) on Osteoclast like cells (OCL).^{7,8}

Collagens are bulk constituents of joint cartilage and bone.^{9,10} In addition to a structural role, collagens also function in the signaling processes in bone and tissue remodeling.^{11,12,13,14,15} Despite these essential component roles in bone and joint structure and function, their modulation in Charcot pathogenesis has not been explored. In this study we hypothesized that in patients with Charcot neuropathy an alteration in collagen

gene expression occurs. To evaluate this, mRNA expression for differing collagen subtypes was determined in bone and cartilage samples of patients with CN of the foot.

METHODS

A prospective single-site study was conducted in patients with active diabetic CN of the foot. Ten patients with active diabetic CN and 10 age and sex matched control individuals without CN or neuropathy were studied. All tissue samples were obtained during surgery through bone samples from seven patients with CN and from 10 control group patients and capsule samples from 10 patients with CN and from 10 control group patients. In 2009 and 2010, two experienced surgeons from the University Hospital of Muenster obtained biopsies from the affected and altered bone and capsule. Samples were then blinded by a random number system. Inclusion criteria for the Charcot group were: reconstruction surgery of the foot because of instability or pseudo-exostoses due to polyneuropathy and CN of the foot. Only patients with Type 2 diabetes were included. Indication for surgery was instability of the foot that could not be treated with total contact casts or orthoses. Patients with osteomyelitis or peripheral vascular disease were excluded. Patients were included in the control group if they underwent foot surgery for other indications without polyneuropathy or signs of CN. All patients provided written consent, and the study was approved by the local ethics board of the University of Muenster. All demands of the declaration of Helsinki were fulfilled.

Total RNA Preparation

Following excision, tissue samples were stabilized in PAX gene Tissue Containers (Qiagen, Hilden, Germany) and stored at -20°C. Prior to homogenization, tissues were briefly shock-frozen in liquid nitrogen. A series of 50 13- μ m cryosections of each tissue sample was homogenized for 2x2 minutes using a ball mill (Mikro Dismembrator U, B Braun Biotech International, Melsungen, Germany), equipped with a stainless steel container and

a ball (1cm in diameter), both pre-cooled with liquid nitrogen. From the tissue homogenate, total RNA was prepared using PAXgene Tissue RNA Kits (Qiagen). RNA was quantified spectrophotometrically using a Nanodrop Photometer (Serva, Heidelberg, Germany) and RNA integrity verified by 1% agarose gel electrophoresis in TAE buffer, followed by staining with SYBR Green II (Lonza, Cologne, Germany). RNA preparations were stored at -80°C.

DNA Microarray Analysis

Comparative gene expression analysis of collagens in the samples from patients with CN and the controls were performed using Agilent SurePrint G3 Human GE Micro-arrays (8x60K format) in combination with a One-Color based hybridization protocol. Duplicate total RNA aliquots (100ng) of each sample were reverse-transcribed into cDNA, converted into cyanine-3-CTP labelled cRNA by in vitro transcription, quality controlled, and hybridized onto the microarrays. Relative fluorescence signals (RLUs) on the micro-arrays were detected using an Agilent DNA Micro-array Scanner. Differential collagen gene expression in CN samples versus controls of more than 3-fold (measured in RLUs) was considered significant.

RESULTS

Study Patient Characteristics

Characteristics of the patients and control group enrolled in this study are detailed in **Table 1**. All patients were of comparable age with a male:female ratio of 7:3 for both groups. All patients in the Charcot group (n=10) had Type 2 diabetes with peripheral sensorimotor neuropathy with active arthropathy. In contrast, none of the patients in the control group had evidence of diabetes and/or neuropathy.

DISCUSSION

In the study we demonstrated a clear alteration of expression of collagens in patients with CN (**Table 2**). Specifically, collagens II, IV, IX, XI, and XVII were noted to be overly

expressed, while collagens XIX und XXII do not rise significantly. Collagen XIX is a minor collagen associated with basement membranes in vascular, neuronal, mesenchymal, and epithelial tissue. Collagen XXII is part of the FACIT protein-family (fibril-associated collagens with interrupted triple-helices).

We provide this evidence for collagen II in terms of mRNA transcription. Our finding of excessive over-expression of the collagen II gene and of its gene product in the capsule of CN as compared to controls is especially intriguing and suggests a possible role for collagens in the process of uncontrolled sterile inflammation of the joints in CN. Further, in this study, we show for the first time a morphological, biochemical, and molecular correlation of crucial pathological findings in diabetic CN of the foot in humans. In our study we found a clear increase in expression

Table 1. Basic data of patients and controls.

