

Title:

Mitochondrial dysfunction is the cause of one of the earliest changes seen on Magnetic Resonance Imaging in Charcot neuroarthropathy - oedema of the small muscles in the foot

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Abstract

The hypothesis laid out in this thesis states that the early changes seen on an MRI imaging in those with early Charcot neuroarthropathy may be due to mitochondrial dysfunction. In a Charcot foot, there is movement between bones. In an attempt to prevent this movement, the small muscles of the foot contract continuously when the foot is weight bearing. This contraction takes energy in the form of ATP. However, the reduction of glucose transport into the muscle cells due to insulin resistance / insufficiency, leads to reduction in the ATP producing capacity of the mitochondria. The ATP depletion affects the cell membrane gradient leading to mitochondrial and cellular swelling. These early cellular changes could then be picked up with MRI imaging as muscle oedema.

Introduction

Charcot neuroarthropathy is an uncommon end organ complication of diabetes that, when it occurs, can have significant life changing consequences [1]. It is often missed in its early stages and by the time it presents to a specialist foot clinic there is often bony deformity and destruction, with a subsequent change in foot shape resulting in changes in plantar pressures. These can increase the risk of ulceration and infection. Ultimately, the risk of limb loss is high [2], and early immobilisation is associated with reduced risk of foot fracture and deformity [3-5]. Thus a diagnosis of a “Charcot foot” has significant impact on the individual – physically and psychologically, as well as on society in general due to the cost of treating the condition [6-8].

The classic way a Charcot foot has been diagnosed is a hot, red, swollen, (painless) foot in a person with diabetes. To date, the best way to diagnose the condition is to suspect the diagnosis, and then do appropriate imaging. If an initial plain radiograph did not show any abnormality, then the most sensitive method has been shown to be Magnetic Resonance Imaging (MRI) [9,10].

Population based studies have estimated a prevalence of Charcot neuroarthropathy at about 0.1-0.5% in people with diabetes, rising to as much as 13% in high risk patients [1]. It normally occurs 8-12 years after diagnosis of diabetes, most frequently during the fifth or sixth decade of life and is more common in men [1]. Other microvascular end organ damage is often present when a Charcot foot is first diagnosed [11,12].

A diagnosis of Charcot foot is frequently missed by referring physicians prior to referral to foot specialists. The delay between problems first noticed by the person with diabetes to diagnosis can average up to 29 weeks [13], and such delays lead to an increased rate of complications [3].

However, some individuals present early and do not necessarily have any overt bony abnormalities on plain imaging – i.e. classified as Eichenholtz stage 0 or 1 [14].

However, these individuals often have a degree of oedema of the soft tissue seen on MRI of the foot and ankle, including the small muscles of the foot [15]. An example of this is seen in Figures 1-3. The significance of this finding is uncertain.

The purpose of this hypothesis paper is to suggest that the soft tissue oedema seen in the small muscles of the foot in an early Charcot foot is a consequence of mitochondrial dysfunction in those with diabetes as a result of insulin insufficiency and/or insulin resistance.

The sequence of events in the hypothesis put forward in this paper is as follows:

1. In a Charcot foot there is movement between the bones
2. In an attempt to prevent the bones from moving, the small muscles of the foot contract to try and keep the bones in place and this contraction occurs at all times when the foot is weight bearing
3. This contraction takes energy and if there is insulin insufficiency and/or resistance then, because insulin is required for glucose uptake in skeletal muscle, intracellular glucose concentrations go down. This reduction in glucose concentrations leads to low ATP concentrations being produced

4. This low ATP concentration leads to the energy required for cellular processes (including muscle contraction) becoming depleted
5. As part of this, cell membranes are unable to maintain their integrity and start to leak. This leads to changes in intracellular ionisation with subsequent protein leak and changes in water content
6. The breakdown of cell membrane integrity leads to muscle swelling, and is the earliest signal picked up on MRI
7. Immobilisation of the bones to prevent the muscles from having to try and keep them still and thus stop contracting

Thus the treatment for an Eichenholtz stage 0 Charcot foot is early intervention with

