Impact of high dose omega-3 polyunsaturated fatty acid treatment on measures of microvascular function and vibration perception in non-alcoholic fatty liver disease: results from the randomised WELCOME trial

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Abbreviations List

- CIMT – Carotid Intima-Media Thickness
- CV - Coefficient of Variation
- DEXA - Dual energy X-ray absorptiometry
- DHA - Docosahexaenoic Acid
- EPA - Eicosapentaenoic Acid
- IR - Insulin Resistance
- MF - Maximum Flux
- MF/RF - The ratio of Maximum Flux (MF) to Resting Flux (RF)
- MR - Microvascular Reactivity
- MRI - Magnetic Resonance Imaging
- MRS - Magnetic Resonance Spectroscopy
- NAFLD - Non-Alcoholic Fatty Liver Disease
- PU - (arbitrary) Perfusion Units
- RF - Resting Flux
- VPT - Vibration Perception Thresholds
- WELCOME - **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy (WELCOME) trial)

Abstract.

Aims/hypothesis. The effect of omega-3 fatty acid treatment on vibration perception thresholds (VPTs) and cutaneous microvascular reactivity (MR) is not known. We tested whether: a) 15-18 months treatment with high dose (4 g/day) docosahexaenoic (DHA)+eicosapentaenoic (EPA) acid improved VPT and MR in patients with non-alcoholic fatty liver disease (NAFLD); and b) there are associations between VPT, MR and metabolic parameters.

Methods. In the completed single centre, randomised, double blind placebo-controlled **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy (WELCOME) trial), we tested the effect of DHA+EPA/placebo (randomised 1:1) on VPT at 125Hz (big toe) and the cutaneous hyperaemic response (forearm) to arterial occlusion (ratio maximum to resting blood flux-MF/RF).

Results. 51 and 49 patients were randomised to placebo and DHA+EPA respectively (mean age 51.4y). 32 subjects had type 2 diabetes. 46 (placebo) and 47 (DHA+EPA) subjects completed the study. There were no serious side effects. In multivariable-adjusted regression models (ITT analyses), DHA+EPA treatment was associated with an increase in VPT (B coefficient 1.49, (95%CI 0.04,2.94), p=0.04). For VPT, the adjusted mean differences (95%CIs) in the placebo and DHA+EPA treatment groups were -0.725 (-1.71,0.25) and +0.767 (-0.21,1.75) $m.s^{-2}$, respectively. With DHA+EPA treatment, there was no change in MF/RF (B coefficient 0.07, (95%CI -0.56,0.70), p=0.84). VPT was independently associated with age (B coefficient 0.019, (95%CI 0.010,0.029), p<0.0001) and MF/RF (B coefficient -0.074, (95%CI -0.132,-0.016, p=0.013), but not diabetes (p=0.38).

Conclusions/interpretation. High dose omega-3 fatty acid treatment did not improve measures of microvascular function or vibration perception. Ageing and microvascular reactivity are associated with a measure of peripheral nerve function.

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Introduction

Peripheral neuropathy and impaired microvascular function are strong risk factors for foot ulceration and impaired wound healing in people with diabetes (1) and both somatic cutaneous sensory fibre neuropathy and microvascular dysfunction are early complications of diabetes mellitus and/or insulin resistance (IR) in obese individuals (2). Screening tests for neuropathy in the clinic include use of a 10 g monofilament and use of a 128 Hz tuning fork (3). Both tests reflect the function of large myelinated sensory nerve fibres and, although the monofilament test has been widely adopted and is easy to use in clinical practice, its sensitivity to detect early impairment in nerve function is limited (4). In contrast, use of vibration perception thresholds (VPTs) allows not only detection of neuropathy but also assessment of the severity of the sensory nerve impairment (5). The prevalence of an abnormal VPT in patients with type 2 diabetes has been shown to be >11% (6) and an abnormal VPT is an excellent predictor of foot ulceration, limb amputation and mortality in patients with type 2 diabetes (7).

Non-invasive assessment of cutaneous microvascular reactivity (MR) has been widely used to assess microvascular endothelial and neurovascular function in patients at increased risk of cardio-metabolic disease (8) and is indicative of MR in other vascular beds (9). Impaired skin MR strongly relates to impaired glucose tolerance, IR and obesity (10;11) and whilst microvascular and neurological function are interlinked in diabetes (12), there is still controversy whether early changes in microvascular function or changes in metabolic parameters have the greater influence on peripheral nerve function (13).

Treatment with high dose long chain omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) improves endothelial function (14). These fatty acids enhance nerve blood flow (15), may improve

vibration perception and clinical symptoms of neuropathy in patients with type 2 diabetes (16), and have been shown to be protective against paclitaxel-induced peripheral neuropathy (17). However, although there is some preliminary evidence of benefit of long chain omega-3 fatty acids in patients with neuropathy, it remains uncertain whether these fatty acids have beneficial effects on VPTs and MF.

Patients with IR and non-alcoholic fatty liver disease (NAFLD) are at increased risk of type 2 diabetes and its complications, including macrovascular disease (18) (19). Additionally, NAFLD is associated with impaired coronary microvascular function (20), increased risk of retinal microvascular disease and prevalence of peripheral neuropathy (21). Since the effect of high dose omega-3 fatty acid treatment on nerve function and microvascular function in high risk patients for type 2 diabetes and its complications is not known, we have tested whether high dose omega-3 fatty acid treatment has beneficial effects on VPTs and MF in this patient group. Specifically, we tested whether 15 to 18 months treatment with high dose DHA+EPA (4 g per day as ethyl esters) produced improvements in VPTs and MR in people with NAFLD, some of whom had type 2 diabetes, but all patients were without clinical evidence of peripheral neuropathy or microvascular disease. In a pre-specified sub-study of the **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy (WELCOME) trial (www.clinicaltrials.gov registration number NCT00760513) (22;23), a randomised double blind placebo-controlled trial, we tested the effect of DHA+EPA treatment on VPT and MR.

Methods

Study design

105 individuals (60 men and 45 women (mean age of 51.4 y)) were studied. The participants formed part of the WELCOME study, a randomised double blind placebo-controlled trial (22) [approved by the local research ethics committee (REC: 08/H0502/165)]. Participants were block randomised by an independent clinical trials pharmacist to identical capsules by mouth of either omega-3 fatty acid ethyl esters (4 g/day Omacor, Pronova, Sandefjord, Norway) or placebo (4 g/day olive oil) for a minimum of 15 months and a maximum of 18 months of treatment. Only the clinical trials pharmacist was unblinded, and randomisation group allocation was concealed from all study members throughout the trial. One gram of Omacor contains 460 mg EPA and 380 mg DHA 380 mg as ethyl esters. Omacor is approved by the Food and Drug Administration and the European Medicines Agency at a dose of 2 to 4 g/day for the treatment of hypertriglyceridaemia. Olive oil placebo contained ~67% oleic acid, ~15% linoleic acid, ~15% palmitic acid. ~2% stearic acid and ~1% alpha linolenic acid. Participants were unpaid and gave their informed written consent. Inclusion criteria were diagnosis of NAFLD based on liver biopsy or presence of hepatic steatosis on ultrasound and exclusion of other liver diseases. Participants were excluded from the final analyses if they had evidence of distal peripheral neuropathy in their feet (n=4), as suggested by their failure to detect a 10 g monofilament (24), or if they had evidence of diabetic eye disease at retinal screening (n=0).

