Metformin is the key factor for elevated plasma GDF-15 levels in type 2 diabetes: a nested, case-control study

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Metformin is the key factor for elevated plasma GDF-15 levels in type 2 diabetes: a nested, case-control study

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Abstract

Produced as a tissue defence-response to hypoxia and inflammation, Growth Differentiation Factor-15 (GDF-15) is raised in subjects on metformin treatment. To gain insight into the relationship of GDF-15 with metformin and major cardiovascular risk factors, we analyzed the data of the SUMMIT cohort (n=1,438), a 4 centres, nested, case-control study aiming at verifying whether biomarkers of atherosclerosis differ according to the presence of type 2 diabetes and cardiovascular disease. While in univariate analysis, major cardiovascular risk factors, with the exception of gender and cholesterol, increased similarly and linearly across GDF-15 quartiles, the independent variables associated with GDF-15, both in subjects with and without diabetes were age, plasma creatinine, NT-proBNP, diuretic use, smoke load, and HbA_{1c}. In subjects with diabetes metformin treatment was associated to a 40% rise in GDF-15, which was independent to the other major factors largely explaining their elevated GDF-15 levels. The relatively higher GDF-15 bioavailability might contribute to explain the protective cardiovascular effects of metformin.

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Introduction

Growth differentiation factor-15 (GDF-15) is a stress protein synthetized by macrophages, cardiomyocytes, adipocytes, and endothelial cells [1]. Expressed in atherosclerotic plaques of carotid and coronary arteries, where it co localizes with macrophages, GDF-15 exerts an inhibitory action on chemotaxis and apoptosis [2]; it is also expressed by cardiomyocytes in response to ischemia also producing in this tissue a limitation of leukocyte recruitment and apoptosis [3]. In cardiomyocytes, GDF-15 expression is also induced by pressure overload with an overall anti-hypertrophic effect [4]. GDF-15 has also been proven to protect in human endothelial cells from high glucose-induced apoptosis [5].

Whether circulating GDF-15 plasma levels reflects the adequacy of the protective tissue responses to chemical and physical biologic stressors, or rather the severity of tissue damage, is still unclear [6]. Elevated plasma concentrations are found in several conditions characterized by low-grade inflammation and accelerated atherosclerosis like type 2 diabetes (T2D), impaired glucose tolerance and insulin resistance [1,6]. Also in patients with heart failure, GDF-15 is significantly upregulated, and its plasma concentrations correlate with the severity of the disease and the prognosis [7]. Although elevated GDF-15 plasma levels have been consistently found in T2D, no information is available on whether the degree of metabolic control has an impact *per se*, whether this is due to their higher prevalence of cardiovascular and/or kidney diseases, or to a different sensitivity to known major GDF-15 modulators. Limited is also the information with regard to the effect of the different glucose lowering treatments; interestingly, in the ORIGIN trial participants among 237 serum biomarkers the only one associated with metformin treatment was GDF-15 [8].

In synthesis, despite its anti-apoptotic, anti-hypertrophic, and anti-inflammatory actions, as a clinical biomarker GDF-15 appears to reflect the severity of cardiovascular disease (CVD) [9] rather than the extent of the activation of the protective mechanisms observed in vitro. Still, if metformin is able to upregulate GDF-15 circulating levels independently of other factors, this might represent a mechanism

through which the drug exerts its effect in terms of cardiovascular disease protection [10]. For this reason, we sought to identify the major determinants of GDF-15 plasma levels separately in subject with and without T2D selected to have a wide range of cardiovascular risk and extent of atherosclerotic disease, and to evaluate whether metformin treatment influences the impact of these factors.

Materials and Methods

Study Population

The volunteers were recruited among the patients and relatives attending the outpatients clinic for metabolic diseases or cardiovascular disease within the SUMMIT multicenter study (http://www.imi-summit.eu) according to the following criteria: a) T2D and clinically manifest CVD, b) T2D without clinical signs of CVD, c) CVD but no T2D, and subjects with neither CVD nor T2D, in a 2:2:1:1 proportion. CVD included any coronary, carotid and peripheral event or positive imaging. Exclusion criteria included atrial fibrillation, malignancy, severe renal impairment (eGFR<30 ml/min/1.73 m^2), or any chronic inflammatory disease. All subjects were treated according to the usual standards of care. The study was approved by the local ethical review boards.

