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Title: Increased circulating granzyme B in type 2 diabetes patients with low-grade systemic inflammation

Article Type: Full length article

Keywords: granzyme B; type 2 diabetes; inflammation; adipose tissue; adipokines; metabolic diseases.

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First Author: Flavia Agata Cimini

Order of Authors: Flavia Agata Cimini; Donatella D'Eliseo; Ilaria Barchetta; Laura Bertoccini; Francesca Velotti, Ph.D., M.D.; Maria Gisella Cavallo

Abstract: In metabolic diseases, like type 2 diabetes (T2D), adipose tissue (AT) is infiltrated by macrophages and other leukocytes -which secrete many bioactive peptides leading to local and systemic low-grade chronic inflammation- and undergoes remodeling and aberrant fibrosis. Granzyme B (GrB) is a serine protease produced by some leukocytes, including cytotoxic lymphocytes and macrophages. It exerts both intracellular apoptotic function and extracellular functions, leading to tissue injury, inflammation and repair. Elevated circulating GrB levels have been found in aging- and inflammation-associated diseases, and a role for GrB in the pathogenesis of several chronic inflammatory diseases has been reported. Aim of this study was to investigate circulating GrB levels in T2D patients in relation to their systemic inflammatory profile and to unravel its correlates. For this cross-sectional study, we recruited 51 consecutive T2D patients referring to our diabetes outpatient clinics (Sapienza University, Rome, Italy) for metabolic evaluations, and 29 sex, age and body mass index comparable non-diabetic subjects as control group. Study participants underwent clinical work-up; fasting blood sampling was performed for routine biochemistry and for inflammatory profile (CRP, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, GM-CSF, adiponectin, WISP1); serum GrB was measured by Human Granzyme-B Platinum Elisa kit (Affymetrix EBIO). We found that T2D patients had serum levels of GrB significantly higher than the control group (10.17±12.57 vs 7.2±14.1 pg/ml, p= 0.03). Moreover, in T2D patients increased GrB correlated with unfavorable inflammatory profile, as described by elevated levels of validated adipokines such as IL-6 (p=0.04), TNF-α (p=0.019) and WISP1 (p=0.005). Furthermore, multivariate linear regression analysis showed that increased GrB was associated with T2D diagnosis independently from possible confounders. In conclusion, our results show that increased levels of circulating GrB are associated with T2D diagnosis and correlates with markers of AT-linked systemic inflammation, suggesting a potential role for GrB in the inflammatory and reactive processes occurring in metabolic diseases.

Dipartimento di Scienze Ecologiche e Biologiche (DEB)

October, 12, 2018

To Editor
Cytokine

Dear Editor,

Please find attached our revised version of the manuscript (**manuscript n.: CYTO-18-544)** entitled "**Increased circulating granzyme B in type 2 diabetes patients with low-grade systemic inflammation**" by *FA Cimini, D D'Eliseo, I Barchetta, L Bertoccini, F Velotti* and MG Cavallo**, that we are submitting for publication as "Research Article" in Cytokine.

We agreed with the comments made by Reviewer 1 and of Reviewer 2. Thus, we made revisions according to them and we included a point-by-point response to the Reviewers' comments.
All revisions are included in the text and are highlighted in red.

We hope you will find our revised version of the manuscript acceptable for publication in Cytokine.

Thank you for your attention,

Sincerely yours,

Francesca Velotti PhD, MD
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Dipartimento di Scienze Ecologiche e Biologiche (DEB)

UNIVERSITÀ DEGLI STUDI DELLA Tuscia

October, 12, 2018

RESPONSES TO REVIEWERS' COMMENTS

Reviewer #1:
Minor issues raised by Reviewer #1:

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Response 1.
We agree with the Reviewer and in *2. Material and Method* section, *2.6. Statistical Analyses* paragraph, we have added the sample size calculation as follows: "*The sample size and power of this study was calculated according to data from the only previous investigation in the literature, which explored circulating GrB in T2D patients vs non-diabetic control [29]. Based on the reported mean ± S.D. of GrB difference found in these two subgroups [29], the sample size of our study allowed us to reach the statistical significance with a power= 0.90 and a-error= 0.01*"

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Response 2.
No ordinal parameters have been evaluated, we apologize for this refuse; this point has been revised in the text.