- a) Immobilisation of the foot to prevent the muscles from having to try and keep the bones still, and thus stop contracting
- b) Improve insulinisation to allow for sufficient glucose uptake to supply the mitochondria with substrate

1. In a Charcot foot there is movement between the bones

There are two main theories concerning the development of a Charcot foot. Firstly, the neuro-traumatic theory describes the damage caused to the distal sensory feedback mechanisms of the foot, leading to repeated trauma and destruction of bones and joints. Repeated trauma produces pro-inflammatory cytokines (RANKL, NF- κ B, and osteoclasts), which lead to local bony resorption [1]. Secondly, the neuro-vascular theory describes the disruption in blood supply regulation due to neuropathy which again leads to bone resorption [16,17].

The structure of the human foot is designed to maintain stable bipedal walking and the mechanisms by which this is achieved involve complex movement patterns between the bones and ligaments. To achieve an efficient gait, fully functioning joints, bones and adequate muscle strength is required [18]. The longitudinal arch is able to compress and recoil thus storing elastic energy that can be released each time the foot has contact with the floor. During the late stance phase stiffening of the midfoot restricts the mobility of the midtarsal joints by inverting the subtalar joint. In early stance phase the subtalar joint is everted and the midtarsal joints become more flexible [19]. In the Charcot foot, osteolysis leads to progressive bone weakening and continuous weight bearing. This then initially leads to microfractures, that if continued cause the bones to fracture leading to joint deformities [6]. These deformities make the process of maintaining foot shape and stability during the gait cycle more challenging.

2. In an attempt to prevent the bones from moving, the small muscles of the foot contract to try and keep the bones in place and this contraction occurs at all times when the foot is weight bearing

Muscles contract in order to try and prevent joint dislocation [16]. Common muscle contractions in the foot include triceps surae (comprising the gastrocnemius and soleus muscles that insert into the calcaneum) and tibialis anterior muscle that inserts into the medial cuneiform and first metatarsal bones of the foot. If their actions were left unopposed would lead to plantar inclination of the calcaneus and dorsal dislocation of cuneiform bone respectively [16]. It has previously been recognised that hyperglycaemia leads to tendons and ligaments denaturing by non-enzymatic collagen glycation, leading to tendon shortening [20]. In addition, the peripheral neuropathy associated with diabetes is associated with weakness and atrophy of the intrinsic muscles of the foot [21]. Combined, these changes can lead to deformity of the joints of the foot which in turn can lead to bone dislocation and fragmentation and a change in the biomechanical properties of the foot [16].

3. This contraction takes energy and if there is insulin insufficiency and/or resistance then because insulin is required for glucose uptake in skeletal muscle intracellular glucose concentrations go down. This reduction in glucose concentrations leads to low ATP concentrations being produced

Insulin binds to the skeletal cell insulin receptor which is autophosphorylated and allows for the binding and subsequent phosphorylation of the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2). IRS binding to the phosphatidylinositol 3-kinase (PI3K) leads to the activation of a PI3K-dependent pathway. Within the pathway protein kinase B promotes the translocation of glucose transporter (GLUT4) to the plasma membrane, which in turn serves for glucose uptake into the muscle cell via facilitated diffusion [22,23].

Glucose is essential for muscle contraction. It has been shown to account for up to 40% of substrate use for oxidative metabolism during exercise [23]. Contracting muscle increases the recruitment of capillaries, which help to increase the surface area available to deliver glucose into the skeletal myocytes [23]. Insulin regulates the cellular supply of energy by activating the diffusion of glucose into myocytes [24]. Insulin has also been shown to increase mitochondrial capacity for oxidative phosphorylation by increasing the concentrations of oxidative enzymes by up to 25% in skeletal muscle [25].