All tests for peripheral neurological and microvascular function were conducted in a temperature controlled room (22 - 24°C). Skin temperature at the toe, if below 25°C, was raised with a heat pad to 25°C prior to vibration testing. Mean (±SD) skin temperature measured at the toe was 28.3 ± 2.5°C (range 25.0 - 35.1°C) and at the forearm was 29.3 ±

0.7°C (range 27.5 - 31.1°C). Participants refrained from caffeine containing drinks, smoking and exercise for ≥ 2 h prior to testing. Measurements were made at baseline and end of study.

Vibrotactile perception

Vibration perception thresholds (VPTs) at 125Hz were measured (Vibrotactile Perception Meter, HVLab Diagnostics Instruments, Southampton, UK) with a 6-mm diameter probe and a 2-mm gap to a 10-mm diameter surround (25). VPTs were determined using the von Békésy method (25): the vibration magnitude alternately increased and decreased at 3 dB/s according to whether the subject felt the vibration. A response button was pressed when the vibration was felt and released when the vibration was not felt. Measurements continued for 30 s or until a minimum of six pairs of reversals had been obtained, after excluding the first pair. Thresholds ($m.s^{-2}$) were determined from the arithmetic averages of the logarithms of the root-mean-square vibration acceleration at the reversals (26). Tests were performed on the pulp of the left great toe at baseline and end of study. The intra-individual coefficient of variation (CV) was 22% measured in 20 volunteers on two occasions.

Microvascular function

Cutaneous microvascular reactivity (MR) was assessed on the ventral surface of the non-dominant forearm arm using laser Doppler fluximetry (Moor VMS LDF2 and DP1T probe, Moor Instruments Ltd, Axminster, UK) (27). Blood flux was recorded continuously before and during the dilator response to transient ischaemia (180 mmHg for 3 min; MoorVMS-PRES). The post occlusive hyperaemic response is an integrated vascular response involving neural, endothelial and vascular smooth muscle activity and is analogous to that used to assess endothelial function in conduit arteries (8). Values for microvascular perfusion in

arbitrary perfusion units (PU) were determined at rest (RF: mean value over the final 5 min before perturbation) and at maximum value after release of the pressure cuff (MF) using the manufacturer's software (MoorVMS-PC software, Moor Instruments Ltd, UK). MR was expressed as the ratio of maximum to resting blood flux (MF/RF). The intra-individual CV measured in the forearm of 10 volunteers on two occasions, 7 days apart, was 15% for RF and 19% for MF/RF.

Biochemical and anthropometric measurements

Measurements were made at baseline and at the end of the intervention period. Glucose, insulin, total cholesterol, HDL-cholesterol and triacylglycerol concentrations were measured in fasting serum or plasma using commercially available kits according to the manufacturers' instructions. HbA_{1c} was measured by high pressure liquid chromatography (Bio-Rad Laboratories, Irvine, CA, USA). HOMA-IR was calculated from fasting insulin and fasting glucose concentrations. Blood pressure was measured in the non-dominant arm after subjects had become acclimatised and had rested for at least 60 min; the mean of three measurements was calculated. Dual-energy X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) were undertaken to assess body fat (total body fat, regional body fat and visceral fat) and liver fat quantity (22). Liver fibrosis was assessed using NAFLD fibrosis score (28) and an additional validated liver fibrosis score (29). Carotid intima-media thickness (CIMT) was measured at both carotid arteries with B-mode ultrasound and a mean value calculated. This measure is a recognised marker of subclinical atherosclerosis (30) and has prognostic value in cardiovascular disease (31). Overall 10 year risk of cardiovascular disease was calculated using the Q-RISK 2011 online calculator (www.qrisk.org).

Statistical analysis

Statistical analyses were undertaken using IBM SPSS Statistics 21.0 (IBM United Kingdom Limited, UK). Data are reported as means and standard deviations for normally distributed variables, or as median and interquartile range for non-normally distributed variables. Where possible, variables that were not normally distributed were normalised by log transformation for parametric statistical analyses. Pearson and Spearman rank correlation coefficients were used to investigate associations between normally and non-normally distributed variables, respectively. In all cases a value of $p<0.05$ was taken to indicate significance.

We tested the independence of associations between baseline factors and the two primary outcomes (VPT and MF/RF) at baseline, by multivariable linear regression. We tested the effects of DHA+EPA treatment on both of the key outcomes of interest (VPT 125Hz difference and MF/RF difference) (the difference represented the change in measurement from baseline to end of study) using multivariable linear regression; and logistic regression for dichotomous outcomes of VPT difference (increased/decreased) and MF/RF (increased/decreased). ANCOVA was also used to assess adjusted mean differences (95%CIs) for both outcomes of interest in the placebo and DHA+EPA groups. These analyses included all participants with complete data (i.e. having baseline and end of study measurements). For all regression models and for ANCOVA, there was also adjustment for baseline measurement of the outcome variable in question, and adjustment also for key covariates and confounders. We also explored the effect of medication usage (statins, metformin, antidepressants and antihypertensive drugs, including calcium antagonists (no patients were taking hydrallazine)).

Results

Characteristics of the trial participants

Figure 1 shows the Consort diagram for recruitment into the study and reasons for withdrawal. Table 1 shows the baseline characteristics of the participants without overt neuropathy or microvascular disease, stratified by diabetes status and by randomisation status. The mean (±SD) age of the 69 (38 men) individuals without diabetes was 51.7±10.9 years and it was 50.1±10.3 years for the 32 (20 men) individuals with type 2 diabetes. 13 participants were current smokers and 3 had known ischaemic heart disease. Of the participants, 41 were taking statins, 36 antihypertensive drugs (9 calcium antagonists), and 27 metformin. VPT 125Hz and MF/RF did not differ between individuals with and without diabetes (Table 1). Of the participants without diabetes ~50% had impaired fasting glucose or impaired glucose tolerance.

Associations of VPT at 125 Hz and MF/RF with cardiovascular and metabolic risk factors at baseline

In univariate analyses, VPT was associated with age (r= 0.507, p=0.0001) and MF/RF (r=-0.301, p=0.002) (Table 2). The scatter plot for the association between VPT and MF/RF is shown in Figure 2. VPT was positively associated with CIMT (r=0.358, p=0.0001). An increase in VPT and a decline in MF/RF were both associated with an increase in Q-RISK (r=0.416, p=0.0001 and r=-0.229, p=0.023, respectively).