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In the model reported in Table 3, the analysis was forced for age, gender and body adiposity – as expressed by BMI and waist circumference- for clinical reasons, because those variables are widely considered among the ones mostly affecting the association between inflammatory markers/mediators and metabolic outcomes, such as T2D. We thank the Reviewer for this comment and we have now described better the multivariate model in *2. Material and Method* section, *2.6. Statistical Analyses* paragraph, as follows: "*The multivariate linear regression analysis was used in order to identify possible determinants of serum GrB concentration, considered as a continuous variable; specifically, the analysis was forced for age, gender and body adiposity – as expressed by BMI and waist circumference- for clinical reasons, since they may exert a major influence in the association between inflammatory markers/mediators and metabolic outcomes, such as T2D*" (see also Response #7).

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As recommended, we have added BMI categories of normal weight, overweight and obese subjects in Table 1. Furthermore, a χ^2-test for trend in proportions has been performed for testing the presence of statistical differences between T2D and non-diabetic individuals within these BMI categories (see Table 1).

6. *All tables: please report the actual p-values, even if not significant (avoid n.s.)*

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As requested, in all tables, we have reported the actual p-values even if not significant.

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We thank the Reviewer for this comment, which allows us to explain better our statistical analysis. In the model reported in Table 3, we forced the analysis for age, gender and body adiposity – as expressed by BMI and waist circumference- for clinical reasons, assuming that these variables are major confounders when investigating the association between a test variable –i.e. GrB concentration- and metabolic outcomes such as T2D. We agree with the Reviewer that, since T2D and controls were matched for age, sex and BMI, these variables do not influence the relationship between "T2D yes/no" variable and the outcome, but in the model in Table 3 the dependent variable to be explored is the circulating GrB concentration and not the presence of T2D. Moreover, as suggested by the Reviewer, we have now analyzed the BMI also as a categorical variable (normal-weight, overweight and obese individuals) by a χ^2-test for trend in proportions, finding a different distribution within these categories between T2D and non-diabetic subjects. Indeed, by adding these variables in the multivariate model, we reinforced the assumption that the association between serum GrB and T2D was not mediated by these characteristics of the study population (see also Response #3). As mentioned above (Response 3), we have now described better the model in *2. Material and Method* section, *2.6. Statistical Analyses* paragraph (see Response #3).

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We agree with Reviewer and we have added additional analysis evaluating the relationship between inflammatory molecules and GrB levels in the whole study population (T2D and non-diabetic controls). Among all these molecules, WISP1 is the only biomarker significantly associated with GrB levels in the entire study sample at the bivariate correlation analysis stratified for the presence of T2D (r= 0.36, p= 0.039, SD 0.13; 95%C.I. 0.05-0.58). This additional result has been now added in the *3. Results and Discussion* section, *3.2. Increased serum GrB correlated with a systemic pro-inflammatory adipokine profile* paragraph, as follows: "*Moreover, the correlation between GrB and multiple inflammatory markers was further explored in the whole study population (T2D and non-diabetic controls) in a bivariate model stratified for the presence of T2D, and it was found that WISP1 was the only biomarker significantly associated with GrB in the entire study sample (r= 0.36, p= 0.039, SD 0.13; 95%C.I. 0.05-0.58)".*

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We are aware that, as suggested by the Reviewer, serum insulin measurement might add information about the metabolic status also in individuals without T2D. However, all the study participants belonging to the control group had fasting plasma glucose values within the normal range (< 100 mg/dl) and none of them had metabolic syndrome or was treated with medications which could influence glyco-metabolic profile, as inclusion criteria. For these reasons, and for the evidence that GrB concentration was not associated with FBI even in the T2D subgroup, we considered the evaluations performed in the control group sufficient as a metabolic screening for the purposes of this study.