Analysis of GDF-15 and NT-proBNP in plasma

Plasma levels of GDF-15 and NT-proBNP were analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD 96x96 reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala. Details of the methods, and the GDF-15 calibration curve used to estimate the approximate absolute concentrations from the relative quantification units (NPX), are available at http://www.olink.com.

Data and Statistical Analysis

Subjects were divided according to the presence of T2D and quartiles of plasma levels of GDF-15 were calculated within each group. Differences across quartiles of GDF-15 were investigated using

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either Chi-squared or Wilcoxon statistics for frequency or continuous values, respectively. *p* ≤0.05 was considered statistically significant. Multiple regression analysis was conducted according to Restricted Maximum likelihood (REML) method using a random effect attribute for the variable center. The strength of the associations was quantified by the logWorth value.

Results

Study population

The study cohort consisted of 1,438 volunteers (937 men and 501 women, 42 to 88 years old), recruited at the 4 centers within the SUMMIT multicenter study (with available GDF-15 data); 448 patients had T2D and clinically manifest CVD, 495 patients had T2D without clinical history or evidence of CVD, 238 patients had CVD but no T2D, and 257 subjects had neither CVD nor T2D.

GDF-15 and clinical data

Median approximate plasma GDF-15 concentrations were higher in presence of CVD or T2D either alone (775 [571-1,000] and 1,000 [736-1,503] pg/L) or in combination (1,290 [903-1,939] pg/L) with respect to absence of both diseases (571 [564-775] pg/L, p<0.001 for all comparisons). Across quartiles of plasma GDF-15, the concentration values increased 3 folds in non-diabetic and 4 folds in T2D (Supplemental **Table 1**). Higher GDF-15 levels were associated, in a quasi-linear fashion in both study groups, with older age, prevalence of hypertension, history or evidence of major atherosclerotic cardiovascular disease, smoking exposure, and systolic blood pressure, while neither gender nor BMI were different (Supplemental **Table 1**). With regard to the ongoing treatments, diuretics and RAS blockers increased across quartiles in both groups and among the subjects with T2D higher GDF-15 values were associated also with a lower prevalence of diet only and a larger use of each and all plasma lowering drugs (metformin, sulphonylureas, DDP-IV inhibitors and insulin).

GDF-15 and biochemical parameters

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Among the biochemical parameters (Supplemental **Table 2**), GDF-15 was associated in both study groups with higher levels of creatinine, NT-proBNP and also HbA_{1c}. On the other hand, we observed an inverse trend with total low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol, which was coupled to higher rates of ongoing statin therapy. No trend was found with plasma triglycerides in either group.

Multivariate analysis

In **Table 1** are shown the variables, among the clinical and the biochemical ones, showing a statistically significant and independent relationship with GDF-15 in multivariate analysis, separately for subjects with and without T2D and in the whole population (with T2D entered as a dummy variable). In subjects without T2D the variables more strongly associated with GDF-15 were, in this order: NT-proBNP, age, serum creatinine, smoke load, diuretic use and HbA_{1c}. In patients with T2D the strongest association was observed with ongoing metformin treatment followed by serum creatinine, NT-proBNP, age, HbA_{1c}, diuretic treatment and smoke load, in this order. In patients with T2D assuming metformin (n=644), approximate absolute plasma GDF-15 levels were 2-fold higher than nondiabetic subjects (1,226 [858-1,939] vs 665 [515-903] ng/L), while T2D not on metformin (n=299) had 40% higher values (903 [665-1,290] ng/L, p<0.0001 for all comparisons). Within the T2D subjects on metformin, the major predictors of GDF-15 were the same as in the T2D subjects not on metformin with the exception of HbA_{1c} that lost is statistical significance. The analysis in the whole study cohort confirmed that metformin was the most powerful predictor, followed by a set of 3 variables of intermediate strength (creatinine, age, and NT-proBNP) and 3 variables of minor strength (smoke load, HbA_{1c}, and diuretic use). Independently from the above-mentioned variables, T2D *per se* had a negligible power as a predictor of GDF-15, while presence of clinically manifest CVD did not enter into the model.

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Discussion

Our data confirm that plasma GDF-15 is raised at the presence of each and all the major risk factors for atherosclerotic CVD with the only exception of plasma LDL-cholesterol. Although still observed after adjusting for ongoing statin therapy, the achieved LDL-cholesterol levels are likely to inversely reflect to the overall cardiovascular risk of the patients. We also confirm [8] that metformin use is associated with elevated GDF-15 plasma levels and demonstrate that its contribution largely explains the consistent finding of increased plasma GDF-15 levels in T2D.

