Metformin Has No Impact on Nitric Oxide Production in Patients With Pre-diabetes

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Abstract

Background: The effect of metformin on vascular system is still not fully understood and the studies performed on animals have suggested possible impact on the nitric oxide (NO) bioavailability. Aim: The evaluation of different doses of metformin impact on NO production in people with pre-diabetes.

Methods: The metformin-naïve patients from one Diabetic Center with new-diagnosed pre-diabetes, without cardio-vascular diseases, were randomised (regarding to the identification number, individual for each inhabitant in the country) for treatment with different doses of metformin (group A 3 x 500 mg, group B 3 x 1000mg) for 15 weeks. The wide panel of L-arginine/NO pathway metabolites concentrations was assessed using the LC-MS/MS.

Results: Between October 2017 and December 2018, 36 individuals were initially randomised to intervention groups, but 25 accomplished the study: 14 patients in group A, 11 in group B; also 11 healthy volunteers were recruited. There was no difference between participants with pre-diabetes and healthy volunteers as regards the baseline characteristics except for fasting glucose and fatty liver. The decrease of L-citrulline concentration only was reported for treatment groups during the intervention period, with no change for the other NO-production related substances.

Conclusions: It was the first study on the *in vivo* release of NO in humans at different metformin doses, in patients with pre-diabetes. Metformin has no impact on NO production irrespective of the dose. The citrulline concentration change can indicate the drug impact on altered enterocytes' condition. The small number of patients is a limitation of the study.

Trial registration: ClinicalTrials; number: NCT03398356; retrospectively registered- date of registration:01 July 2018; https://register.clinicaltrials.gov/prs/app/action/SelectProtocol?sid=S0007ODV&selectaction=Edit&uid=U0003WJC&ts=4&cx=1y9eeb

Background

Metformin is widely used to treat diabetes mellitus and, in some countries, also pre-diabetic conditions. The medicine reduces blood glucose level but results from UK Prospective Diabetes Study (UKPDS) (1) it was suggested also, that metformin reduces cardiovascular (CV) risk, regardless of the hypoglycemic effect. The Diabetes Prevention Program (DPP), was the first to demonstrated, that metformin may have a beneficial effect also on coronary atherosclerosis in a pre-diabetic population (2). Since the pleiotropic effect of the drug has been proposed, some researches are carried out to clarify the mechanisms responsible for this effect (3, 4). The understanding how the drug works can not only explain previous observations but also contribute to the use of the molecule in other diseases. Flosins can serve as an example. They were initially used only as anti-glycemic drugs, and are now becoming increasingly applied by cardiologists for the protection of heart failure.

There are many disorders, typical for diabetes and pre-diabetes conditions, characterized by insulin-resistance and connected with higher CV risk in these groups of patients, that metformin can potentially correct. They are mainly involved in endothelial damage via oxidative stress and inflammation (5, 6). One of the proposed gripping point of the metformin, seems to be the effect of the molecule on the production of NO (7, 8).

Gluco- and lipo-toxicity, that are typical for insulin resistance, are responsible for reactive oxygen species (ROS) over-production, preceding injury of the vessels wall. Hyperglycemia evokes increased production of advanced glycation end-products (AGEs) and is responsible for increased expression of their receptors-Receptor for AGEs (RAGE). It leads to the accumulation of these products on the vessel's wall, as well as the alteration of its structure and function. One of the first sign of the endothelial failure is a decrease in endothelial NO synthase (eNOS) expression with lower nitric oxide (NO) release (9). Activation of protein kinase C (PKC), that is another glycemic-related factor, also decreases eNOS and finally NO production (10, 11). As the insulin can also modulate the activity of eNOS, insulin resistance even without the high glucose concentrations which characterised diabetes, can be responsible for improper NO release (12, 13). Furthermore the free fatty acids (FFAs) induce inflammatory response with the inhibition of eNOS and reduced NO production (14, 15) and it is well-known, that lipid disorder is also typical for insulin resistance. The next component of the metabolic syndrome which is hypertension, can damage endothelial cells directly and provoke its dysfunction (16), like probably many others, yet unknown, factors derived from bad habits, obesity/overweight and aging. Thus there are many components found in pre-diabetes that can contribute to poorer nitric oxide availability and consequently to a circulatory system malfunction (17, 18).