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We agree with the Reviewer that increased GrB levels have been found in T1D and have been suggested to reflect the autoimmune process in this condition. We know that a fraction of patients diagnosed with T2D have an autoimmune component, as revealed by the presence of autoantibodies (LADA). For these reasons, to rule out the possible presence of LADA subjects in our population, we screened the whole diabetic population for the diagnosis of LADA by measuring anti-GAD antibodies, and we found that none of them resulted to be positive. Therefore, to better describe our study population, in *2. Material and Method* section, *2.1. Study subject* paragraph, we added the information as follows: "*Since 2-12% of T2D are affected by latent autoimmune diabetes of the adult (LADA) [32-35], the whole study population was screened for LADA by measuring serum anti-glutamic acid decarboxylase (anti-GAD) antibodies to exclude possible misdiagnosis*".

Reviewer #2:

Minor comments raised by Reviewer #2:

1) *Since reference 29 also studied granzyme B with similar results in some areas, this study needs to be discussed much more thoroughly in relation to the present data!*

Response 1.

The study cited in reference 29 by El Mesallamy et al. reported, according to our results, increased GrB in T2D patients compared to non-diabetic subjects. Moreover, in contrast to our findings (Figure 4), the Authors also reported a significant correlation between GrB levels and BMI, waist circumference, fasting plasma glucose, HbA1c%, fasting plasma insulin and HOMA-IR (Table 3). We think that this discrepancy might be due to the metabolic characteristics of the population of T2D patients studied, in that, in contrast to our population of T2D patients (Table 1), El Mesallamy et al. measured GrB levels in patients with extremely poor glycemic control (Table 1), that is an inflammatory situation that might represent a possible confounder *per se*. In addition, we have noticed substantial differences in the serum GrB concentrations measured in our and in their study populations, especially in relation to standard deviations, may be due to the different assays used. Indeed, to discuss much more thoroughly the results of El Mesallamy et al., in the *3. Result and Discussion* section, *3.1. Increased serum GrB was associated with T2D diagnosis* paragraph, we wrote as follows: "*Only one study, to day, has investigated GrB in T2D patients, and an association between increased GrB and T2D diagnosis has been reported [29]. However, this study is only partially comparable with our investigation, because, in contrast to T2D patients in our analysis (Table 1), the study was performed in a population of obese patients in extremely poor glycemic control (FBG: 245.40+11.35; HbA1c%: 10.56+0.3), which is an inflammatory situation that might represent a possible confounder per se. In fact, in contrast to our results (Table 4), the Authors also reported a significant correlation between GrB levels and BMI, waist circumference, fasting plasma glucose, HbA1c% and HOMA-IR in TD2 patients [29]*."

2) *In the tables (especially table 4), the authors should harmonize the number of decimal places, e.g. the p value in table 4 is rounded to between one and three decimal places.*

Response 2.

As suggested, we have now harmonized the number of decimal places in all the tables.

Highlights

-Circulating Granzyme B is increased in type 2 diabetes patients compared to non-diabetic subjects

-Increased circulating Granzyme B is associated with type 2 diabetes diagnosis independently from possible confounders

-Increased serum Granzyme B correlated with a systemic pro-inflammatory adipokine profile

-Increased serum Granzyme B correlated with a systemic inflammatory profile linked to adipose tissue dysfunction

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Increased circulating granzyme B in type 2 diabetes patients with low-grade systemic inflammation

Flavia Agata Cimini[a], Donatella D'Eliseo[a,b], Ilaria Barchetta[a], Laura Bertoccini[a], Francesca Velotti[b*] and Maria Gisella Cavallo[a*]

[a] *Department of Experimental Medicine, Sapienza University of Rome, Viale Regina Elena 324, 00161 Rome, Italy;* [b] *Department of Ecological and Biological Sciences (DEB), Tuscia University, 01100 Viterbo, Italy*

***Corresponding authors** at: Department of Experimental Medicine, Sapienza University of Rome, Viale Regina Elena 324, 00161 Rome, (M. G. Cavallo); DEB, Tuscia University, Loc. Riello, 01100 Viterbo, Italy (F. Velotti);

