ANTI-COLLAGEN TYPE IV ANTIBODIES AND THE DEVELOPMENT OF MICROVASCULAR COMPLICATIONS IN DIABETIC PATIENTS WITH ARTERIAL HYPERTENSION

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SUMMARY

Background and Aims: Thickening of basement membrane in capillaries and small vessels is a well-known finding and important in the progression of diabetic microangiopathy. Patients with diabetes mellitus and arterial hypertension are at higher risk of vascular disease.

Material and methods: To monitor the metabolism of the basement membrane protein collagen type IV (CIV) in type 2 diabetes mellitus (T2DM), serum levels of antibodies to CIV (ACIV) IgG, IgM and IgA were measured using an ELISA method in 93 patients with type 2 diabetes mellitus and arterial hypertension (AH) (mean age $61,4\pm11,3$ years, diabetes duration $9,88\pm3,12$ years; hypertension duration $9,28\pm4,98$). These values were compared to serum antibodies to CIV in 42 age and sex matched controls. Diabetics were divided in two groups according to presence- Group 1 (n=67) or absence- Group 2 (n=26) of microangiopathy.

Results: Patients with T2DM and AH showed statistically significant higher levels of ACIV IgG in comparison to healthy controls (0.30 ± 0.12 vs. 0.21 ± 0.08) (p=0.0001). Group 1 showed significantly hihger levels of ACIV IgG than Group 2 (0.32 ± 0.13 vs. 0.24 ± 0.08) (p=0.009) and healthy controls (0.32 ± 0.13 vs. 0.21 ± 0.08) (p=0.0001). ACIV IgG are statistically significant higher in diabetics with retinopathy than this without (0.33 ± 0.10 vs. 0.26 ± 0.13) (p=0.04). ACIV IgG correlates with diabetes duration (r=0.49); (p=0.0004), retinopathy (r=0.20); (p=0.05) and BMI (r=-0.24); (p=0.05). Serum ACIV IgM and IgA levels in patients with T2DM and AH were lower than these in controls, but the differences are not statistically significant.

Conclusion: Our study showed a relationship between elevation of serum levels of ACIV IgG in diabetics and development of microangiopathy.

Key words: Diabetes mellitus, arterial hypertension, anti-collagen type IgG, micrangiopathy

INTRODUCTION

Clinical manifestations of microvascular and macrovascular involvement in diabetes include retinopathy, nephropathy and accelerated atherosclerosis, possible leading to blindness, renal failure, myocardial infarction, stroke and limb amputation. These target organ manifestations show common pathological characteristics underlying all vascular diseases; however, each complication also has some specific features depending on the tissue (s) involved, with important implications for prevention and treatment Mario and Pugliese, 2001 [1]. This diversity is supported by the distinct epidemiological characteristics of vascular complications, which occur with a different prevalence in diabetic subjects Toeller et al. 1999, Laasko 1999 [2]. The incidence of vascular complications also varies with the site involved. Macroangiopathy is sometimes present at the time of diagnosis, particularly in type 2 (non-insulin-dependent) diabetes mellitus [2], whereas microvascular disease usually developed several years after the onset of diabetes Krolewski et al. 1987 [3], Hanefeld et al. 1996 [4]. However, while the incidence of retinopathy increases with diabetes duration, that of nephropathy reaches a peak after about 15 years and declines thereafter. The rate of development and the severity of vascular injury are also dependent on the individual background; as a result, some people show only initial lesions, whereas others progress towards end-stage disease, despite a similar degree of metabolic derangement.

Arterial hypertension and diabetic vascular complications are connected with an elevated degradation of elastic tissue. As a result collagen type IV (CIV) are released in the circulated blood, which are a pathological stimulus for an increased production of antibodies to CIV (ACIV). Because it is very important to find characteristics of pathological activation of collagen turnover in diabetics we studied diabetic patients with arterial hypertension who demonstrated vascular complications.

In diabetes mellitus, thickening of basement membrane in capillaries and small vessels is a well-known finding and important in the progression of diabetic microangiopathy. Collagen type IV is uniquely present in basement membranes and represents their predominant structural element Paulsson 1992 [5], Kuhn 1995 [6]. Metabolic alteration of CIV occurs in micro- or

macrovascular basement membrane of diabetic patients.