The nitric oxide is crucial for proper functioning of endothelium, and impairment of its secretion is connected with CV complications as the molecule is responsible for vasodilatation, having an impact on the tone of the vessel's wall and increasing blood flow (19). Several authors (20–22) reported the influence of metformin on NO bioavailability and release in rodents. The analysis of the connection between the adenosine monophosphate (AMP) activated protein kinase (AMPK) and the NO production (7, 8), was proposed to explain how the metformin, which affects the AMPK production, could protect the vessels. As the metformin improves insulin sensitivity, also this path should be considered with regard to the fact that a study on human showed relationship between eNOS, NO and insulin resistance (23). However, the direct impact of metformin on the process of the NO release in human is still not clear. Among the most popular approaches one can enumerate the assessment of NO and its bioavailability with the evaluation of intermediates in NO synthesis pathway and inhibitors of nitric oxide synthases (NOS) enzymes, the measurement of L-arginine and/or asymmetric dimethylarginine.

Methods

The study evaluated the effect of different doses of metformin on the intermediates of NO biosynthesis in people with pre-diabetes.

Patients were recruited from Diabetes Centre between October 2017 and December 2018, after the agreement (no: ST.C310.17.009) with Wroclaw Medical University Bioethical Committee was concluded, and patients' written consent was obtained. The full trial protocol, the flow-chart of the patients, as well as detailed inclusion and exclusion criteria can be accessed at ClinicalTrials.gov Registration webpage.

The metformin-naïve patients (age: 40–65 yo) with new-diagnosed pre-diabetes and without previously diagnosed cardiovascular diseases, were treated during three weeks with an increasing dose of the immediate-release (regular form) metformin to 3 × 500 mg, if no contraindication was present, and then randomly assigned to: group A – continuation of a dose of 3 × 500 mg or group B – dose increased to: 3 × 1000 mg (also by titration). After 12 weeks of the different dose treatment mentioned above, group B returned to the dose of 3 × 500 mg for the next three weeks. Thus the total treatment period lasted 15 weeks with a fixed dose of 3 × 500 mg for group A, and variable dose (with max dosage of 3 × 1000 mg) for group B. During titration period (by phone) and blood collection visits (personally), patients were asked about the compliance. They were also encouraged to contact in any case of the problem with metformin tolerance. If potential side effects appeared in group B, the patients could take a lower dose (3 × 500 mg, if well-tolerated) thus, being assigned to group A. If the smallest dosage (3 × 500 mg) was not well tolerated, the patient was excluded from the study.

The statistician and the team, responsible for biochemical and statistical analysis, had no information on the dose administered to patients (single-blinded study). The nurse randomised patients regarding to the identification number (ID): even or odd second digit, which is personal and individual for each inhabitant in the country.

Pre-diabetes was defined as impaired fasting glucose (IFG) or/and impaired glucose tolerance (IGT) according to the local, as well as the European Association's for the Study of Diabetes (EASD) criteria (24). The results of fasting glucose (FG) and oral glucose tolerance test (OGTT) had been obtained before the patient from treatment groups started the study.

Before the administration of the first dose of the metformin and after: 6, 12, 15 weeks of the treatment, the blood concentration of metformin, as well as the compounds related to the production of nitric oxide were assessed for group A and B. The wide panel of L-arginine/NO pathway metabolites (L-arginine, L-citrulline, ADMA – asymmetric dimethylarginine, DMA – dimethylamine, SDMA – symmetric dimethylarginine, ornithine) were assessed by a recently developed novel assay (25), using the liquid chromatography-mass spectrometry technique (LC-MS/MS).

The admissible delay in taking blood for lab-testing was set at four days. The blood samples were taken randomly (both: time and gaps between metformin administration were not defined).