Once glucose enters the myocytes with the aid of insulin, mitochondria serve as the primary site of its metabolism and thus ATP production [25]. Insulin resistance leads to impaired insulin mediated glucose transport into the muscle cells [24]. However,

with under-insulinisation and resultant hyperglycaemia the excess glucose that is taken up by the muscle cells is preferentially metabolised by hexokinase, an enzyme with a much higher affinity for glucose, and rapidly transforms glucose into glucose-6-phosphate. When glucose levels rise in diabetes, the hexokinase pathway is saturated forcing more glucose down the sorbitol pathway where enzyme aldose reductase converts the glucose to fructose via sorbitol using the enzyme sorbitol dehydrogenase. However, the conversion of sorbitol to fructose is very slow, leading to build up of sorbitol. This reaction used NADPH as a proton donor. However, NADPH is also used by glutathione reductase to convert reduced glutathione (GSH) to glutathione disulphide (GSSG). GSH is important because it removes hydrogen peroxide. The excessive use of NADPH by the sorbitol pathway, results in lower levels of GSH and thus higher concentrations of hydrogen peroxide. The hydrogen peroxide is produced by normal mitochondrial respiration that produces free oxygen radicals that are themselves converted to hydrogen peroxide by superoxide dismutase. When levels of hydrogen peroxide build up, the Fenton reaction liberates free radical superhydroxide that is damaging to the surrounding tissues (so called 'oxidative stress'). Thus, high glucose concentrations ultimately lead to a reduction in superoxide removal. It is these superoxide molecules that have been implicated in damaging strands of DNA. This in turn leads to reductions in intracellular NAD, thus slowing the rate of conversion of sorbitol to fructose, leading to a greater build-up of sorbitol. Sorbitol diffuses very slowly out through cell membranes, thus intracellular accumulation ultimately leads to irreversible cell damage and cell death.

Further damaging processes also occur as a result of high intracellular glucose. As mentioned, once the glucose is converted to glucose-6-phosphate, most of it is

metabolised to fructose-6-phosphate. This molecule is metabolised in 2 main ways. Firstly, fructose-6-phosphate is converted into glyceraldehyde-3-phosphate. This is metabolised into the highly damaging methylglyoxal. This molecule, as well as other reactive glucose metabolites, non-enzymatically attaches to other proteins, resulting in the accelerated formation of advanced glycation end-products (AGEs) and so, cellular dysfunction. In addition, these proteins may extravasate leading to progressive microvascular occlusion – leading to an inability to recruit more capillaries in times of need. AGEs can affect muscle function in 2 main ways. Firstly, protein glycation affects structure and normal biological function. Secondly the binding of AGE to cell surface receptors (RAGE) leads to activation of an inflammatory intracellular signalling cascade, increasing the oxidative stress.

The second pathway for fructose -6-phosphate is as a metabolite for the hexosamine pathway. This converts fructose-6-phosphate into uridine diphosphate-*N*-acetylhexosamine (UDP-GlcNAc). This molecule has an affinity for serine and threonine residues on proteins such as Sp1. The binding of UDP-GlcNAc to these proteins in turn adversely affects their structure and function and has been suggested as a contributory factor to the inflammation and endothelial injury seen in hyperglycaemia.

The intracellular hyperglycaemia and activation of the aldose reductase pathway described above is also associated with activation of intracellular signalling pathways mediated by the mitogen-activated protein (MAP) kinases. These kinases are also activated by high levels of oxidised low density lipoprotein (LDL) and RAGE. These molecules perform key roles in the intracellular signalling pathways, with at least two

(p38 and JNK) being activated in the face of intracellular hyperglycaemia. Activation is associated with phosphorylation of a variety of proteins, including ion channels, disrupting their function, and eventually causing measurable changes in cell damage.

The resistance mechanisms could include defects in phosphorylation of the insulin receptors or abnormalities in the GLUT4 function [24]. Mitochondrial dysfunction is observed in high energy requiring tissues such as muscle tissue [26]. Patients with type 2 diabetes have smaller, dysfunctional mitochondria [27] and do not increase their muscle mitochondrial ATP production in the presence of insulin [25]. It has been shown that mitochondrial dysfunction is a marker of impaired response to insulin in people with diabetes [28,29]. Studies involving people with type 1 diabetes show that degradation proteins of mitochondrial function are upregulated [30]. In addition to the abnormal metabolic pathways described above, in people with type 1 diabetes reduced insulin action in insulin resistant states causes muscle mitochondrial dysfunction, with multiple defects in mitochondrial gene transcript concentrations leading to reduced maximal ATP production [31,32]. Structurally, muscles of people with diabetes appear fragmented when compared to healthy subjects that have a large network of fused mitochondria [26]. Type I muscle fibres, which have high oxidative enzyme capacities, have more GLUT-4 transporters than type II muscle fibres. Insulin resistant muscles however have lower percentages of type I muscle fibres and lower GLUT-4 protein within their muscle fibres [25].