	Charcot Group (n=10)	Control Group (n=10)
Male:Female	7:3	7:3
Age (median)	60.2 years range: 45-62 years	64.4 years range: 47-81 years
Type 2 diabetes	10	0
Polyneuropathy	10	0
Eichenholtz I (acute)	6	0
Eichenholtz II	4	0
Eichenholtz III	0	0

Table 2. Quantity of mRNA expression of different types of collagens in bone and in capsular tissue of patients with CN and control individuals without neuropathy or diabetes.

Bone Tissue (n=7)	Charcot (RLUs)	Control (RLUs)	Odds Ratio (increase in expression)
Collagen XVII A1	721.00	13.73	52.5
Collagen VI A5	22.52	3.82	5.9
Capsular Tissue (n=10)	Charcot (RLUs)	Control (RLUs)	Odds Ratio (increase in expression)
Collagen II A1	3,109.83	25.41	122.4
Collagen IX A3	1,466.60	505.72	2.9
Collagen XI A2	389.48	41.00	9.5
Collagen XIX A1	15.62	2.33	6.7
Collagen XXII A1	28.50	4.57	5.4

Numbers indicate mRNA expression x-fold compared to control group.

of collagen II in the joint capsule of patients with CN, with increases detected in collagens XI, XIX, and XXII, but no increase in the expression of collagen IX.

In most of the cases acute CN is unilateral without systemic signs of inflammation (eg, fever, rise of CRP) in contrast to systemic inflammatory diseases such as rheumatoid arthritis. That is why the specimens for this study were taken from the site of maximum involvement (inflammation).

While collagen II represents the principal molecular component in mammals, collagens VI and XI contribute to the mature matrix.⁹ Collagen II has been shown to increase the rate of DNA and proteoglycan synthesis of chondrocytes stimulated by transforming growth factor β 1.¹² Collagen II is significantly increased in callus bone healing committed by pleiotrophin, playing a role in the differentiation of mesenchymal stroma cells.¹⁶ Collagen breakdown is considered to play a critical role in the development of osteoarthritis.¹¹ Our findings suggest that collagen II hyper-expression in CN is likely contributory to a vicious cycle of a sterile inflammatory process resulting in the acceleration and perpetuation of cartilage matrix and possibly bone degradation. This too is in accordance with the cell culture work of Klatt and colleagues who provided experimental evidence that collagen II induces matrix metalloproteinases and pro-inflammatory cytokines, which in turn leads to a release of collagen II fragments from mature collagen II fibers (**Figure 3**).¹³

Collagens II, IX, and XI form a three-dimensional heteropolymer that plays a decisive role in the stability of extracellular matrix of cartilage (**Figure 2**).¹⁰ Collagen IX is essential for the “cross-linking” in the II:IX:XI heteropolymer,⁹ and therefore plays the key role for stabilizing this molecule as an interfibrillar network bonding agent.¹⁰ Early-onset osteoarthritis is seen if collagen IX is missing (**Figure 2**).¹⁰ Jakkula showed that gene mutations of collagens II, IX, and XI play a role in the development of early onset arthritis.¹⁴ This corresponds to the well-known radiologically detectable remodeling processes with uncontrolled lysis, osteogenesis,

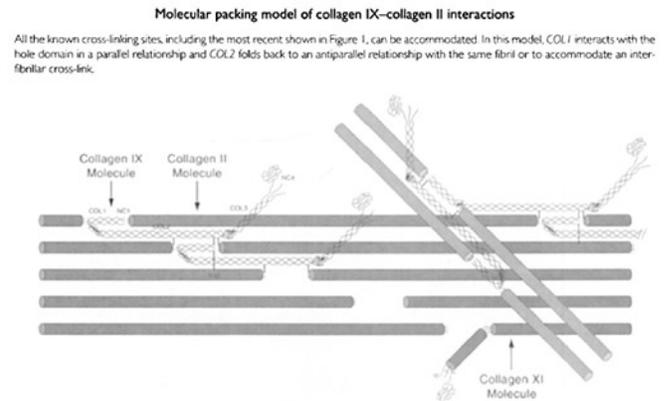


Figure 2a. Collagen II:IX:XI macromolecule and mRNA expression of collagens (from Eyre et al. 2002)