The blood samples from matched healthy volunteers (group C) were taken once to compare the initial parameters as this group was not treated with metformin.

The basic parameters for all three groups (A,B,C) like: lipid profile, HbA1c etc. (Table no1) were obtained from patients' medical records and considered relevant, if they had been taken, up to six months earlier.

The specialistic parameters like metformin concentration or concentration of the compounds related to the production of nitric oxide were assessed in the laboratory of the Department of Medical Biochemistry at Wroclaw Medical University.

The Statistica 13 programme was used with cut-off point for statistical significance (P) at 0.05. In order to determine the significance, statistical tests were used in accordance with the distribution of variables and the nature of the data (Student t test, Welch's test, Mann-Whitney U test, chi – square test, the Wilcoxon signed-rank test, Kruskal – Wallis test, Friedman test, F test).

The funding source had no involvement in any part of the study conduction. The corresponding author confirms that had full access to all the data in the study and assumes final responsibility for the decision to submit it for publication.

Results

During the above mentioned period 36 individuals suffering from pre-diabetes gave their written consents to participate in the study, and were randomly assigned to group A or B. During the project period, 11 healthy volunteers, from the aforementioned practice, decided to participate as a control group. Finally, 25 individuals out of 36 patients assigned to the treatment groups finished the study because of formal or informal withdrawal of consent due to different reasons, or because of disqualification by the researcher due to unacceptable medicine side-effects. The reasons for discontinuing participation in the project were as follows: loss of contact with three participants, bad tolerance of the metformin (even the smallest dose) for three participants, and other reasons for five participants (e.g. medication regimen or the patient found the study schedule too much absorbing). Two patients (both women), formerly assigned to group B, were moved to group A, because of bad metformin tolerance after the dose was increased, according to their schedule (a few loose stools a day), which changed when the dose was lowered (to 3 × 500 mg). Thus 14 patients in group A and 11 individuals in group B accomplished the study. None of the patients who could be assigned to the groups, reported significant side effects of the drug and compliance to the metformin was assessed at the level of 100% in both groups, based on telephone relation or personal declaration during visits.

To compare the treatment groups (A, B) and healthy volunteers (C) regarding to the demographic parameters, as well as potential cardiovascular risk factors, the basic characteristic was done. There was no difference between the groups (A,B,C) according to the basic characteristics, except for the FG level which was higher and liver steatosis that appeared to be more frequent in patients with pre-diabetes when compared to healthy participants (p = 0,00001 and p = 0.003, respectively), (Table no 1). The concentration of the compounds related to the production of nitric oxide before the treatment was similar, comparing the patients with pre-diabetes (from both groups taken together) to healthy volunteers (A and

B vs C): for arginine p = 0.34; ADMA p = 0.13; SDMA p = 0.44; citrulline p = 0.88; DMA p = 0.78; ornithine p = 0.78.

There were no differences for metformin concentrations between groups: A vs B after 12 weeks (when groups differed as regards, the dosage) and after 15 weeks (when patients from both groups were taking again the same dose) of the therapy (p = 0.14 and p = 0.53 respectively). There was no difference between metformin concentration among patients from group A (p = 0.53), but there was a statistical significance among patients from group B when comparing the 6,12 and 15 weeks treatment period (p = 0.024), Table no 2.

There were no differences between groups A,B,C at the beginning of the therapy, and between groups A and B during the therapy period as regards the concentration of parameters involved in NO production: ornithine, L-arginine, ADMA, DMA and SDMA. Before the therapy, there were no differences among L-citrulline concentrations between all three groups (p = 0.22). The decrease of L-citrulline concentration was reported for both intervention groups during the treatment period, with significantly important change only for group A (p = 0.029). The concentration of these parameters, as well as the value of *p* are shown in Table no 3.

Discussion

Synthesis and bioavailability of nitric oxide – a free radical and a key vasodilator – are crucial for the proper functioning of vascular endothelium. The NO deficiency is a prerequisite for and a hallmark of endothelial dysfunction, which is a pathology preceding the development of cardiovascular diseases, that are under the influence of risk factors such as obesity, hypertension, and carbohydrate disturbances.