E-mail address: gisella.cavallo@uniroma1.it (M.G. Cavallo); velotti@unitus.it (F. Velotti)

ABSTRACT

In metabolic diseases, like type 2 diabetes (T2D), adipose tissue (AT) is infiltrated by macrophages and other leukocytes -which secrete many bioactive peptides leading to local and systemic low-grade chronic inflammation- and undergoes remodeling and aberrant fibrosis. Granzyme B (GrB) is a serine protease produced by some leukocytes, including cytotoxic lymphocytes and macrophages. It exerts both intracellular apoptotic function and extracellular functions, leading to tissue injury, inflammation and repair. Elevated circulating GrB levels have been found in aging- and inflammation-associated diseases and a role for GrB in the pathogenesis of several chronic inflammatory diseases has been reported. Aims of this study were to investigate circulating GrB levels in T2D patients in relation to their systemic inflammatory profile and to unravel its correlates. For this cross-sectional study, we recruited 51 consecutive T2D patients referring to our diabetes outpatient clinics (Sapienza University, Rome, Italy) for metabolic evaluations, and 29 sex, age and body mass index comparable non-diabetic subjects as control group. Study participants underwent clinical work-up; fasting blood sampling was performed for routine biochemistry and for inflammatory profile (CRP, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, GM-CSF, adiponectin, WISP1); serum GrB was measured by Human Granzyme-B Platinum Elisa kit (Affymetrix EBIO). We found that T2D patients had serum levels of GrB significantly higher than the control group (10.17±12.6 *vs* 7.2±14.1 pg/ml, p= 0.03). Moreover, in T2D patients increased GrB correlated with

unfavorable inflammatory profile, as described by elevated levels of validated adipokines such as IL-6 (p=0.04), TNF-α (p=0.019) and WISP1 (p=0.005). Furthermore, multivariate linear regression analysis showed that increased GrB was associated with T2D diagnosis independently from possible confounders. In conclusion, our results show that increased levels of circulating GrB are associated with T2D diagnosis and correlates with markers of AT-linked systemic inflammation, suggesting a potential role for GrB in the inflammatory and reactive processes occurring in metabolic diseases.

Keywords: granzyme B, type 2 diabetes, inflammation, adipose tissue, adipokines, metabolic diseases.

Abbreviations: granzyme B (GrB), type 2 diabetes (T2D), adipose tissue (AT), extracellular matrix (ECM), cardiovascular disease (CVD), body mass index (BMI), cytotoxic T lymphocytes (CTL), natural killer (NK), tumor necrosis factor (TNF), interleukin (IL), Wnt1-inducible signaling pathway protein 1 (WISP1), interferon (IFN), granulocyte/monocyte-colony stimulating factor (GM-CSF), systolic blood pressure (SBP), diastolic blood pressure (DBP), high-density lipoprotein (HDL), low-density lipoprotein, (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), C reactive protein (CRP), fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), statistical package for social sciences (SPSS), standard deviation (S.D.), confidence interval (C.I.), magnetic resonance imaging (MRI)

1. Introduction

Type 2 diabetes (T2D) is the most common endocrine disease and its incidence is increasing worldwide [1]. It is a progressive metabolic disorder characterized by a combination of insulin resistance and defects in insulin secretion. Low-grade systemic inflammation is considered to play a key role in the development of T2D and T2D-associated complications such as atherosclerosis and cardiovascular diseases (CVD) [2]. Low-grade systemic inflammation likely takes origin from a dysfunctional adipose tissue (AT), where a bulk of infiltrating inflammatory cells (like macrophages, cytotoxic T lymphocytes-CTLs and natural killer-NK cells) are activated and release several bioactive peptides, including pro-apoptotic and pro-inflammatory molecules [3-6]. In this context, AT undergoes adipocyte death and tissue remodeling, with abnormal collagen deposition and aberrant fibrosis [6,7]. In dysmetabolic patients, the presence of inflammatory molecules in dysfunctional AT results in elevated serum levels of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, WISP1, and low serum levels of anti-inflammatory molecules such as adiponectin [8-11]. Thus, increased pro-inflammatory mediators in AT exert local effects on AT structure and function, as well as systemic effects on other organs such as blood vessels and heart.