When all the relevant variables were entered in a multivariate model, actually a similar pattern was observed in both study groups and the whole cohort, with NT-proBNP, age and serum creatinine emerging as the most powerful predictors (**Table 1**). The major difference was in the strength of metformin in subjects with T2D, which was well above and also independent of any other variable being responsible for a 40% rise in GDF-15. Interestingly, HbA$_{1c}$, though with a smaller effect, was associated with higher GDF-15 values in both T2D and non-T2D, and this is a novel finding. Notably, this association was lost in the patients on metformin treatment (ß=0.003±0.002) while it was present in T2D subjects not on metformin (ß=0.007±0.001), implying that the drug might prevent the fall in GDF-15 that is expected to occur with the improvement of metabolic control. Noteworthy, the presence of CVD does not appear to contribute to the rise in GDF-15 once age, serum creatinine, NT-proBNP and metformin use are taken into account. This suggests that GDF-15, rather than reflecting overall atherosclerosis, marks the presence of kidney and cardiac damage. As shown in **figure 1**, the relationship between GDF-15 and NT-proBNP is similar in those with or without T2D, but in T2D on metformin it is shifted upright. The higher plasma GDF-15 levels for the same degree of cardiac dysfunction might explain the reduced myocardial vulnerability to ischemia and remodeling observed in experimental studies with metformin treatment [10]; GDF-15 could be included in the number of known mechanisms [10] through which metformin produces its beneficial clinical effects.

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One possible explanation for the association of GDF-15 with metformin treatment, and also with HbA$_{1c}$, could be that this growth factor is also a powerful marker of presence and severity of mitochondrial disorders [11] and/or hypoxia [3]. In fact, both metformin and hyperglycemia are known to interfere with mitochondrial bioenergetics. Moreover, high glucose in human umbilical endothelial cells [5] and in HepG2 cells [12] is able to promote GDF-15 expression and secretion, and in non-diabetic subjects glucose ingestion is associated with an elevation of plasma GDF-15 [13].

We confirm that current smoking is associated with elevated GDF-15 [14], but being the association with smoke load stronger than the current smoking status, we suggest that it is chronic damage on pulmonary structures [15] that result in a persistent GDF-15 upregulation. Similarly, since circulating GDF-15 levels are strongly correlated with its intra-renal expression [16], we interpret the association with plasma creatinine as a reflection of chronic kidney damage.

We acknowledge that our cohort has peculiar characteristics and therefore we cannot extend our conclusion to the general population. However, its enrichment with patients with CVD and risk factors offered the opportunity to explore the full range of pathophysiologic variability of the different variables related to CVD. Clearly our analysis is cross-sectional and the subjects were treated with an intensity that is expected to be proportional to the severity of their diseases. While this might have hampered our ability to detect the relevance of GDF-15 in the natural context of each atherosclerosis-related disease/condition it also represented an opportunity to gain information on real world outpatients with regard to residual and unexplained CVD risk.

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List of abbreviations

T2D: type 2 diabetes
CVD: cardiovascular disease
BMI: body mass index
GDF-15: Growth Differentiation Factor-15
HbA$_{1c}$: glycated hemoglobin
NT-proBNP: N-terminal pro-brain natriuretic peptide

Declarations

- **Ethics approval and consent to participate.** Ethics, consent and permissions: the study has been approved by the local ethic committee at each center and all subjects gave their informed consent to participate.
- **Consent for publication:** we obtained consent to publish from the participant

- **Availability of data and material.** All authors have read the manuscript and gave their consent for the publication, all participants were informed that personal data were permanently stored in an anonymous from and might have been used in such a form for scientific publications. The datasets used and/or analyzed during the current study are available from the SUMMIT consortium according to the established data sharing and publication policy described in details at http://www.imi-summit.eu.

- **Competing interests.** The authors declare that they have no competing interest to disclose with regard to the content of the manuscript.