Despite the previous suggestions (7, 8, 20–22), that metformin can impact NO release, we have not shown this in our interventional study, which involved individuals with pre-diabetes. The metformin had no impact on NO release irrespective of the dose applied. To rule out the failure of a metformin administration, besides the patient verbal compliance declaration, the serum drug concentration was also determined, for the purpose of the study. Taking into consideration that our research was based on a small group of patients, what should be regarded as the limitation of the study, it was hard to find, the trends that can support the theory of the effect of metformin on changes in NO production *in vivo* in human. If such an impact would be found, the changes in all measured parameters would be observed.

The NO is synthesised by nitric oxide synthases from L-arginine with L-citrulline being the other product of the reaction. There are three isoforms of the enzyme: constitutively expressed endothelial (eNOS) and neuronal (nNOS) isoforms, and inducible isoform (iNOS). The activity of NOS enzymes is regulated by methylated derivatives of arginine. The asymmetric dimethylarginine is believed to be a strong, and symmetric dimethylarginine a weak enzyme inhibitor. Both ADMA and SDMA compete with L-arginine for transporters, and hence their accumulation decreases NO production by diminishing L-arginine availability for the NOS enzymes. ADMA and SDMA pool is regulated at the level of their synthesis, conducted by the protein arginine methyltransferases (PRMTs) and degradation. While ADMA is mostly

catabolised to L-citrulline and dimethylamine by dimethylarginine dimethylaminohydrolases (DDAHs), SDMA is preferentially excreted with urine. L-citrulline may be used in arginine-citrulline cycle to satisfy the body demand for L-arginine. In our study we have observed citrulline depletion which was statistically important only for group A, with a downward trend for group B. The trend, with no significant difference among group B during treatment time, may result from the fact that this group was smaller than group A which, with a small number of samples in general, could have affected the *p* value and thus needs further observation. Since there were no differences between the groups as regards the concentration of L-citrulline, it appears that it is affected by metformin intake in general and not its dose, although also in this case, a small number of samples may play a role. When considering the reasons for this change, for only one molecule of the urea cycle, we considered both the fate of this amino acid in the body and the potential impact of metformin on it. The citrulline is produced by intestinal mucosa (26) and is rather poorly present in food (27). Because of its unique metabolism, the plasma concentration of the citrulline was proposed as a reliable marker of gut function, with 20-micromol/L threshold for permanent intestinal failure in the patients with short bowel syndrome (28). The sensitivity, specificity and predictive value of the citrulline concentration for this disease were high (92%, 90%, 95% respectively), and as the authors summarised, their concentration was more reliable indicator for permanent vs transient intestinal failure differentiation when compared to the previously proposed anatomic variables (28). None of the patients who participated in our study suffered from significant, intestinal disorders. Thus in the case of citrulline depletion, which was found already after six weeks, from the moment when the participants started treatment with metformin, its occurrence suggests the impact of the medicine on altered enterocyte work. The previous studies have shown that metformin is taken up from the intestine by plasma monoamine transporter and organic cation transporter 3, and then it is transported into the bloodstream (about 50% orally administrated dose is absorbed) to finally target the tissue (29, 30). It was found that at the gut level, metformin may have an impact on glucose concentration by impairment of glucose uptake in the small intestine (31), but also alteration of the intestinal microbiome (32). The enterocytes are the first-line cells where the highest metformin concentration may be expected, what can explain the characteristic gastro-intestinal side effects of the drug (33). The impact of the drug on citrulline, the amino acid produced inside the enterocytes, which was observed in our study, can reveal the possible action of the metformin on these cells. As none of the patients declared significant side effects of the metformin, the role of the lowering of citrulline blood concentration during treatment, which probably derived from impaired enterocytes activity, should be the topic of research as it probably can reveal the citrulline as a marker of intestine “damage”. It is particularly interesting taking into account the fact that the citrulline concentration range that has been determined in our study (see Table no 3), was below the mentioned threshold for intestinal failure in patients with short bowel syndrome. Perhaps there is a threshold, that is lower than for individuals with bowel disease, which can explain side effect of the metformin in the patients who develop such a condition.