The contractile properties of skeletal muscle rely on muscle mitochondria which constantly adapt to meet new demands [19]. To maintain healthy mitochondria, there

must be a balance between mitochondrial biogenesis and mitophagy, but also between ATP and reactive oxygen species production [33]. Alterations to the energy supply of the mitochondria affect the balance between biogenesis and mitophagy [30]. Mitochondrial oxidative function is controlled by mammalian target of rapamycin (mTOR) [34]. mTOR balances energy metabolism by affecting mitochondrial transcription regulation via PPAR γ coactivator 1 α , which in turn regulates mitochondrial biogenesis [30]. Insulin regulates mTOR activity [30], highlighting its importance in the ATP-dependent processes of the body. Protective measures involve upregulation of signals to initiate mitophagy and ensure that damaged mitochondria are removed from the tissue [30]. For example, insulin deprivation leading to reactive oxygen species formation within the cell stimulates the activation of Parkinson protein 7 (PARK7/DJ-1) which in turn regulates mitophagy [35].

During metabolic stress induced by endurance exercise mitochondrial biogenesis can be increased. However in chronic disease, such as diabetes, it has been shown to be decreased [19]. The inflammation associated with decreased mitochondrial numbers associates with a reduction of oxidative phosphorylation, leading to further reduced cellular metabolism and impaired muscle function [36,37].

4. This low ATP concentration leads to the energy required for cellular processes (including muscle contraction) becoming depleted

ATP production requires oxidation of NADH or FADH₂ and oxidative phosphorylation of ADP to ATP. NADH and FADH₂ are generated during Krebs cycle of glucose, glycolysis and beta oxidation of fatty acids [38]. Electrons from NADH and FADH₂ are transferred to respiratory chain complexes and the final product is water. Protons produced during oxidation are pumped into the inter-mitochondrial membranes through respiratory complexes I, III, and IV, thus producing an electrochemical gradient [38]. The gradient drives ATP synthase to produce ATP from ADP [38]. There is constant utilisation within the cells and in intense or prolonged exercise the ATP concentrations are reduced [23]. NADH oxidase of the mitochondrial electron transport chain within the muscle is reduced in subjects with type 2 diabetes [39]. Therefore in both resting and energy requiring states, it may be reasonable to assume that the ATP producing capacity of people with diabetes would be affected.

ATP production maintenance within the muscle is so important, that myofibres contain two types of mitochondrial populations, the subsarcolemmal mitochondria and intermyofibrillar mitochondria [40]. These are interconnected and rapidly distribute energy through myofibres in order to provide sufficient amounts of energy to keep up with rapidly changing requirements [40].

In a study comparing skeletal muscle mitochondrial physiology between insulin deprived streptozotocin (STZ) mice and non-diabetic controls, the level of mitochondrial complexes I, II, and V were reduced in the STZ treated mice [35]. The

effects of insulin deprivation lead to reduction in mitochondrial proteins, including the mitochondrial complexes and adenine nucleotide translocases. Adding to this, translocation elongation factor involved in the regulation of mitochondrial protein synthesis was downregulated. Higher mitochondrial leak and lower phosphorylation efficiency was noted, along with overall mitochondrial ATP synthesis reduction [35]. These effects were reversed by insulin replacement [35].

These authors also found that uncoupled respiration led to higher reactive oxygen species production, which in turn would lead to DNA damage and protein degradation. These findings are supported by another study that found that ADP-stimulated respiration with pyruvate and malate in vastus lateralis muscles in people with type 2 diabetes was reduced and highlighted the reduction of functional capacity in those patients [41].