There was no significant association between either VPT 125Hz or MF/RF and measures of obesity, glycaemic status or insulin resistance; although in individuals specifically without diabetes, MF/RF was negatively correlated with HOMA-IR (r=-0.326, p=0.006, n=69).

Stratification by diabetes status suggested that in individuals with diabetes, an increase in VPT was associated with an increase in duration of diabetes (r=0.485, p=0.005).

A multivariable regression model with baseline VPT as the outcome variable, and age, sex, MF/RF, diabetes status, liver fibrosis score and CIMT as exposure variables, showed that all of these together explained ~35% of the variance in VPT (R^2= 0.35, p<0.0001, adjusted R^2=0.30) (Table 3). Use of statins, metformin, antidepressants or all antihypertensive drugs (including calcium antagonists) was not independently associated with VPT at 125Hz. The model was also not affected by adjustment for skin temperature (data not shown). We repeated the model replacing diabetes status with insulin resistance (HOMA-IR). In this model, age, sex, MF/RF, HOMA-IR, liver fibrosis score and CIMT explained ~33% of the variance in sensory nerve function (R^2= 0.325, p=0.0001, adjusted R^2=0.276) in the whole cohort.

We repeated the regression model in Table 3, replacing VPT with MF/RF as the new outcome. In a model that included VPT, age, diabetes status, liver fibrosis score, use of calcium antagonists and CIMT as exposure variables, female sex (p=0.010), VPT (p=0.011) and use of calcium antagonists (p<0.0001) were significant, (R^2= 0.27, adjusted R^2=0.21, p<0.001). Use of other anti-hypertensive agents was not associated with MF/RF. Replacing diabetes status with HOMA-IR as an exposure variable did not improve the model (R^2= 0.23, adjusted R^2=0.17, p=0.003) and HOMA-IR was not independently associated with MF/RF (p=0.97).

Effect of omega-3 polyunsaturated fatty acid treatment on VPT at 125 Hz and MF/RF

Table 1 shows the baseline characteristics of the participants without overt neuropathy or microvascular disease, by randomisation status. At baseline, by chance VPT was higher in participants randomised to placebo compared with DHA+EPA and to take account of this difference the regression models were adjusted for baseline measurement. Table 4 shows the results of multivariable regression modelling testing the effects of the DHA+EPA intervention on both outcomes (i.e. VPT difference or MF/RF difference). In regression modelling adjusting for key potential confounders, (age, sex, VPT at baseline, MF/RF, diabetes (y/n), a liver fibrosis marker, and mean CIMT) there was a small increase (worsening) in VPT difference with DHA+EPA treatment. The model explained ~33% of the variance in VPT difference (R^2=0.33, p<0.0001, adjusted R^2=0.27). The model was slightly improved when we replaced diabetes (y/n) with HOMA-IR as a measure of insulin resistance (R^2=0.37, p<0.0001, adjusted R^2=0.29). None of the tested medications affected the model or were associated with either outcome. Since there was a significant, and unexpected, effect of DHA+EPA to increase VPT at the end of the study, we assessed adjusted mean differences in placebo and DHA+EPA treatment groups. The adjusted mean differences (95%CIs) in the placebo and DHA+EPA treatment groups were -0.725 (-1.71,0.25) and +0.767 (-0.21,1.75) respectively. These data were in keeping with the unstandardized B coefficient from the regression modelling analyses shown in Table 4 showing the treatment effect (i.e 1.492). We also assessed the OR (95%CI) for a worsening of VPT difference with DHA+EPA treatment. These data (OR 2.47, (95%CI 0.97,6.32), p=0.058) were also consistent with the results from multivariable linear regression modelling and ANCOVA. Next we tested whether there was a DHA+EPA treatment interaction with baseline VPT measurement. These analyses (Table 4) showed there was a significant association between the interaction term and VPT difference.

We repeated the regression model replacing VPT difference with MF/RF difference as the outcome (Table 4). Variables included in the model explained 35% of the variance in MF/RF difference (R^2=0.35, p<0.0001, adjusted R^2=0.28) but there was no effect of the DHA+EPA intervention (p=0.84) (Table 4). Figure 3 shows individual baseline and end of study measurements for VPT and MF/RF for each participant. Stratifying by diabetes status, there was no specific benefit of DHA+EPA treatment on VPT (p=0.36) or MF/RF (p=0.53) in people with diabetes.

Discussion

Our study is the first randomised double blind placebo controlled trial to examine the effects of the highest licensed dose of omega-3 fatty acids on measures of vibration perception and microvascular function. Our novel data show that there was no benefit from high dose DHA+EPA treatment for 15-18 months, on vibration perception threshold or microvascular reactivity. In fact, our results show that with DHA+EPA treatment there was a small, albeit significant, increase in VPT between baseline and end of study (Table 4) that is unlikely to be of clinical relevance. Although we show an independent association between VPT and MF/RF at baseline (Table 3), this association was weaker at the end of the study (data not shown). We consider that the change in the association between these two parameters (between baseline and end of study) may have occurred due to two factors (illustrated in Table 4): a) the significant interaction between DHA+EPA treatment and baseline VPT, and the association of both factors with change in VPT during the study, and b) the DHA+EPA treatment-mediated increase in end of study VPT.

Whilst there is general agreement that microvascular and neurological function are interlinked in diabetes, there is still controversy over the pathogenesis of neuropathy and uncertainty remains as to whether metabolic or vascular risk factors (including impaired MR) are more important in influencing VPTs in people at risk of diabetic foot ulceration (13). In addressing this uncertainty, our results suggest that ageing and early changes in microvascular function have a greater influence on peripheral nerve function than metabolic parameters.

We do not have measurements of DHA or EPA tissue enrichment in nerves or the microvasculature. However, we have shown that the DHA+EPA treatment caused excellent tissue enrichment in erythrocytes and there was good adherence to the intervention (all participants consumed ~75% of their allocated capsules) (23). Whilst we did not undertake

prior sample size calculations for this pre-specified sub-study, our results provided no hint of improvement with the intervention (Table 4). A retrospective power calculation showed that with the number of patients completing the trial and α=0.05, we had 99% power to detect a 20% change in MF/RF. Although, previously, it has been suggested that six weeks treatment with DHA had beneficial effects on forearm blood flow in obese individuals (14) and that omega-3 fatty acid treatment (in a non-randomised study) improved vibration perception in 21 patients with type 2 diabetes (16), prior to our study these benefits of DHA+EPA had not been tested in randomised double blind placebo-controlled trials lasting over 12 months.