The maximum therapeutic (glucose lowering) effect of the drug is observed when about 2,000 mg/day is taken. Nevertheless, some authors indicate that a number of patients could respond better to higher doses (34, 35). Based on this information, we decided to compare different, extreme doses of the

medication based on the recommendations and with regard to the half-life-time of the regular form of the drug. As it was mentioned before, the assessment of the medicine concentration was useful to confirm that patients were taking the drug regularly, but could not explain its effect on the concentration of substances associated with the production of nitric oxide, which remained unchanged. Although the drug concentration at the level of 3 × 500 mg vs 3 × 1000 mg dose differed in our study, the distinction between the serum concentration of the drug at these doses (group A vs B) was insignificant. Given the small number of patients, this can be explained by the limited number of samples.

Our study has confirmed, demonstrated a few months ago in this year, the lack of metformin effect on the production of NO on mice (36), and additionally has discovered the possible mechanism of action of the drug at the level of enterocytes. The clinical aspect of the last observation requires clarification.

Conclusions

This is the first study which assessed in vivo release of NO in humans. Metformin had no impact on NO production irrespective of the dose. The citrulline concentration change can indicate the drug impact on altered enterocytes' condition.

Abbreviations

ADMA – asymmetric dimethylarginine

AGEs- advanced glycation end-products

AMP- adenosine monophosphate

AMPK- AMP activated protein kinase

CV-cardiovascular

DDAHs -dimethylamine by dimethylarginine dimethylaminohydrolases

DMA – dimethylamine

DPP- Diabetes Prevention Program

EASD- European Association for the Study of Diabetes

eNOS- endothelial NO synthase

FG- fasting glucose

FPG- fasting plasma glucose

ID- identification number

IFG- impaired fasting glycaemia

IGT- impaired glucose tolerance

iNOS-inducible NOS

LC-MS- liquid chromatography-mass spectrometry

NO- nitric oxide

nNOS- neuronal NOS

OGTT- oral glucose test tolerance

PKC- protein kinase C

PRMTs- protein arginine methyltransferases

RAGE–- Receptor for AGEs

ROS- reactive oxygen species

SDMA – symmetric dimethylarginine

UKPDS- United Kingdom Prospective Diabetes Study

Declarations

Compliance with Ethics Guidelines- The study was performed in accordance with the Helsinki Declaration of 1964, and its later amendments. The study was approved by the Wroclaw Medical University Ethics Committee (approval number: ST.C310.17.009). Written informed consent was obtained from all the participants.

Consent for publication- not applicable

The data will be available via ClinicalTrials website, as well as will be available from the corresponding author on reasonable request.

Conflict of interest: The principal researcher was a lecturer for the following companies: AstraZeneca, Sanofi, NovoNordisk, Merck, Boehringer Ingelheim, Mundipharma. For the rest of the authors there is no conflict of interest.

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The author's contribution: ES- conception and study design, collecting patients' data and data analysis, literature review, manuscript preparing; PF- laboratory tests, data analysis; BK- statistical analysis,

participation in manuscript preparing; KS- collecting patients' data, literature review, data analysis, participation in manuscript preparing; JW- participation in data analysis; AG-participation in study conception and data analysis, participation in manuscript preparing

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Authors information: ES- is a diabetologist and vascular medicine specialist and concerns mainly on prevention of the diabetes and vascular complications

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Tables

Table 1. Characteristics of the study groups.