Insulin regulates mixed muscle protein synthesis, however in diabetes and in aging the function and synthesis of mitochondrial protein is reduced [42]. High insulin concentrations have been shown to stimulate mitochondrial protein synthesis within the muscle when infused with essential amino acids [42]. On a cellular level, insulin replacement in STZ mice restored mitochondrial protein concentrations and reduced reactive oxygen species production to concentrations close to the non-diabetic group - but not fully [35].

In patients with type 1 diabetes the insulin deficiency leads to a reduction in ATP production in the muscles, but treatment with insulin allows for the maintenance of mitochondrial function within muscles [30]. Insulin infusions have been shown to

increase mitochondrial capacity for oxidative phosphorylation within skeletal muscle [25]. Previous work has shown that short term hyperinsulinaemia stimulated myocellular ATP production in healthy subjects but not in those with type 1 diabetes mellitus, who had 42% lower intramyocellular glucose-6-phosphate levels suggesting an impairment of glucose disposal [43]. Mitochondrial ATP production did not change in the muscles of those with type 2 diabetes mellitus [25]. Mitochondrial oxidative enzymes citrate synthase activity and COX activity increased with insulin infusions within the skeletal muscle. The functional synthesis rate of mitochondrial proteins also increases which may in turn lead to increased ATP producing units [25].

5. As part of this, cell membranes are unable to maintain their integrity and start to leak. This leads to changes in intracellular ionisation with subsequent protein leak and changes in water content

It is evident that proteins play a key role in maintaining the integrity of the cell membrane [44]. Lack of insulin increases the breakdown of proteins and this effect is more prominent within skeletal muscle [42]. Highly reactive oxygen species produced within muscle cells during low energy states lead to protein degradation and cause DNA damage [35]. When ATP concentrations decrease dramatically during states of insulin deficiency, it results in apoptosis [45]. The cells lose their ability to maintain intracellular homeostasis of ions and water, which can lead to rupture of the plasma membrane and leakage of the cytoplasm into the extracellular environment [45]. The level of ATP depletion triggering apoptosis has been studied, and depletion of ATP concentration of over 50% was found to be the trigger [46].

A key factor in maintaining cellular integrity is the regulation of ion gradients against cell membranes. The ion pumps responsible are ATPases (Ca^{2+} ATPase, Na^+/K^+ ATPase, mitochondrial H^+ (F1) ATPase, H^+/K^+ ATPase) which utilise ATP to maintain ion balance across the cell membrane [47]. When oxidative phosphorylation fails and intracellular glucose concentrations are depleted, cells become depleted of ATP [46]. Ion pumps and other metabolic processes use up the remaining ATP, and then the Na^+/K^+ pump activity starts to become affected leading to disruption of the ratio of ions across the cell membrane [46]. Any compromise in the cell membrane functioning can have a detrimental effect on the cell [48]. Disruptions in the plasma membranes are particularly high in skeletal muscle due to continuous contraction

and lengthening occurring in physiological states [48]. These disruptions are resealed by utilisation of extracellular calcium ions and thus prevent triggering of cell death in large numbers [48]. Specifically the cell utilises calcium triggered exocytosis to deliver excess membrane to form a membrane patch at the site of injury [48]. However during ATP depletion, calcium homeostasis can also be disrupted [46], therefore could affect the ability of the cells to repair damaged cell membranes.

Various proteins have been implicated in the formation of the patch to prevent the disruption of the cell membrane [44]. Annexins are a large protein family that bind phospholipids in a calcium dependent manner and regulate endocytosis and exocytosis that lead to stabilisation of the membrane compartments [44]. Annexin A6 has been shown to accumulate the fastest at the injury site (within 20 seconds) [44]. Combined with dysferlin they act to formulate a repair patch, at the damaged cell membrane sites and increase membrane resealing [44].

Energy depletion has also been implicated in the breakdown of the cell membrane lipids [46]. In early studies of renal epithelial cells, it was found that depletion of ATP within the cells was much faster when glucose was absent. When examining the cell morphology of cell cultures depleted of glucose, it was found that no cells had normal morphology [46]. Instead cell swelling, mitochondrial swelling and disrupted cell membrane were observed [46].