Elastin and collagen are the main proteins of vascular wall. An important factor in the development of vascular wall alterations is degradation of the extracellular matrix's major protein – collagen Rosenbloom et al., 1993 [7]. Basement membranes regulate the functions of many cells and mediate the interactions between different tissues Paulsson, 1992 [5]; Kuhn 1995 [6]. Collagen type IV constitutes the major component of basement membranes Schuppan and Riecken, 1990 [8]. Measurement of serum antibodies to fragments of CIV Balashova et al., 2000 [9]; Nicoloff et al., 2002 [10] is now possible and enables changes to be detected.

Because collagen type IV is uniquely present in basement membranes and represents their predominant structural element [6, 8] several immunological methods for detection of circulating CIV were developed Risteli L. Et al. 1987 [11]. The detection of circulating CIV in the serum of diabetic subjects Alland A , Matsumoto E, Nicoloff G [12, 13, 14] shows that the CIV macromolecule is not fully degraded by collagenases in vivo. These CIV may become a stimulus for immunocompetent cells and result in the production of anti-CIV antibodies.

There is data for detection of serum CIV in animals or humans with arterial hypertension Hasslacher et al., 1987 [15]; Yano et al., 1997 [16]. These CIV fragments may become an increased stimulus for immunocompetent cells for pathological production of anti-CIV antibodies.

Investigators Susumu et al, 2003 [20] measured serum anti-type IV collagen antibody (IgG) in diabetic patients and they found that serum levels of the anti-type IV collagen antibody were significantly higher in diabetics than these in the nondiabetics. However, there was no relationship between the levels of urinary albumin and the serum levels of anti-type IV collagen antibody.

We investigated anti-collagen type IV antibodies in children with essential hypertension and patients showed significantly higher levels of collagen type IV IgM while collagen type IV IgG levels were lower than these in controls Nicoloff et al, 2006 [21].

In the present investigation we studied whether the serum levels of antibodies (IgG, IgM and IgA) to type IV collagen are related with the presence of diabetic microangiopathy. This study establishes normal serum levels of ACIV in healthy subjects and compares them to these of patients with diabetes mellitus and arterial hypertension. The changes observed are correlated with diabetic microvascular complications.

MATERIAL AND METHODS
Subjects
The experimental group consisted of 93 patients (37 men, 56 women) with type 2 diabetes mellitus and arterial hypertension (mean age 61,4±11,3 years, diabetes duration 9,88±3,12 years; hypertension duration 9,28±4,98). These values were compared to serum antibodies to CIV in 42 age and sex matched controls with no family history of diabetes, atherosclerosis or emphysema. The controls were equally distributed to match the diabetic ages. Diabetics were divided in two groups according to presence- Group 1 (n=67) or absence- Group 2 (n=26) of microvascular complications (Table 1). Group 1 cosisted of 39% men and 61% women. Fifty-five percent were smokers and 45% non-smokers. Group 2 consisted of 42% men and 58% women. Fifty-eight percent were smokers and 42% non-smokers. Controls consisted of 45% men and 55% women. Twenty-seven percent were smokers and 73% non-smokers (Table 2). Microalbuminuria was defined as a persistent urinary albumin excretion rate (AER) in the range of 20 and 200 µg/min in sterile urine. None of the patients had a diagnosis of renal disease unrelated to diabetes during the follow up.

Clinical draft
All patients were residing in the vicinity of the Pleven University Hospital. Subjects' sera were taken from October 2011 to May 2012. Ethical approval was obtained from the local research ethics committee and all subjects gave an informed written consent prior to the study.

Procedure
Serum antibodies (IgG, IgM and IgA) to CIV were measured by an enzyme-linked immunosorbent assay (ELISA). In brief, each well of the microtiter plate was sensitized with 100 µl of 10 µg/ml of human CIV (SIGMA, USA) at room temperature for 3 h, followed by an overnight incubation at 4oC. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA, SIGMA, USA). Then, 100 µl serum sample (diluted 1:10), was placed in each well of a microtiter plate, and incubated for 1 h at 37oC. After washing three times, 100 µl of immunoconjugates (anti-human immunoglobulin peroxidase conjugates (SIGMA, USA) to heavy chain of IgG, IgM and IgA) were added to each well for 1 h at 37'C. All immunoconjugates were diluted 1:10,000 with PBS containing 1% BSA and 0.05% Tween 20. The plate was incubated for 1 h at 37oC. o-Phenylenediamine (0.4 mg/ml) was added to citrate buffer, and 100 µl of this solution was added to each well and allowed to react for 30 min. The reaction was stopped by adding 50 µl 4 M H_2SO_4 to each well and the optical density was measured with a Microelisa Reader 210 (Organon Teknika, Belgium) at a wavelength of 492 nm. Anti-CIV levels greater than the mean±2SD of the healthy controls were designated positive.