Group	Metformin 3 x 500mg	Metformin 3 x 1000mg N= 11	Control	p
Parameter	N= 14		N= 11	
age: mean (years)	50,64	55,73	49,91	
M	50,50	57,00	49,00	0,18
range	40-64	43-66	41-64	
SD	8,85	8,27	8,37	
Sex/women (%)	5 (35,71)	3 (27,27)	6 (54,54)	0,40
Body mass:mean(kg)	96,71	92,36	80,82	
M	98,00	92,00	80	0,31
range	76-123	78-110	57-122	
SD	13,74	9,73	20,64	
[1]BMI: mean (kg/m2)	31,49	30,75	26,99	
M	30,85	30,40	27,60	0,38
range	24,60-40,20	25,3-37,20	19,6-35,20	
SD	5,26	4,00	5,43	
creatinine: mean(mg/dl)	0,86	0,89	0,73	
M	0,83	0,88	0,72	
range	0,61-1,20	0,59-1,16	0,71-0,78	0,21
SD	0,18	0,16	0,03	
[2]AlAt: mean(U/l)	28,13	35,45	19,11	
M	27,50	35	17	
range	8-61	14-86	6-40	0,16
SD	14,01	20,51	12,06	
[3]TCL: mean (mg/dl)	207,21	184,91	201,00	
M	191	181	207	0,42
range	146-323	133-237	146-239	
SD	50,22	34,19	27,47	
[4]TG: mean (mg/dl)	133,29	136,82	137,09	

M	102	110	130	0,81
range	55-242	72-301	73-242	
SD	62,77	66,63	49,20	
[5]LDL: mean (mg/dl)	125,29	106,95	115,70	
M	123,8	105	124	0,22
range	92,4-207	81-157	83,4-139	
SD	30,79	23,78	19,91	
[6]HDL: mean (mg/dl)	49,86	50,73	57,91	
M	48,5	53	61	0,56
range	34-80	32-74	38-83	
SD	12,07	14,35	16,82	
[7]FPG: mean (mg/dl)	109,39	107,23	88,81	
M	104,5	105,7	88	0,00001
range	90,8-125,6	100,2-119,2	79,5-95,4	
SD	11,13	5,95	4,44	
Liver steatosis:				
N (%)	7 (50)	9 (81,82)	1 (9,09)	0,003
Nicotinism:				
N (%)	3 (21,43)	4 (36,36)	2 (18,18)	0,57
Hypertension:				
N (%)	7 (50)	6 (54,55)	3 (27,27)	0,38
Family history for diabetes:				
N (%)	7 (50)	4 (36,36)	3 (27,27)	0,5

[1]BMI-body mass; [2]AlAt-alanine transaminase, [3]TCL-total cholesterol level, [4]TG-triglycerides, [5]LDL-low density lipoprotein, [6]HDL-high density lipoprotein, [7]FPG-fasting plasma glucose;

Table 2. Metformin concentration change during treatment.

Metformindosage Group	3 x 500 mg after 6 weeks treatment (titration + full dose)	→3 x 1000 mg after next 6 weeks treatment with (group B) or without (group A) dosage increasing (12 weeks of total treatment)	3 x 500 mg after next 3 weeks	p
A, N=14				
mean (µM)	4,36	5,09	4,66	
M	4,42	5,03	4,85	0,53
range	1,61-9,53	1,67-8,09	0-15,10	
SD	2,36	2,29	3,73	
B, N=11				
mean (µM)	4,25	7,42	4,01	
M	4,82	8,44	3,42	0,024
range	0-9,26	0-14,90	0-10,80	
SD	2,58	4,77	4,01	

Table 3. Change in parameter levels during treatment depending on the metformin dose.

Group	Metformin 3 x 500mg	Metformin 3 x 1000mg	Control	p
Parameter(μM)	N=14	N=11	N=11	
L-Arginine1, mean	114,29	110,37	122,11	0,58
M	113,25	104,20	110,74	
Range	79,37-161,40	68,9-169,28	94,51-192,18	
SD	28,15	27,63	27,96	
L-Arginine2, mean	112,18	111,72	No intervention	0,73
M	89,10	105,43		
Range	67,24-255,64	67,44-185,45		
SD	53,42	36,89		
L-Arginine3, mean	107,72	97,69	No intervention	0,37
M	106,29	82,45		
Range	76,97-130,89	51,55-184,27		
SD	13,93	38,76		
L-Arginine4, mean	104,72	103,76	No intervention	0,94
M	95,07	106,07		
Range	63,76-168,56	67,10-142,04		
SD	34,21	19,76		
p	0.52	0.69		
[1]ADMA1, mean	0,51	0,53	0,56	0,23
M	0,47	0,52	0,55	
Range	0,43-0,71	0,42-0,77	0,44-0,77	
SD	0,09	0,09	0,10	
ADMA2, mean	0,51	0,57	No intervention	0,18
M	0,47	0,57		
Range	0,41-0,79	0,41-0,84		
SD	0,11	0,11		
ADMA3, mean	0,52	0,55		