Granzyme B (GrB) is a serine protease traditionally considered as a molecule specifically expressed by cytotoxic lymphocytes (like CTL and NK cells) to cause apoptosis in tumor and virally infected target cells [12, 13]. Currently, it has been well established that GrB is also expressed by non-lymphoid cells, including inflammatory macrophages [14], and that exerts both intracellular and extracellular functions [15-18]. Thereby, GrB can promote diverse processes, such as cellular apoptosis, as well as extracellular matrix (ECM) remodeling, cytokine activation, vascular permeability and immune cell transmigration, leading to tissue injury, inflammation and repair [19]. Data in the literature demonstrated that GrB is over-expressed during inflammation, accumulates in the extracellular space in tissues and in body fluids (e.g., plasma, synovial fluid) derived from patients with aging- and chronic inflammation-associated diseases, showing its implication in the pathogenesis of multiple chronic inflammatory diseases [19-21]. In particular, a role for GrB in CVD and tissue fibrosis has been reported [22-27]. In addition, increased GrB has been described in AT of obese mice [28] and in the serum of obese dysmetabolic subjects and adolescent polycystic ovary syndrome patients with insulin resistance and increased CVD risk [29, 30], suggesting a role for GrB in inflammation associated with obesity and insulin resistance. However, little is known about a possible relationship between GrB, T2D and dysfunctional AT-linked systemic inflammation.

In the current study, we analyzed GrB levels in the serum of patients with T2D. Then, to extend our investigation on a possible role of GrB in the inflammation associated to T2D, we measured circulating levels of several cytokines and inflammatory bioactive peptides, and we analyzed whether a correlation existed between circulating GrB and the systemic inflammatory profile considered the signature of dysfunctional AT.

2. Materials and methods

2.1. Study subjects

We enrolled 51 consecutive T2D patients referring to our diabetes outpatient clinics at Sapienza University of Rome, Italy, for metabolic evaluations. T2D was diagnosed according to the American Diabetes Association 2009 criteria [31]. Since 2-12% of T2D are affected by latent autoimmune diabetes of the adult (LADA) [32-35], the whole study population was screened for LADA by measuring serum anti-glutamic acid decarboxylase (anti-GAD) antibodies to exclude possible misdiagnosis. Inclusion criteria were as follows: (a) male and female aged >18; (b) Caucasian ethnicity; (c) full acceptance of informed consent to the study. Exclusion criteria were as follows: (a) severe psychiatric illness; (b) dialysis and/or end-stage renal disease; (c) cirrhosis; (d) active cancer of any type; (e) chronic treatment with corticosteroids. We also enrolled 29 sex, age and body mass index (BMI) comparable metabolic healthy subjects as a control group.

This study was reviewed and approved by the Ethics Committee of Sapienza University of Rome and conducted in conformance with the Helsinki Declaration. A written informed consent was obtained from all subjects before participating to the study.

2.2. Clinical work up and Laboratory Determinations

The study population underwent clinical work up, including medical history collection, physical examination with calculation of BMI (kg/m^2), normal weight, overweight and obese individuals (number and percentage), waist circumference (cm), systemic [systolic (SBP), diastolic (DBP), mmHg] blood pressure and fasting blood sampling to assess total cholesterol (mg/dl), high-density lipoprotein (HDL, mg/dl), low-density lipoprotein (LDL, mg/dl), triglycerides (mg/dl), aspartate aminotransferase (AST, IU/l), alanine aminotransferase (ALT, IU/l), gamma-glutamyl transferase (GGT, mg/dl), creatinine (mg/dl) and C reactive protein (CRP, mg/dl) by standard laboratory methods. The glycometabolic profile has been evaluated by measuring fasting blood glucose (FBG,

mg/dl), insulin (IU/l, PANTEC srl; Italy) and glycosylated hemoglobin (HbA1c, % - mmol/l). The homeostasis model assessments of insulin resistance (HOMA-IR) and insulin secretion (HOMA-β%) have been calculated as previously described [36]. Clinical and biochemical characteristics of patients with T2D and of controls are shown in Table 1.