Ophthalmoscopy through dilated pupils was carried out in all patients with diabetes mellitus to assess the presence of retinopathy; all patients were examined by the same ophthalmogist. Hemoglobin A1c was measured using high-pressure liquid chromatography (normal range 4-6%). Total serum cholesterol and triglyceride concentrations were measured by enzyme assay (Boehringer Mannheim, Mannheim, Germany). Arterial blood pressure was measured

using a standard mercury sphygmomanometer, to the nearest 2 mm Hg, in the dominant arm after at least 10 min rest in the supine position. AER was determined by nephelometry using a commercial kit containing specific antibody (Behringwerke, Marburg, Germany).

Statistical analyses

All values are expressed as mean±SD. Statistical analyses were done using the computer programs EXCEL and STATGRAPHICS plus for WINDOWS. The Student t-test and ANOVA were used to assess the differences between groups. Correlation and regression analyses were also used. The level of significance was determined as $p < 0.05$.

RESULTS

Patients with T2DM and AH showed statistically significant higher levels of ACIV IgG in comparison to healthy controls (0.30±0.12 vs. 0.21±0.08) (p=0.0001). Group 1 showed significantly hihger levels of ACIV IgG than Group 2 (0.32±0.13 vs. 0.24±0.08) (p=0.009) and healthy controls (0.32±0.13 vs. 0.21±0.08) (p=0.0001). Group 2 showed higher levels of ACIV IgG than controls (0.24±0.08 vs. 0.21±0.08) (p=0,05). ACIV IgG correlates with diabetes duration (r=0.49); (p=0.0004), retinopathy (r=0.20); (p=0.05) and BMI (r=-0.24); (p=0.05) (Table 3, Fig. 1). All diabetic patients (n=93) were divided in two subgroups depending on data for diabetic retinopathy: patients with retinopathy (n=20) and patients without retinopathy (n=73) according to European Association for the Study of Diabetes (EASD) Classification. Levels of ACIV IgG are statistically significant higher in diabetics with retinopathy than this without (0,33±0,10 vs. 0,26±0,13) (p=0.04) (Table 4, Fig. 2).

Serum ACIV IgM and IgA levels in patients with T2DM and AH were lower than these in controls, but the differences are not statistically significant (Table 5 and 6).

DISCUSSION

Epidemiological studies have shown a strong relationship between obesity and cardiovascular disease, and between obesity and specific cardiovascular risk factors such as hypertension and diabetes mellitus. In diabetic patients morbidity and mortality are mainly related to the presence of late complications, namely macro- and microangiopathy. Diabetes mellitus (both Type 1 and Type 2) is a major risk factor for cardiovascular disease. In the Framingham Study, the risk of cardiovascular disease was doubled by the presence of diabetes The Framingham study 1979 [17].

Collagens are known to be immunogenic in animals, and antibodies to various collagens are present in human serum in several diseases, particularly those of autoimmune origin Moreland et al., 1991 [18]; Yang et al., 1993 [19]; Daskalova et al., 1997 [22]. The detection of serum CIV in animals or humans with arterial hypertension Hasslacher et al., 1987 [15]; Yano et al., 1997 [16] shows that CIV macromolecule is not fully degraded by collagenases *in vivo*.

These CIV fragments may become an increased stimulus for immunocompetent cells for pathological production of anti-CIV antibodies. Changes in the levels and pattern of antibodies to CIV may occur with time.