M	0,50	0,52	No intervention	0,73
Range	0,39-0,72	0,39-0,92		
SD	0,09	0,14		
ADMA4, mean	0,50	0,52		
M	0,49	0,49		0,64
Range	0,40-0,68	0,37-0,71	No intervention	
SD	0,08	0,10		
p	0,92	0,2		
^{2}SDMA1, mean	0,39	0,41	0,42	
M	0,38	0,37	0,44	0,61
Range	0,29-0,50	0,29-0,59	0,31-0,52	
SD	0,07	0,10	0,08	
SDMA2, mean	0,40	0,45		
M	0,41	0,44	No intervention	0,18
Range	0,28-0,50	0,34-0,62		
SD	0,08	0,08		
SDMA3, mean	0,41	0,42		
M	0,39	0,39	No intervention	0,93
Range	0,3-0,56	0,31-0,62		
SD	0,08	0,08		
SDMA4, mean	0,39	0,39		
M	041	0,39		
Range	0,24-0,49	0,30-0,53	No intervention	0,98
SD	0,07	0,06		
p	0,41	0,12		
^{3}DMA1, mean	1,77	1,74	1,67	
M	1,54	1,57	1,47	
Range	1,07-3,24	1,12-2,60	1,27-2,32	0,85
SD	0,71	0,48	0,40	

DMA2, mean	1,71	2,07		
M	1,75	1,94	No intervention	
Range	1,17-2,09	1,43-3,16		0,05
SD	0,31	0,52		
DMA3, mean	1,63	1,89		
M	1,55	1,85	No intervention	
Range	1,35-2,36	1,41-2,67		0,10
SD	0,28	0,43		
DMA4, mean	1,62	1,84		
M	1,59	1,64		
Range	1,04-2,30	1,06-3,64	No intervention	
SD	0,34	0,68		0,56
p	0,5	0,12		
Citrulline1, mean	34,10	38,33	36,45	
M	31,56	37,05	34,80	
Range	22,41-52,47	25,57-53,66	24,38-52,73	0,22
SD	9,84	7,49	8,82	
Citrulline2 mean	21,73	28,08		
M	21,57	27,93	No intervention	
Range	11,61-36,33	11,11-50,45		0,10
SD,	6,66	11,31		
Citrulline3 mean	25,95	27,01		
M	25,45	19,37	No intervention	
Range	12,81-46,81	13,68-52,93		0,84
SD	9,48	15,38		
Citrulline4, mean	26,93	29,77		
M	26,24	33,03	No intervention	
Range	13,62-43,81	11,28-48,71		0,50
SD	9,71	11,26		

p	0,029	0,14		
Ornithine1, mean	34,77	37,12	35,57	0,92
M	34,89	37,20	32,61	
Range	13,62-60,19	11,59-76,23	19,66-53,54	
SD	11,58	15,71	11,09	
Ornithine2, mean	34,28	38,84	No intervention	0,26
M	34,23	37,23		
Range	16,87-48,97	28,64-61,81		
SD	9,80	9,58		
Ornithine3, mean	31,92	34,41	No intervention	0,52
M	30,11	30,76		
Range	23,45-45,84	20,33-61,64		
SD	6,75	11,42		
Ornithine4, mean	34,09	34,49	No intervention	0,85
M	34,49	33,88		
Range	25,45-43,40	14,49-53,32		
SD,	4,43	10,62		
p	0,69	0,41		

[1]ADMA-asymmetric dimethylarginine; , [2]SDMA-symmetric dimethylarginine;[3]DMA-dimethylamine