Importantly, the association between VPT and MF/RF at baseline remained significant after adjusting for potential confounders such as age, sex, diabetes status, obesity, NAFLD severity or CIMT. In our study, stratifying by diabetes status, there was no association between VPT and insulin resistance (HOMA-IR) in people with diabetes. There was also no association between VPT and current glycaemic control (HbA_{1c}) but there was a significant association between VPT and HOMA-IR in people who did not have diabetes (r=0.342, p=0.005; n=67). This latter finding most likely reflects the fact that HOMA-IR measurements are an inaccurate estimate of insulin resistance in people with diabetes who have pancreatic beta-cell failure. Our findings are also consistent with the results of a study of 156 individuals with peripheral neuropathy and diabetes, where there was no association between neuropathy and glycaemic control (12). These findings and our data, taken together, suggest strongly that peripheral neurological function is related more to a measure of microvascular health than to metabolic risk factors, with the exception of insulin resistance. Microvascular dysfunction has been proposed to be a link between obesity, IR and hypertension (32) and a reduced microvascular dilator and exchange capacity has consistently been reported by us and by others in individuals with features of the metabolic syndrome (33-36). With respect to a link

between VPT and HOMA-IR, it is important to note that the DHA+EPA intervention did not improve HOMA-IR (data not shown but available from the authors).

Peripheral neuropathy is associated with cardiovascular disease (37) and increased vibration thresholds have been shown to be a risk factor for mortality with diabetes (38). We showed for the first time, a strong association between VPT at 125Hz and CIMT (Table 2). CIMT is a reliable marker of pre-clinical atherosclerosis and NAFLD is an independent predictor of an increased CIMT (39). Endothelial dysfunction may occur early in NAFLD (40) and it has been suggested that the mechanisms associated with arterial thickening could impair blood flow and initiate endoneurial hypoxia, thought to play a significant role in causing peripheral neuropathy in diabetes (41).

We are not able to explain what aspect of ageing underpins the association between age and VPT. Sensory perception decreases with age and higher mechanical and thermal sensory perception thresholds have been observed in older people (42), but whether any decrease in perception occurs as a result of changes in the brain, spinal cord or peripheral nerves or receptors is uncertain. Our finding that higher VPTs were associated with increased duration of diabetes is consistent with results reported by Shun *et al.* (43) in people with type 2 diabetes. These authors additionally reported that diabetes duration was negatively associated with epidermal denervation, an early event in people with diabetes (44). It is plausible that insulin resistance and advanced protein glycation (45) combine with deleterious changes in nerve perfusion through components of inflammation and oxidative stress (46) and may be responsible for the increase in VPT, but this is speculation and further research is needed.

Limitations

The current study has strengths and limitations. The main strength is that we have undertaken a randomised, double blind placebo controlled trial testing the effects of highly purified long chain omega-3 fatty acids lasting 15-18 months. We cannot overlook the fact that perfusion of skin capillaries primarily serves the purpose of thermoregulation, whereas those in deeper tissues (e.g. skeletal muscle) are much more closely linked to metabolic demand but nevertheless, the ability to perform minimally invasive in vivo mechanistic studies in human skin can inform our understanding of how disease states adversely affect vascular function. There is no ideal biomarker for diagnosis of neuropathy in diabetes. We have used neurothesiometry (VPT) to derive a quantitative measurement of peripheral nerve function and VPT at 125Hz is neuroselective for large myelinated sensory nerve fibres. Recently, it has been shown that nerve dysfunction in large nerve fibres occurs in individuals with IGT compared to health controls (47). Although, dysfunction in small nerve fibres may precede large nerve fibre dysfunction in diabetic neuropathy(13;48), since we measured VPT we cannot comment on the effect of DHA+EPA on small nerve fibre function or on the relationship between MR, ageing and HOMA-IR and small nerve fibre function. We also cannot be sure of the effect of duration of diabetes on VPT in our cohort, as identification of the date of onset of type 2 diabetes is often imprecise. Additionally, we undertook assessment of VPT in the foot and microvascular function in the arm. It was technically challenging in this obese cohort to measure microvascular function in the lower leg and it is well accepted that the cutaneous arm microcirculation provides a well validated index of microvascular function indicative of MR in other, less accessible vascular beds (9;49).

Conclusions

In summary, treatment with the highest licensed dose of DHA+EPA (as Omacor/Lovaza) did not improve VPT or MR in patients with NAFLD. In a high risk patient group, without evidence of overt peripheral neuropathy, both ageing and a measure of microvascular

reactivity were independently associated with VPT. VPT is related more to a measure of microvascular health than to metabolic risk factors, with the exception of insulin resistance.

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Our findings were presented as an abstract at Diabetes UK (2015). Some of the preliminary data were also presented as abstracts at the British Microcirculation Society (2011 and 2012) and Diabetes UK (2011).

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Duality of Interest

PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin. None of the other authors has any disclosures.

Contribution Statement

All the named authors fulfil all three International Committee of Medical Journal Editors (ICMJE) uniform requirements for authorship of this manuscript. KMcC, CDB, GFC and MJG made a substantial contribution to the conception and design of the study. KMcC, ES and LB made a substantial contribution to the acquisition of the data. CDB, GFC, KMcC, PCC and MJG conducted the analysis and interpretation of data. CDB and GFC drafted the manuscript, PCC, MJG, KMcC, ES, and LB helped to revise it critically for important intellectual content. All the contributing authors approved the final version to be published. CDB is responsible for the integrity of the work as a whole and is the guarantor of this work.

Figure legends

Figure 1. Consort diagram. VPT, vibration perception test; MF/RF microvascular reactivity test (the ratio of maximum to resting blood flux). N=5 participants were excluded (neuropathy=4, and not randomised because of illness=1).

Figure 2. Scatter plot showing the relationship between baseline vibration perception threshold (log_{10} VPT) at 125Hz and cutaneous microvascular reactivity (log_{10} MF/RF) (the ratio of maximum to resting blood flux).

Figure 3. Effect of 15-18 months treatment with docosahexanoic acid+eicosapentanoic acid (DHA+EPA) ethyl esters (4 g/day) or placebo on cutaneous microvascular dilator capacity and vibration perception threshold at 125Hz. Figure A and C represent the placebo group and B and D represent the treatment group. Baseline and end of study median measurements for each group are indicated by a horizontal line.