Another important communication system exists between mitochondria and the endoplasmic reticulum. This takes place via mitochondrial associated endoplasmic reticulum membranes (MAMs) [49]. The structure of MAMs is supported by FUNDC1

proteins within the mitochondria [49]. It has been found that MAM concentrations and FUNDC1 expression are elevated in cardiac myocytes of diabetic mice [49]. An important observation is that MAM overexpression causes aberrant calcium signalling and in turn leads to mitochondrial dysfunction. When glucose concentrations are lower to establish normoglycaemia, MAM levels are reduced and calcium concentrations return to baseline within 8 hours [49]. This highlights the importance of establishing good glycaemic control to try and enable resolution of mitochondrial dysfunction in people with diabetes.

Endurance exercise training leads to increases of mitochondrial volume of up to 50% in training interventions of a few weeks in previously untrained subjects [50]. Bedrest and microgravity conditions lead to losses of both myofibrillar and mitochondrial volume, likely as a consequence of the decrease in metabolic and mechanical stress on muscle tissue. Permanent severe hypoxia leads to a loss of muscle mass and muscle oxidative capacity; however, hypoxia signalling events are triggered, which lead to distinct reprogramming phenomena of the transcriptome of the muscle cells.

6. The breakdown of cell membrane integrity leads to muscle swelling, and is the earliest signal picked up on MRI

Initial assessment of Charcot neuroarthropathy is undertaken within plain radiography [51]. There are three stages of the neuroarthropathy (I-III) that were described by Eichenholtz, which represent the natural evolution of the condition. However in stage 0, which was later added, there is no bony deformity and no changes that can be identified on plain radiograph [51]. MRI images are superior in detecting early changes [52] and are the most sensitive method to achieve this [51]. Muscle oedema can correspond to areas of low to intermediate signal in T1 weighted images and high signal in T2 weighted images [52].

7. Immobilisation of the bones to prevent the muscles from having to try and keep them still and thus stop contracting

Early diagnosis and treatment of Charcot stage 0 is crucial in preventing bony destruction and deformity [51]. Offloading and immobilisation of the affected foot at stage 0 can help reduce progression to stage 1 [51]. A retrospective cohort study evaluating acute Charcot foot treatment in 71 patients, demonstrated that diagnosis and treatment of Charcot foot at stage 0 is more efficient [53]. The treatment instigated was complete immobilisation and offloading with subsequent total contact cast. Even though the time to heal was not significantly shorter between patients with stage 0 and stage 1 disease, the rate of healing was different [53]. 70% of patients with stage 0 disease healed without deformity, whilst only 32% of patients with stage 1 disease healed without deformity [53]. This highlights the importance of diagnosing and treating Charcot neuroarthropathy at stage 0.

In summary

The hypothesis laid out in this thesis states that the early changes seen on an MRI scan in those with early Charcot maybe due to mitochondrial dysfunction. The reduction of glucose transport into the muscle cells due to insufficiency of insulin, leads to reduction in the ATP producing capacity of the mitochondria. With dysfunctional mitochondria being present in people with diabetes, these effects of energy depletion would be expected to be exacerbated. The ATP depletion affects the cell membrane gradient leading to mitochondrial and cellular swelling. These early cellular changes could then be picked up with MRI imaging as muscle oedema. What remains unknown however, is do these changes occur in those who develop Charcot who do not have diabetes. Thus further work is needed – such as skeletal muscle biopsies of those with early Charcot foot, and those with Charcot due to other reasons.

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Legend to Figures

Figure 1

AP plain radiograph of the weight bearing right foot showing no obvious bony abnormality (The same patient is seen in all three figures and the images were all done in the same week)

Figure 2

Lateral plain radiograph of the weight bearing right foot showing no obvious bony abnormality (The same patient is seen in all three figures and the images were all done in the same week)

Figure 3

Magnetic Resonance Image showing no bony abnormality. There is oedema seen within the soft tissues of the dorsal aspect of the foot as well as the muscles of the sole of the foot. (The same patient is seen in all three figures and the images were all done in the same week)