Type IV collagen, which is one of the major constituents of basement membranes and fibrous tissue, is composed of a 7S collagen domain formed by four aminoterminal ends linked together in an antiparallel arrangement, and a globular carboxyterminal cross linking domain Sato et al., 1997 [23]. Four or five collagen molecules, lenghtwise disposed and linked with glycoproteins, form collagen microfibrile Stanley RG, 2009 [24]. Collagen catabolism is not fully studied rather than its synsthesis Labasse A, 2007 [25]. Collagen catabolism is a complex and multistage process, running not only at intracellular level, but also at extracellular matrix. Intracellular collagen destruction starts with activation of highly specific collagenase, which interacts with collagen molecules Weis et al, 2001 [26].

The object of the study of Yano et al. 1997 [16] was to evaluate the serum 7S domain of type IV collagen (7S-collagen) levels in patients with essential hypertension and in type 2 diabetic patients with or without hypertension and to investigate the relationship between the type IV collagen metabolism and the arterial blood pressure. Serum 7S-collagen levels in 18 patients with essential hypertension were significantly higher than in 24 normal subjects. There was a significant correlation between the serum 7S-collagen levels and the systolic blood pressure in cases with essential hypertension. In a recent study we measured levels of ACIV antibodies in patients with diabetes mellitus and arterial hypertension. Patients with microvascular complications showed significantly higher levels of ACIV IgG in comparison with other groups. Patients with type 2 diabetes mellitus are at high risk of diabetic microangiopathy and vascular disease. The angiopathic complications usually developed after several years of type 2 diabetes Klein et al. 1984 [27]. In the present study, we found a significant relationship between serum levels of ACIV IgG and development of diabetic retinopathy.

One possible explanation is that the first immunoglobulin synthesised during the early phase of a pathologically activated immune response is IgM. The immune system then switches on to producte IgG. The elevation of ACIVAbs of the IgM is therefore the first indicator of the pathological turnover of collagen and the development of microvascular complications. Since the cells responsible for the cell-mediated immune response (phagocytes, T-effector cells, NK-cells, etc) have more receptors for the Fc-fragments of IgM, they will react preferentially with immobilized and soluble immune complexes formed with IgM ACIV Abs. The presence of ACIV Abs could lead to a variety of pathological processes including immune complexes formation.

We found that ACIV IgG are statistically significant

higher in diabetics with retinopathy. Probably, patients developed microangiopathy before our detection and they are not in the very early phase of microvascular disease. The elevation of anti-CIV of IgG type is therefore the next indicator of the pathological turnover of CIV.

There is evidence that proteins, including immunoglobulins, accumulate in the matrix lining the walls of small blood vessels in diabetic subjects Brownlee et al. 1983 [28]. IgG binds preferentially to basement membranes, which contain AGE Brownlee et al. 1993 [29]. Prolonged hyperglycaemia results in irreversible cross-linkage of collagen containing AGE Airaksinen et al. 1993 [30]. Immunoglobulins bind to collagen-AGE complex, increasing attachment sites for other proteins and expanding the matrix. Macrophages react with new epitopes on CIV molecule (CIV with AGE) and release monokines, tumor necrosis factor and interleukin-1. The last is an increased non-specific pathological stimulus for production of more anti-CIV IgG, which will react with the new epitopes and may be, will cross-react with "normal "epitopes of CIV molecule. Glycation of basement membrane CIV is a major process leading to diabetic microvascular complications. Increased synthesis or increased degradation could cause the elevated serum levels of CIV which are strong pathological stimulus for production of specific antibodies found in our study, mainly in the patients with retinopathy.

S. Tisheva founds that in pre-hypertension patients compared to healthy persons, quantities of elastin degradation peptides were significantly increased. This is a character of an accelerated elastolisa, and the changes happen before hypertension determination. In modarate arterial hypertension persons, elastin turnover is disbalanced. Increased EDP quantities and high levels of anti-elastin antibodies can be stated. AH patients compared to pre-hypertension patients and controls, the constellation of significantly higher EDP quantities and IgA-antielastin antibodies levels, of IgA-collagen type IV antibodies, of IgG collagen type I and of IgG collagen type III antibodies, is a destroyed integrity sign of fibril structures of vessel wall, 2004 [34].

E. Mekenyan investigates elastin and collagen turnover in patients with metabolic syndrome and founds correlation between anti-elastin, anti-tropoelastin and collagen type IV IgG antibodies and serum triglycerides levels and correlation between anti-AGEs IgG and IgM and triglycerides, which prooves association between vascular aging and athergenic dyslipidemia, characterising metabolic syndrome, 2012 [35].