Table 1. Baseline characteristics according to diabetes status and according to randomisation group

	No Diabetes n=69	Diabetes n=32	P value	Randomised to Placebo n= 51	Randomised to DHA+EPA n=49	*P* value
Sex (M/F)	38/31	20/12		34/17	24/25	
Age (y)	51.7±10.9	50.1±10.3	0.66	53.8±9.7	48.2±11.1	0.27
BMI (kg/m^2)	31.3(5.0)	35.2(7.5)	0.004	31.7(5.2)	32.8(7.1)	0.42
Duration of Diabetes (y)		5(7)		6(5)	3(5)	0.45
Diabetes				17	15	
Current smoker	10	3	0.54	6	7	0.71
10 Year Cardiovascular Disease Risk(QRISK2, %)	8.5(12.9)	15.0(14.9)	0.014	10.9(13.6)	8.5(13.4)	0.26
SBP (mmHg)	137(22)	138(20)	0.77	137(17)	135(26)	1.0
CIMT mean (mm)	0.65 (0.12)	0.65(0.13)	0.87	0.66(0.9)	0.62(0.13)	0.23

Cholesterol (mmol/l)	4.6(1.4)	4.3(2.3)	0.0009	4.6(1.4)	4.9(1.3)	0.07
Low-density lipoprotein Cholesterol (mmol/l)	2.9(1.2)	2.6(1.0)	0.013	2.8(1.0)	3.0(1.3)	0.19
High-density lipoprotein Cholesterol (mmol/l)	1.1(0.4)	1.0(0.3)	0.11	1.0(0.3)	1.0(0.4)	0.42
Cholesterol/HDL	5.1±1.5	4.7±1.5	0.23	4.6±1.4	5.2±1.6	0.11
Triacylglycerol (mmol/l)	1.8(1.3)	1.5(1.0)	0.62	1.5(1.0)	1.8(1.2)	0.55
Total body fat (DXA, %)	38.1±7.5	37.9±7.0	0.48	35.7±6.9	39.9±7.1	0.38
Subcutaneous Fat (MRI, %)	32.8± 9.6	31.6±10.1	0.56	29.9±9.4	34.9±10.2	0.14
Visceral Fat (MRI, %)	16.2±4.6	16.7± 5.1	0.64	17.0±4.6	15.5±5.0	0.30
Liver Fat	26(26.3)	23.3(21.5)	0.54	24.0(20.1)	23.5(33.9)	0.92

(MRS, %)						
Liver fibrosis score [29]	24.2(26.0)	-0.6(2.0)	0.0001	-1.8(1.8)	-1.8(2.1)	0.92
NAFLD fibrosis score [28]	8.7(0.8)	9.1(0.8)	0.01	9.0(0.9)	8.7(1.0)	0.42
Insulin Resistance (HOMA-IR)	2.6(2.4)	4.6(5.0)	0.002	2.8(3.2)	3.2(3.2)	0.60
HbA_{1C} (%)	5.8(0.6)	7.6(2.6)	<0.0001	6.1(1.6)	5.9(1.2)	0.13
[mmol/mol]	40 (6.6)	60 (28.4)		43.2 (17.5)	41.8 (13.1)	
Vibration Threshold (125Hz) ($m.s^{-2}$)	3.8(5.5)	4.2(7.5)	0.48	5.8(6.5)	3.4(4.0)	0.026
Resting skin blood flow (RF) (PU)	10.7(6.8)	12.1(6.8)	0.0630	12.4(7.8)	10.4(8.1)	0.110
Microvascular reactivity (MF/RF)	4.2(1.9)	4.7(2.5)	0.3218	4.3(2.0)	4.2(2.4)	1.0

Data are presented as means±SD or medians(IQR).

Table 2. Univariate associations between vibration perception threshold (VPT) or microvascular reactivity (MF/RF) and anthropometric and biochemical risk factors at baseline

	Vibration perception threshold (VPT) at 125 Hz r value (*p* value)	Microvascular reactivity (MF/RF) r value (*p* value)
Age (years)	0.507 (0.0001)	-0.179 (0.073)
BMI (kg/m^2) [a]	-0.156 (0.123)	0.074 (0.465)
Duration of Diabetes (years)* (n=32)	0.567 (0.001)	-0.281 (0.119)
CVD Risk (%) [a]	0.416 (0.0001)	-0.229 (0.023
Systolic BP (mmHg)	0.046 (0.654)	-0.163 (0.164)
CIMT (mm)	0.358 (0.0001)	-0.092 (0.368)
Cholesterol/HDL	-0.122 (0.229)	-0.041 (0.686)
Total Body Fat (DXA, %) [a]	-0.029 (0.776)	0.029 (0.777)
Subcutaneous fat (MRI, %) [a]	-0.106 (0.315)	0.088 (0.402)
Visceral fat (MRI, %)	0.114 (0.281)	-0.132 (0.209)
Liver Fat (MRS, %) [a]	0.088 (0.395)	-0.044 (0.667)
Liver Fibrosis score [29]	0.146 (0.155)	0.065 (0.526)
NAFLD fibrosis score [28] [a]	0.200 (0.047)	-0.087 (0.387)
Insulin Resistance (HOMA-IR)	0.070 (0.507)	-0.103 (0.324)
HbA_{1c} (%) [a]	0.073 (0.474)	0.110 (0.275)

(mmol/mol)		
Vibratory perception threshold 125Hz ($m.s^{-2}$)	-	-0.301 (0.002)
Microvascular reactivity (MF/RF)	-0.301 (0.002)	-

Data are Pearson or [a]Spearman correlation coefficients. N=101

Table 3. Multivariable regression model with vibration perception threshold as the outcome and key covariates and potential confounders as explanatory variables.

	Unstandardised β coefficient	95%CI	*p*
Age (y)	0.019	0.010, 0.029	0.0001
Male sex	0.031	-0.142, 0.203	0.72
Microvascular reactivity (MF/RF)	-0.074	-0.132, -0.016	0.013
Diabetes status	0.080	-0.102, 0.262	0.38
Liver fibrosis score [29] (AU)	0.017	-0.089,0.123	0.75
CIMT (mm)	0.544	-0.469, 1.557	0.29
[a]Antihypertensive (Calcium antagonists)	-0.268	-0.593, 0.057	0.11

R^2 for the model = 0.35 (P<0.001); adjusted R^2 = 0.30 (p<0.001). [a]Other anti-hypertensives were not associated with VPT.

Table 4 Multivariable linear regression modelling testing the effects of treatment on each of the primary outcomes, adjusting for baseline measurement of each outcome, and key covariates and potential confounders.

	Primary outcomes	
	Vibration perception threshold (VPT $m.s^{-2}$) Change from baseline to end of study	**Microvascular reactivity (MF/RF) Change from baseline to end of study**
Independent variables	**Unstandardised B coefficient (95% CI) *p* value**	**Unstandardised B coefficient (95% CI) *p* value**
Treatment Docosahexanoic acid + Eicosapentanoic acid	1.492 (0.04,2.94) 0.04	0.07 (-0.56,0.70) 0.84
Age (y)	0.04 (-0.04,0.13) 0.32	-0.03 (-0.06,0.01) 0.16
Male sex	-1.07 (-2.53,0.39) 0.15	0.60 (-0.06,1.25) 0.08
Diabetes status	1.36 (-0.11,2.83) 0.07	-0.14(-0.81,0.53) 0.68
VPT ($m.s^{-2}$)	-0.30 (-0.44,-0.17) <0.0001	0.02 (-0.11,0.15) 0.77
MF/RF	-0.17 (-0.63,0.29) 0.47	-0.63 (-0.84,0.42) <0.0001
[a]Liver fibrosis score	-0.23 (-1.16,0.70) 0.63	0.03 (-0.38,0.44) 0.89
Mean CIMT (mm)	-4.16 (-12.40,4.07) 0.32	0.02 (-3.72,3.75) 0.99

Treatment x baseline VPT ($m.s^{-2}$) Or Treatment x baseline MF/RF	0.31 (0.06,0.56) 0.015	0.16 (-0.24,0.57) 0.42

Multivariable regression models for all subjects completing the randomised double blind placebo-controlled trial testing the effect of DHA+EPA treatment on each of the two primary outcomes. Placebo group n=46, treatment group n=47. Each regression model was adjusted for age, sex, outcome variable value at baseline (i.e. VPT or MF/RF), plus diabetes status (yes/no), a marker of NAFLD severity ([a]liver fibrosis score, see reference 29) and a marker of pre-clinical macrovascular disease status (mean carotid intima medial thickness (CIMT) of left and right common carotid arteries).