Table 1. Clinical and biochemical characteristics of T2D patients and controls.

	T2D (n=51)	**Controls (n=29)**	***p*-value**
Gender (M/F)	32/19	16/13	0.62*
Age (years)	58±11	47±12	0.38
BMI (kg/m^2)	29.1±6.5	26.5±4.8	0.09
Normal weight, overweight and obese individuals (number and percentage)	8/21/22 17%-41%-42%	16/8/5 55%-28%-17%	0.001*
Waist circunferences (cm)	103.4±13.5	95.4±17.9	0.08
SBP (mmHg)	132.5±15.1	124.4±16.3	0.31
DBP (mmHg)	81.8±9.4	76.6±9.1	0.38
FBG	142.7±50.6	88.7±11.6	0.001
HbA1c (% - mmol/mol)	7.1±1.2 - 54±5	-	-
Triglycerides (mg/dl)	114.2±61.4	101.7±92.3	0.12
Total Cholesterol (mg/dl)	185.1±26	178.5±38.7	0.24
HDL (mg/dl)	50.3±14.7	54.7±17.8	0.56
LDL (mg/dl)	115.4±23.1	99.7±27.2	0.28
AST (IU/L)	20.5±7.6	19.2±4.9	0.45
ALT (IU/L)	25.7±11.8	22.8±11.3	0.40
GGT (IU/L)	21.9±14	17.1±5.5	0.28
Fasting Plasma Insulin (μU/L)	12±3.3	-	-
HOMA-IR	4.1±1.5	-	-
HOMA β %	75.2±49.8	-	-

Values are expressed as mean±S.D. T test for independent sample test was applied. *χ^2 test applied; *p*-values <0.05 are considered significant.

2.3. Biological material preparation

Vein blood samples were drawn from subjects after overnight fasting. Serum was separated by centrifugation (2500 g, 10 min.), divided into several aliquots (to avoid thawing-freezing cycle) and kept at −70°C for further examination.

2.4. Cytokine measurement

Systemic inflammatory profile was performed in all study participants by measuring the serum concentration of a panel of cytokines, such as IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, GM-CSF, by MultiplexTM (Biorad).

2.5. Adiponectin, WISP-1 and GrB measurement

Circulating adiponectin, Wnt1-inducible signaling pathway protein 1 (WISP1) and GrB were measured by specific enzyme-linked immunoassorbent assays, such as Tema Ricerca s.r.l., RayBiotech Inc. and Human Granzyme B Platinum –Kit Elisa-Affymetrix EBIO, respectively.

2.6. Statistical analyses

The IBM statistical package for social sciences (SPSS) statistics (version 17) was used to perform all the analyses (IBM, Armonk, NY). The sample size and power of this study was calculated according to data from the only previous investigation in the literature, which explored circulating GrB in T2D patients vs non-diabetic control [29]. Based on the reported mean ± S.D. of GrB difference found in these two subgroups [29], the sample size of our study allowed us to reach the statistical significance with a power= 0.90 and a-error= 0.01. Student's T-test for continuous variables and χ^2 test for categorical variables were performed to compare mean values between two independent groups. Skewed variables underwent logarithmic transformation before the analyses. Correlations between continuous variables were calculated by Pearson's coefficient, whereas Spearman's coefficient was used for dichotomic parameters. Correlation coefficients were reported as *r* values in the text and tables. The multivariate linear regression analysis was used in order to identify possible determinants of serum GrB concentration, considered as a continuous variable; specifically, the analysis was forced for age, gender and body adiposity – as expressed by BMI and waist circumference- for clinical reasons, since they may exert a major influence in the association between inflammatory markers/mediators and metabolic outcomes, such as T2D. Data are shown as mean ± standard deviation (S.D.); a p-value < 0.05 was considered statistically significant in all the analyses, with a 95% confidence interval (C.I.).