In our study we found increased serum levels of anti-CIV IgG in diabetics with retinopathy. These findings are supported by the results of Balashova et al. 2000 [11] among patients with preclinical, nonproliferative, preproliferative, and proliferative diabetic retinopathy. The authors found that diabetic retinopathy is characterized by a notable increase in antibody-dependent immune response, associated with appearance of antibodies to collagen of the II and IV types in the lachrymal fluid and serum, with the "local" reactions predominating. The level of reactions of cellular autoimmune response (tumor necrosis factor-alpha) and cell-to-cell reactions in the lachrymal fluid and serum was low. According to the authors increased level of circulating immune complexes in the serum and almost complete absence of free antibodies to collagen in the blood may be indicative of formation of pathogenic immune complexes precipitating on vascular walls and in other tissues.

ACIV IgG antibodies showed a significant relationship to diabetes duration and BMI. The elevated mean levels of triglycerides and total cholesterol, out of the normal range, found during the study correlate with the development of diabetic vascular complications as described Steiner, 1997 [31]. It is well known that hypertriglyceridaemia is the most common abnormalities in poorly controlled diabetes.

In our study it was not possible to detect the "active" phase of vascular disease, because of patients' diabetes duration 9,88±3,12 years. During this "active" phase the levels of IgM ACIV were probably elevated, while during the chronic phase the levels of this type of ACIV antibody were decreased and only the level of ACIV IgG was increased.

Our study showed a relationship between serum levels of ACIV IgG and development of diabetic retnopathy. The presence of ACIV antibodies could lead to a variety of pathological processes including immune complexes formation, complement activation and K cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) activation, all of which may contribute to the progression of collagen destruction of arterial wall. Furthermore, collagen antigen present at the damaged arterial sites retains selectively antigen specific T- Peterszegi et al. 1997 [32, 33] and B-lymphocytes which would aggravate the response.

Identification of markers would allow more aggressiveness in the achievement of tight glycaemic control and search for initial lesions in those patients who are predisposed to complications than in those who are not. The understanding of the relation between hyperglycemia, the altered pattern of expression of various mediators and the dysregulated vascular remodeling at the level of the target tissue would allow design of therapeutic approach targeted to the individual tissue and to the relevant biochemical, functional and structural alterations.

In conclusion, our results suggest an association between levels of ACIV IgG and the development of vascular wall lesions. Elevation of ACIV IgG may indicate increased collagen degradation and development of microvascular complications. However, a larger study is necessary for clarification of these possibilities.

Table 1. Clinical data of patients with T2DM and AH

CLINICAL DATA	Group 1	Group 2	Controls
Age	62,5±12,58	60,4±8,4	58,9±7,56
Gender (M/F)	26/41	11/15	10/12
Mean diabetes duration	9,30±5,36	9,16±7,59	N/A
Mean hypertension duration	9,50±7,63	8,68±7,26	N/A
HbA1c	*7,63±2,03	7,27±1,63	N/A
SBP (mmHg)	142,83±18,05	140,58±20,51	114,29±15,74
DBP (mmHg)	82,23±11,52	81,35±11,96	72,5±10,4
BMI	29,62±4,99	28,42±3,96	22,61±2,27
TCL (mmol/l)	*5,26±1,40	5,18±0,93	3,99±0,65
HDL (mmol/l)	*0,88±0,30	0,93±0,30	0,96±0,20
LDL (mmol/l)	3,18±1,19	3,16±1,09	2,43±0,64
TGL (mmol/l)	2,91±1,68	2,53±1,49	1,31±0,61
Insulin dose (U/kg/24h)	2,57±0,52	2,03±0,93	N/A
MAU (μg/min)	*78,94±52,87	8,53±4,69	N/A
MAU	**(n=43)**	-	
Retinopathy	**(n=20)**	-	
Neuropathy	**(n=4)**	-	
Smokers	37/67	15/26	16/42
Number	**67**	**26**	**42**

Group 1- patients with microvascular complications (n=67); Group 2- patients without microvascular complications (n= 26);   Controls (n=42)   **All Data are Mean ±SD**