Reference List

1. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ (2011) Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes Care 34: 2220-2224

2. Herman RM, Brower JB, Stoddard DG, et al (2007) Prevalence of somatic small fiber neuropathy in obesity. Int.J.Obes.(Lond) 31: 226-235

3. Tan LS (2010) The clinical use of the 10g monofilament and its limitations: a review. Diabetes Res.Clin.Pract. 90: 1-7

4. Richard JL, Reilhes L, Buvry S, Goletto M, Faillie JL (2014) Screening patients at risk for diabetic foot ulceration: a comparison between measurement of vibration perception threshold and 10-g monofilament test. Int.Wound.J. 11: 147-151

5. Young MJ, Breddy JL, Veves A, Boulton AJ (1994) The prediction of diabetic neuropathic foot ulceration using vibration perception thresholds. A prospective study. Diabetes Care 17: 557-560

6. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE (1994) Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J.Am.Coll.Cardiol. 24: 471-476

7. Crawford F, Inkster M, Kleijnen J, Fahey T (2007) Predicting foot ulcers in patients with diabetes: a systematic review and meta-analysis. QJM. 100: 65-86

8. Roustit M, Cracowski JL (2013) Assessment of endothelial and neurovascular function in human skin microcirculation. Trends Pharmacol.Sci. 34: 373-384

9. Holowatz LA, Thompson-Torgerson CS, Kenney WL (2008) The human cutaneous circulation as a model of generalized microvascular function. J Appl.Physiol (1985.) 105: 370-372

10. de Jongh RT, Serne EH, Ijzerman RG, de VG, Stehouwer CD (2004) Impaired microvascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance. Circulation 109: 2529-2535

11. Levy BI, Schiffrin EL, Mourad JJ, et al (2008) Impaired tissue perfusion: a pathology common to hypertension, obesity, and diabetes mellitus. Circulation 118: 968-976

12. Karvestedt L, Martensson E, Grill V, et al (2009) Peripheral sensory neuropathy associates with micro- or macroangiopathy: results from a population-based study of type 2 diabetic patients in Sweden. Diabetes Care 32: 317-322

13. Tesfaye S, Selvarajah D (2012) Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy. Diabetes Metab Res.Rev. 28 Suppl 1: 8-14

14. Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ (2000) Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. Circulation 102: 1264-1269

15. Coste TC, Gerbi A, Vague P, Pieroni G, Raccah D (2003) Neuroprotective effect of docosahexaenoic acid-enriched phospholipids in experimental diabetic neuropathy. Diabetes 52: 2578-2585

16. Okuda Y, Mizutani M, Ogawa M, et al (1996) Long-term effects of eicosapentaenoic acid on diabetic peripheral neuropathy and serum lipids in patients with type II diabetes mellitus. J.Diabetes Complications 10: 280-287

17. Ghoreishi Z, Esfahani A, Djazayeri A, et al (2012) Omega-3 fatty acids are protective against paclitaxel-induced peripheral neuropathy: a randomized double-blind placebo controlled trial. BMC.Cancer 12: 355

18. Targher G, Byrne CD (2013) Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. J.Clin.Endocrinol.Metab 98: 483-495

19. Bhatia LS, Curzen NP, Calder PC, Byrne CD (2012) Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? Eur.Heart J. 33: 1190-1200

20. Pinarbasi B, Demir K, Oflaz H, et al (2012) Measurement of the coronary flow velocity reserve in patients with non-alcoholic fatty liver disease. Turk.J.Gastroenterol. 23: 720-726

21. Lv WS, Sun RX, Gao YY, et al (2013) Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes. World J Gastroenterol. 19: 3134-3142

22. Scorletti E, Bhatia L, McCormick KG, et al (2014) Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chain omega-3 fatty treatment in non-alcoholic fatty liver disease. Contemp.Clin.Trials 37: 301-311

23. Scorletti E, Bhatia L, McCormick KG, et al (2014) Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology 60: 1211-1221

24. Spruce MC, Bowling FL (2012) Diabetic foot screening: new technology versus 10g monofilament. Int.J.Low Extrem.Wounds. 11: 43-48

25. Gu C, Griffin MJ (2013) Spatial summation of vibrotactile sensations at the foot. Med.Eng Phys. 35: 1221-1227

26. Gu C, Griffin MJ (2011) Vibrotactile thresholds at the sole of the foot: effect of vibration frequency and contact location. Somatosens.Mot.Res. 28: 86-93

27. Clough GF, Turzyniecka M, Walter L, et al (2009) Muscle microvascular dysfunction in central obesity is related to muscle insulin insensitivity but is not reversed by high-dose statin treatment. Diabetes 58: 1185-1191

28. Angulo P, Hui JM, Marchesini G, et al (2007) The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 45: 846-854

29. Guha IN, Parkes J, Roderick P, et al (2008) Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology 47: 455-460

30. Polak JF, Pencina MJ, O'Leary DH, D'Agostino RB (2011) Common carotid artery intima-media thickness progression as a predictor of stroke in multi-ethnic study of atherosclerosis. Stroke 42: 3017-3021

31. Simon A, Megnien JL, Chironi G (2010) The value of carotid intima-media thickness for predicting cardiovascular risk. Arterioscler.Thromb.Vasc.Biol. 30: 182-185

32. Karaca U, Schram MT, Houben AJ, Muris DM, Stehouwer CD (2014) Microvascular dysfunction as a link between obesity, insulin resistance and hypertension. Diabetes Res.Clin.Pract. 103: 382-387

33. Clough GF, L'esperance V, Turzyniecka M, et al (2011) Functional Dilator Capacity is Independently Associated with Insulin Sensitivity and Age in Central Obesity and is not Improved by High Dose Statin Treatment. Microcirculation 18: 74-84

34. Turzyniecka M, Wild SH, Krentz AJ, et al (2009) Skeletal muscle microvascular exchange capacity is associated with hyperglycaemia in subjects with central obesity. Diabet.Med 26: 1112-1119