3. Results and Discussion

3.1. Increased serum GrB was associated with T2D diagnosis

The investigation of GrB levels in the serum of patients with T2D showed that significantly higher circulating levels of GrB were present in T2D patients compared to control group (Table 2; 10.2±12.6 *vs* 7.2±14.1 pg/ml, p= 0.03). In order to identify independent determinants of increased GrB levels in the entire study population, we performed a multivariate linear regression analysis showing that increased serum GrB levels were associated with T2D diagnosis after adjusting for the possible clinical confounders, such as age, gender and body adiposity (Table 3; β: 0.829, p=0.008). Only one study, to day, has investigated GrB in T2D patients, and an association between increased GrB and T2D diagnosis has been reported [29]. However, this study is only partially comparable with our investigation, because, in contrast to T2D patients in our analysis (Table 1), the study was performed in a population of obese patients in extremely poor glycemic control (FBG: 245.40+11.35; HbA1c%: 10.56+0.3), which is an inflammatory situation that might represent a possible confounder per se. In fact, in contrast to our results (Table 4), the Authors also reported a significant correlation between GrB levels and BMI, waist circumference, fasting plasma glucose, HbA1c% and HOMA-IR in TD2 patients [29].

3.2. Increased serum GrB correlated with a systemic pro-inflammatory adipokine profile

The systemic inflammatory profile was investigated in all study participants by measuring the serum concentration of a panel of inflammatory cytokines and other bioactive mediators. As expected, circulating levels of pro-inflammatory adipokine, such as IL-6, IL-8, TNF-α and WISP1 were significantly increased (Table 2; p=0.03, p=0.02, p=0.03, p=0.01, respectively) in T2D patients, whereas adiponectin, a well-known anti-inflammatory adipokine, was decreased compared to the control group (Table 2; p=0.03) [8, 9, 37]. No significant alteration in the level of circulating cytokines such as IL-2, IL-4, IL-10, IFN-γ, GM-CSF was observed in patients with T2D when compared to the control group (Table 2). Interestingly, when we evaluated the relationship between the levels of GrB and the inflammatory molecule profile in T2D patients, we found that increased circulating GrB correlated with higher serum levels of IL-6 (p=0.04) and TNF-α (p=0.02) (Table 4), circulating markers of inflammation and AT dysfunction [38]. No correlation was observed with IL-8 levels (Table 4; p=0.06), even if we observed a trend not reaching statistical significance very

likely due to the reduced sample size of patients. In addition, increased levels of circulating GrB in T2D patients also correlated with higher serum levels of WISP1 (Table 4; *p*=0.005). Moreover, the correlation between GrB and multiple inflammatory markers was further explored in the whole study population (T2D and non-diabetic controls) in a bivariate model stratified for the presence of T2D, and it was found that WISP1 was the only biomarker significantly associated with GrB in the entire study sample (r= 0.36, p= 0.039, SD 0.13; 95%C.I. 0.05-0.58). This is a very interesting finding, since WISP1 is a novel adipokine, widely expressed in inflamed visceral AT, released by fully differentiated human adipocytes, which stimulates cytokine responses in dysmetabolic subjects [39]. Circulating concentration of WISP1 is known to correlate with its expression level in AT [39]. Barchetta et al. have recently demonstrated that circulating levels of WISP1 are increased in obese patients and are directly related to adiposity, independently of the glycemic status or insulin resistance, and that are strongly associated with magnetic resonance imaging (MRI) signal abnormalities of visceral AT [10]. In light of our results and data in the literature, reporting increased GrB expression in AT of obese mice [28] and a role of GrB in the promotion of tissue injury, inflammation and repair [15-19, 27], we suggest that GrB might be a player in the remodeling of inflamed AT and thus a potential circulating marker of AT dysfunction in dysmetabolic patients.

Table 2 . Granzyme B levels and inflammatory profile of T2D patients and controls.