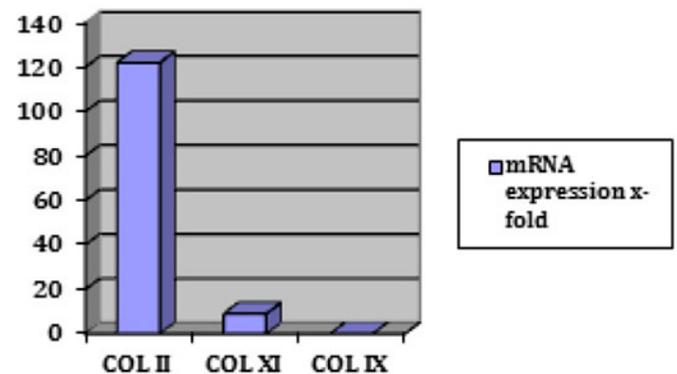


Figure 2b. Table of mRNA expression

and remodeling of bone and gives a probable explanation for the instability of Charcot feet. The imbalanced hyper-expression of collagen II and XI without hyper-expression of collagen IX could lead to a massive production of unstable II:IX:XI heteropolymers missing the essential “cross-linking” (**Figure 3**).

These unstable heteropolymers possibly break down as a result of molecular processes associated with the continuous weight-bearing and microtrauma, resulting in, or further driving, the postulated vicious cycle. This is consistent with the hypotheses of Jeffcoate and von Virchow stating that microtrauma triggers acute stages of CN resulting in an inflammatory cascade (**Figure 3**). In the former scheme for this cycle of CN, the new formation of bone, which can be seen as typical signs for Eichenholtz stage I in x-rays, had been missing.⁶ This study reveals, for the first time, the potential role of collagens in the progressive pathogenesis of CN and is hypothesis-generating as to the exact molecular

bone turnover. Also, an excessive increase in the pyridinoline cross-linked carboxy-terminal telopeptide domain of type 1 collagen was detected in these blood samples, which indicates increased osteoclast activity.

CONCLUSION

In this study we demonstrated significant over-expression of a range of collagen subtypes as correlate for the disturbed formation of regenerate cartilage and the disturbed remodeling of bone and soft tissue in intraoperative samples of CN. Destruction and proliferation represent unique alterations in CN visible in x-rays as amorphous and irregular formation of bone especially in Eichenholtz stage I, which still could not be shown in tissue or be explained so far. This study provides biochemical bridging this gap. For CN of the foot this increased expression of collagens may be a significant mechanistic contributor to the initiation or maintenance of pathogenesis.

Additionally, the finding of the absence of collagen IX in II:IX:XI, collagen heteropolymers raises the possibility that this may be a driver of inflammation. In work by others collagen IX has already been shown to play the key role as an interfibrillar network bonding or stabilizing agent.¹⁰ As such, the breakdown of unstable collagen II:IX:XI heteropolymers could start a vicious cycle of inflammation, since the role of released collagens in a signaling process for inflammatory diseases such as osteoarthritis have already been shown. Conversely, the finding that with immobilization and reduced micro-trauma the inflammatory process is halted or reversed suggest that a mechano-transductive molecular mechanism is likely a contributor to this collagen IX – collagen II:IX:XI heteropolymer interaction. On a clinical level this could explain, for instance, why the destructive process stops, when the affected joint is immobilized for example in a total contact cast. Further studies are needed to elucidate details of this process.

On a clinical level, our study suggests that CN may be further characterized, and possibly staged, by determining expression of collagen subtypes. This may serve as a valuable

diagnostic and research tool. As the observed changes in collagen mRNA species appear to be characteristic for CN, these may serve as a CN gene expression signature. Ex vivo analysis of this CN signature (e.g., by RT-PCR of intra-operative tissue samples) may have important clinical implications with regard to diagnosis, staging, and future therapies of this disease.

Strengths of this study are that this is the first study, to the knowledge of the authors, with in vivo samples to show the role of collagens in CN and the first study with in vivo samples supporting the inflammation theory of W Jeffcoate of 2005.

These results could be a new approach and possible use for clinical tests, while valid tests for CN are still missing. This study represents translational research, with a possible impact on clinical care.

Limitations are that more research with more samples are necessary, and the hypothesis for the inflammation vicious cycle should be controlled in new studies.

Contribution of the authors:

U Illgner initiated the study with HH Wetz, wrote the draft, collected samples, and is the corresponding author.

T Budny collected samples, corrected the draft.

DG Armstrong corrected and supervised the draft.

G Brunner DNA microarray analysis, corrected the draft.

M Slepian corrected the draft, supervision, biology.

W Scherbaum corrected the draft, supervision.

HH Wetz co-initiated the study, corrected the draft.

Data Sharing Statement: There is no additional data.

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