35. De Jongh RT, Serne EH, IJzerman RG, Jorstad HT, Stehouwer CD (2008) Impaired local microvascular vasodilatory effects of insulin and reduced skin microvascular vasomotion in obese women. Microvasc.Res. 75: 256-262

36. de Boer MP, Meijer RI, Wijnstok NJ, et al (2012) Microvascular dysfunction: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. Microcirculation 19: 5-18

37. Tesfaye S, Boulton AJ, Dyck PJ, et al (2010) Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 33: 2285-2293

38. Young LH, Wackers FJ, Chyun DA, et al (2009) Cardiac outcomes after screening for asymptomatic coronary artery disease in patients with type 2 diabetes: the DIAD study: a randomized controlled trial. JAMA 301: 1547-1555

39. Oni ET, Agatston AS, Blaha MJ, et al (2013) A systematic review: burden and severity of subclinical cardiovascular disease among those with nonalcoholic fatty liver; should we care? Atherosclerosis 230: 258-267

40. Villanova N, Moscatiello S, Ramilli S, et al (2005) Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. Hepatology 42: 473-480

41. Yokoyama H, Yokota Y, Tada J, Kanno S (2007) Diabetic neuropathy is closely associated with arterial stiffening and thickness in Type 2 diabetes. Diabet.Med. 24: 1329-1335

42. Seah SA, Griffin MJ (2008) Normal values for thermotactile and vibrotactile thresholds in males and females. Int.Arch.Occup.Environ.Health 81: 535-543

43. Shun CT, Chang YC, Wu HP, et al (2004) Skin denervation in type 2 diabetes: correlations with diabetic duration and functional impairments. Brain 127: 1593-1605

44. Sumner CJ, Sheth S, Griffin JW, Cornblath DR, Polydefkis M (2003) The spectrum of neuropathy in diabetes and impaired glucose tolerance. Neurology 60: 108-111

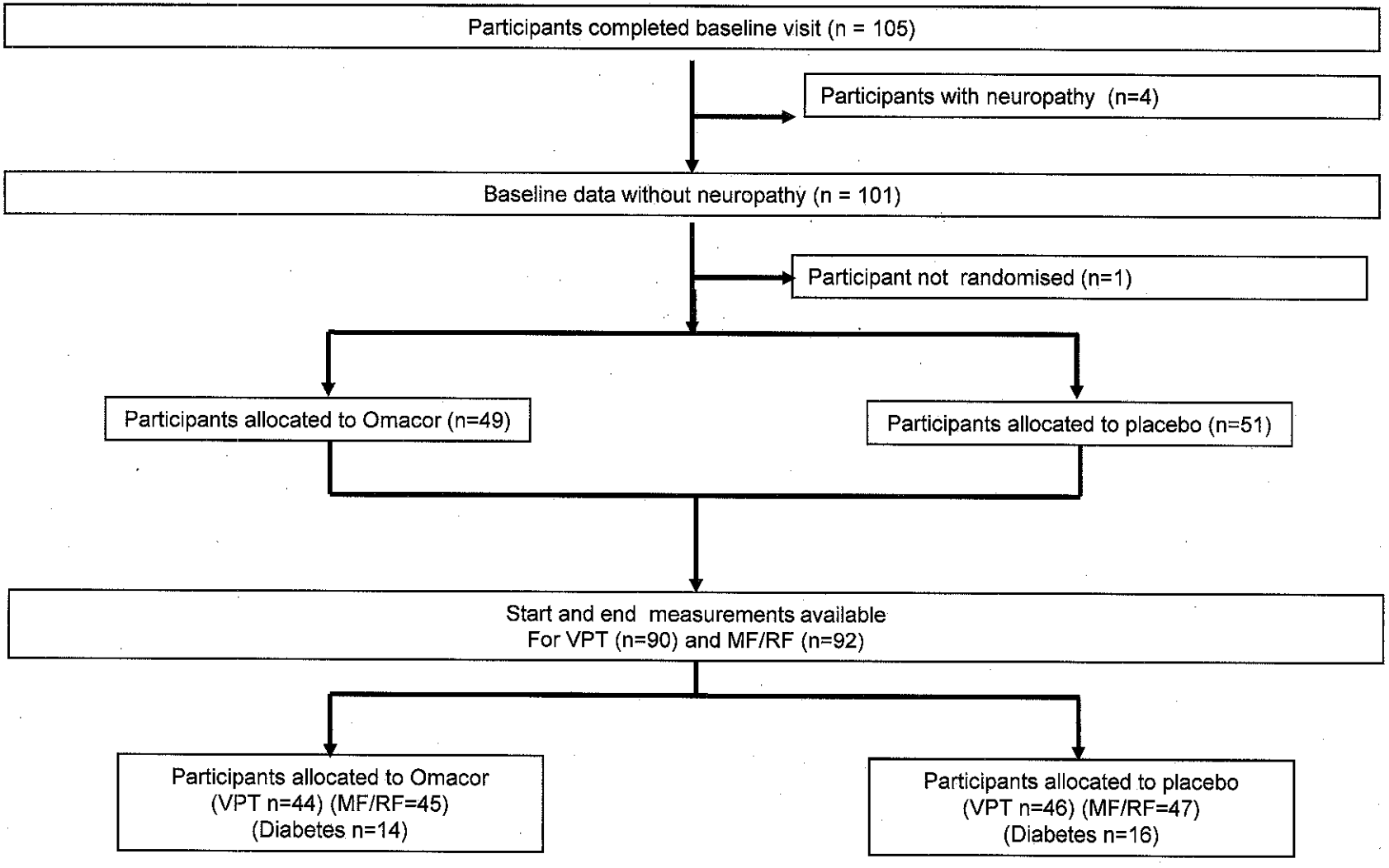
45. Sveen KA, Karime B, Jorum E, et al (2013) Small- and large-fiber neuropathy after 40 years of type 1 diabetes: associations with glycemic control and advanced protein glycation: the Oslo Study. Diabetes Care 36: 3712-3717

46. Byrne CD (2010) Fatty liver: role of inflammation and fatty acid nutrition. Prostaglandins Leukot.Essent.Fatty Acids 82: 265-271

47. Im S, Kim SR, Park JH, Kim YS, Park GY (2012) Assessment of the medial dorsal cutaneous, dorsal sural, and medial plantar nerves in impaired glucose tolerance and diabetic patients with normal sural and superficial peroneal nerve responses. Diabetes Care 35: 834-839

48. Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL (2012) Diabetic neuropathy: clinical manifestations and current treatments. Lancet Neurol. 11: 521-534