	T2D (n=51)	**Controls (n=29)**	***p*-value**
IL-2 (pg/ml)	2.7±4.5	3.3±2.1	0.18
IL-4 (pg/ml)	3.4±1.4	4.1±3.7	0.09
IL-6 (pg/ml)	22±14.5	14.7±8.3	0.03
IL-8 (pg/ml)	63.4±22.4	27.5±16.8	0.02
IL-10 (pg/ml)	5.1±2.2	6.3±5	0.15
TNF-α (pg/ml)	37.1±23.4	20.2±11.9	0.03
IFN-γ (pg/ml)	1±2.2	0.9±1.8	0.12
GM-CSF (pg/ml)	1.5±1.8	2.5±2.1	0.08
WISP1 (ng/ml)	453.7±988.7	230.3±330.8	0.01
CRP (mg/L)	5.1±2.4	2.7±0.6	0.04
Adiponectin (ng/ml)	5.4±3.3	11.6±4.7	0.03
Granzyme B (pg/ml)	**10.2±12.6**	**7.2±14.1**	**0.03**

Values are expressed as mean±S.D.; T test for independent sample test was applied; *p*-values <0.05 are considered significant.

Table 3. Granzyme B level is the dependent variable in T2D patients.

-Multivariate linear regression analysis.

	Unstandardized		Standardized		
	ß	Standard Deviation Error	ß	T	p-value
(Costante)	1,176	1,081		1,088	0,280
T2D (yes/no)	**0,829**	**0,304**	**0,335**	**2,727**	**0,008**
Waist circumference	-0,006	0,015	-0,090	-0,429	0,669
BMI	0,028	0,045	0,123	0,629	0,531
Age	-0,008	0,011	-0,080	-0,757	0,451
Sex	-0,111	0,265	-0,049	-0,417	0,678

R	R^2	Corrected R^2	Standard Deviation Error
0,469	0,220	0,128	0,981

Table 4. Correlation of serum granzyme B levels and inflammatory profile in T2D subjects.

-Bivariate correlation analyses.

	Correlation coefficient	***p*-value**
Sex	0.07	0.51
Age	0.04	0.82
BMI	-0.06	0.56
Waist circumference	-0.1	0.24
SBP	0.15	0.38
DBP	0.09	0.41
Total Cholesterol	0.07	0.46
HDL	-0.1	0.93
LDL	0.23	0.22
Triglycerides	0.08	0.41
FBG	0.3	0.09
HbA1c	0.25	0.15
HOMA-IR	0.14	0.37
HOMA-ß%	-0.08	0.68
IL-2	0.04	0.35
IL-4	0.07	0.43
IL-6	**0.33**	**0.04**
IL-8	0.1	0.06
IL-10	- 0.26	0.07
TNF-α	**0.22**	**0.02**
IFN-γ	0.04	0.77
GM-CSF	0.03	0.85
WISP1	**0.29**	**0.00**
CRP	0.18	0.09
ADIPONECTIN	-0.05	0.69

4. Conclusions

Here, we showed that increased levels of circulating GrB associated with the presence of T2D and correlated with markers of dysfunctional AT-linked systemic inflammation. This is the first study specifically designed to investigate circulating GrB in an extensively metabolically characterized population of subjects with T2D and we observed an independent association between increased systemic GrB levels and T2D diagnosis. Moreover, the analysis of the relation between serum GrB levels and the systemic inflammatory profile, revealed that increased GrB correlated with the concentration of circulating markers of low-grade systemic inflammation related to AT dysfunction such as IL-6, TNF-α and WISP1. We acknowledge that the cross-sectional design of our study does not allow to establish a causal nexus between increased serum levels of GrB and AT dysfunction, however it highlights the potential role of GrB as inflammatory mediator implicated in mechanisms underlying T2D development and progression. Therefore, further studies with a longitudinal design are warranted to clarify the role of GrB in AT remodeling in metabolic diseases.

Conflicts of interest

The authors do not have any proprietary, financial, professional or other personal conflicts of interest.

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