Table 2. Percentage of smokers on groups

Group 1		Group 2		Controls	
Men	Smokers	Men	Smokers	Men	Smokers
39%	55%	42%	58%	45%	27%
Women	Non-smokers	Women	Non-smokers	Women	Non-smokers
61%	45%	58%	42%	55%	73%

Table 3. Serum ACIV IgG in patients with T2DM and AH

Groups	ACIV IgG (ng/ml)		Comparison	
	Mean ±SD	Group 1	Group 2	All diabetics
All diabetics	0.30±0.12	NS	P=0.01	-
Group 1	0.32±0.13	-	P=0.009	NS
Group 2	0.24±0.08	P=0.009	-	P=0.01
Controls	0.21±0.08	P=0.0001	P=0.05	P=0.0001

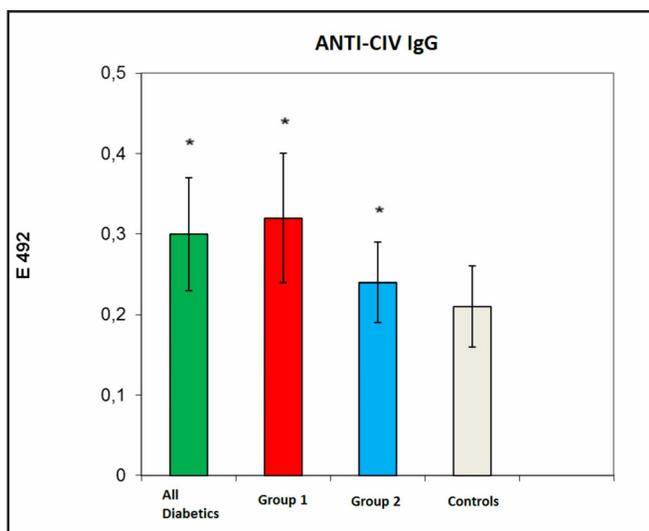**Fig. 1.** Serum ACIV IgG in patients with T2DM and AH

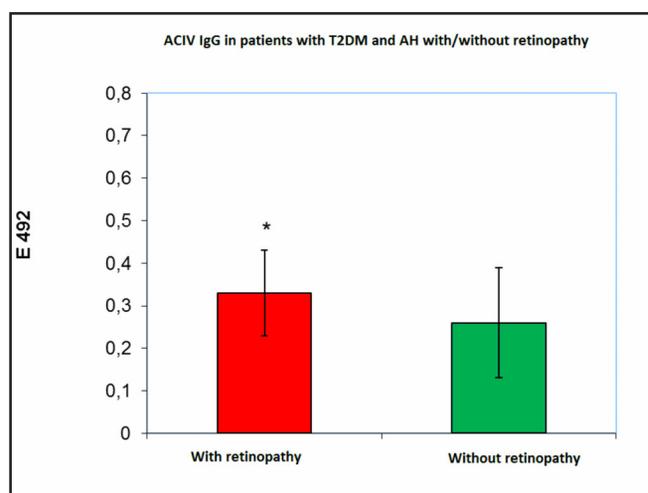**Fig. 2.** ACIV IgG are statistically significant higher in diabetics with retinopathy than this without (p=0.04).

Table 4. Serum ACIV IgG in patients with T2DM and AH. Patients are divided in two subgroups depending on data for diabetic retinopathy

	ACIV IgG (ng/ml)	Comparison	
		(With)	(Without)
With retinopathy (n=20)	0.33±0.10	-	P=0.04
Without retinopathy (n=73)	0.26±0.13	P=0.04	-

Table 5. Serum ACIV IgM in patients with T2DM and AH

Groups	ACIV IgM (ng/ml)		Comparison		
	Mean ±SD	Group 1	Group 2	All diabetics
All diabetics	0.18±0.07	NS	NS	-
Group 1	0.19±0.01	-	NS	NS
Group 2	0.17±0.05	NS	-	NS
Controls	0.21±0.04	NS	NS	NS

Table 6. Serum ACIV IgA in patients with T2DM and AH

Groups	ACIV IgA (ng/ml)		Comparison		
	Mean ±SD	Group 1	Group 2	All diabetics
All diabetics	0.15±0.03	NS	NS	-
Group 1	0.13±0.02	-	NS	NS
Group 2	0.11±0.07	NS	-	NS
Controls	0.17±0.06	NS	NS	NS

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