49. Corretti MC, Anderson TJ, Benjamin EJ, et al (2002) Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am.Coll.Cardiol. 39: 257-265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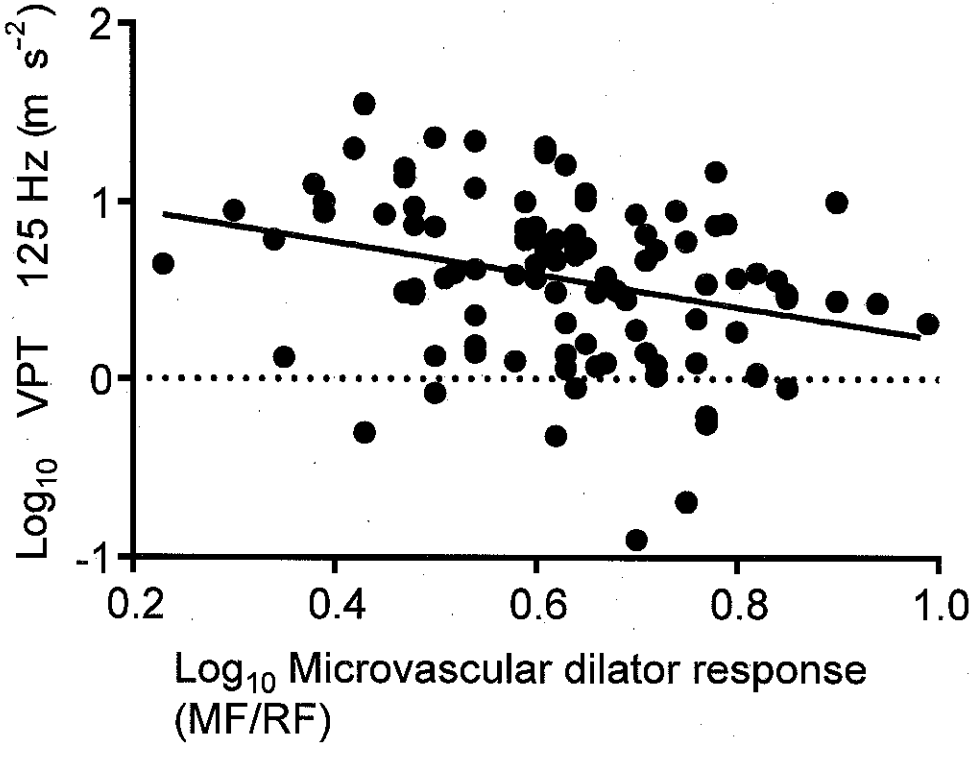
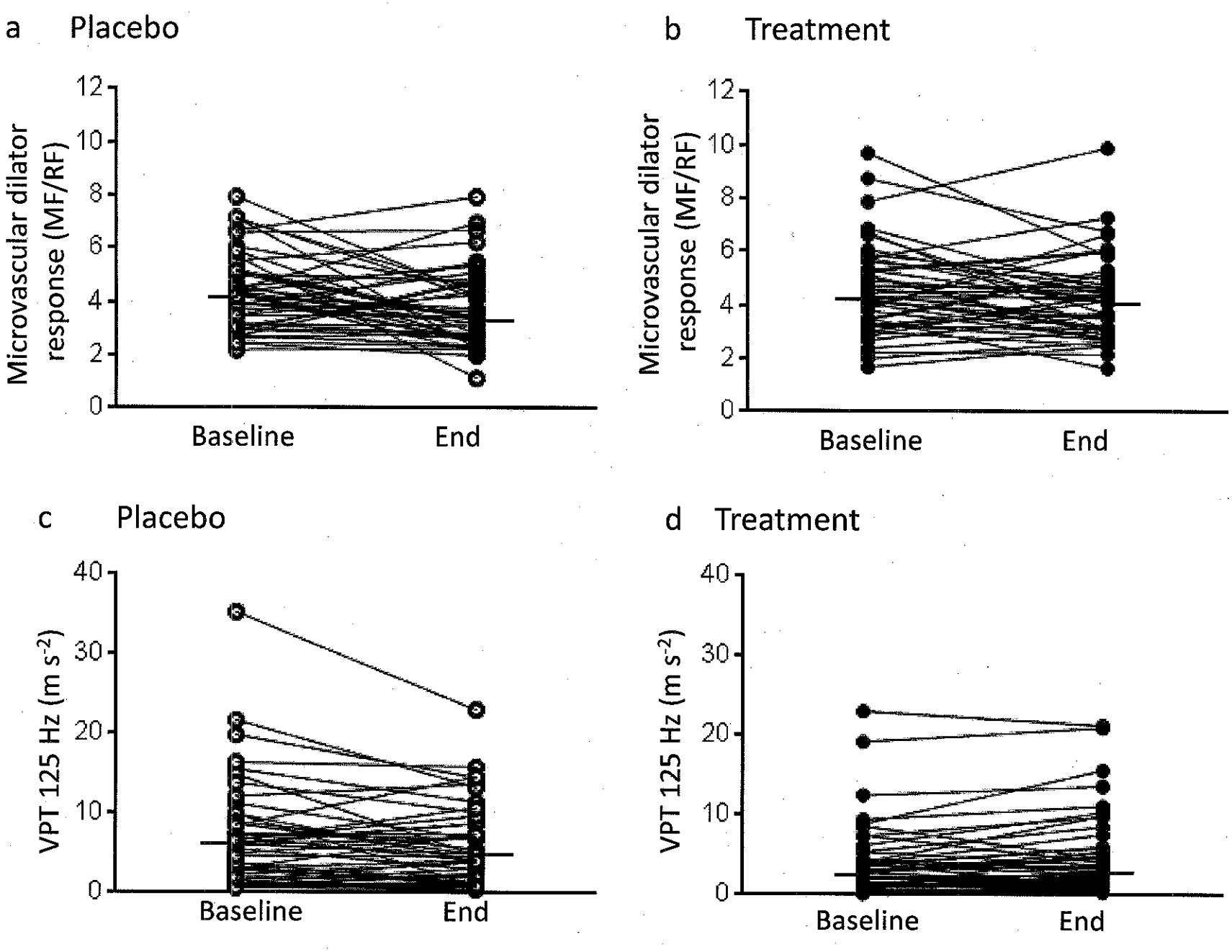
Participants with neuropathy (n=4)
Baseline data without neuropathy (n = 101)
Participant not randomised (n=1)
Participants allocated to Omacor (n=49)
Participants allocated to placebo (n=51)
Start and end measurements available
For VPT (n=90) and MF/RF (n=92)
Participants allocated to Omacor
(VPT n=44) (MF/RF=45)
(Diabetes n=14)
Participants allocated to placebo
(VPT n=46) (MF/RF=47)
(Diabetes n=16)


Log10 VPT 125 Hz (m s-2)
2
1
0
-1
0.2
0.4
0.6
0.8
1.0
Log10 Microvascular dilator response
(MF/RF)


a Placebo
b Treatment
c Placebo
d Treatment
Microvascular dilator response (MF/RF)
VPT 125 Hz (m s-2)
0
2
4
6
8
10
12
20
30
40
Baseline
End