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- **Authors' contributions.** AN: Study design, data analysis, manuscript revision, LN: Manuscript preparation, data analysis, EV: Study conduction, database building, data analysis, AS: Patients recruitment, manuscript revision, FK: Patients recruitment, manuscript revision, KG: Patients recruitment, database building, PE: Study conduction, database building, HCL: Study conduction, database building, FD: Study conduction, database building, IG: Study conduction, database building, MP: Study conduction, database building, JN: Study coordination, manuscript revision.
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Legends to figures

Figure 1 - Scatterplot of GDF-15 and NT-proBNP values in the non diabetic population (a) and in the

subject with type 2 diabetes (b) according to whether were on metformin treatment (dark gray symbols)

or not (light gray symbols).

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Table 1 - Multivariate predictors of plasma GDF-15 among the clinical characteristics, the biochemical variables

Variable (n)	LogWorth	ß	Statistics [§]
No diabetes			
NT-proBNP (AU)	11.5	*0.169*	*<0.0001*
Age (yrs)	10.0	*0.020*	*<0.0001*
Creatinine (µmol/L)	7.7	*0.007*	*0.0002*
LnPackyears	4.5	*0.060*	*0.0001*
Diuretic (yes/no)	3.2	*0.110*	*0.0006*
HbA$_{1c}$ (mmol/mol)	2.6	*0.017*	*0.0026*
Whole model R^2 = 0.56			
Type 2 diabetes			
Metformin (yes/no)	37.8	*0.318*	*<0.0001*
Creatinine (µmol/L)	13.5	*0.005*	*<0.0001*
NT-proBNP (AU)	9.4	*0.145*	*<0.0001*
Age (yrs)	8.4	*0.019*	*<0.0001*
HbA$_{1c}$ (mmol/mol)	4.1	*0.006*	*<0.0001*
LnPackyears	2.6	*0.038*	*0.0023*
Diuretic (yes/no)	2.3	*0.068*	*0.0053*
Whole model R^2 = 0.42			
All			
Metformin	42.8	*0.323*	*<0.0001*
NT-proBNP (AU)	23.7	*0.018*	*<0.0001*
Creatinine (µmol/L)	16.3	*0.005*	*<0.0001*
Age (yrs)	15.1	*0.017*	*<0.0001*
LnPackyears	7.2	*0.054*	*<0.0001*
HbA$_{1c}$ (mmol/mol)	5.2	*0.007*	*0.0001*
Diuretic (yes/no)	5.0	*0.079*	*0.0001*
Diabetes (yes/no)	1.9	*0.074*	*0.0129*
Whole model R^2 = 0.51			

[§] Multiple regression analysis was conducted according to Restricted Maximum likelihood (REML) method with centre entered as a random variable, $p \leq 0.05$ was considered statistically significant. ß coefficients are not standardized, the strength of the association is provided by the LogWorth value. The dependent variable GDF-15 is expressed in NPX units that approximates the log transformed value of plasma concentration. Due to sparse missing values the models were run in 482 subjects without diabetes, 892 subjects with type 2 diabetes and in 1,374 subjects in the whole study group.

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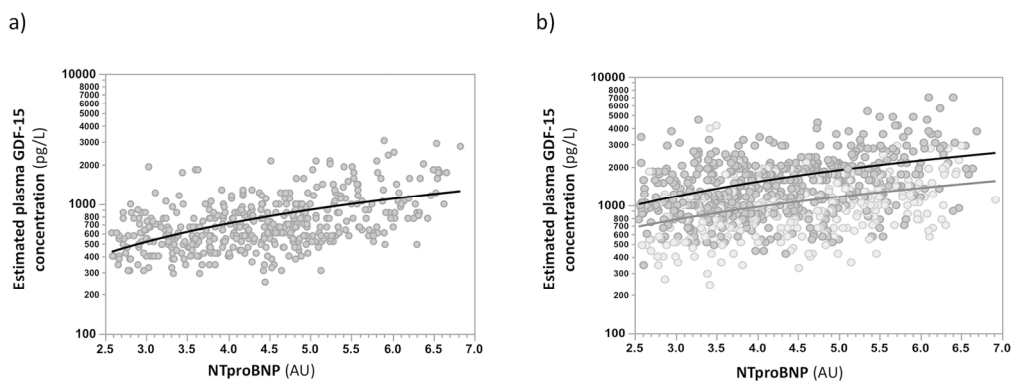48
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Figure 1 - Scatterplot of GDF-15 and NT-proBNP values in the non diabetic population (a) and in the subject with type 2 diabetes (b) according to whether were on metformin treatment (dark gray symbols) or not (light gray symbols).

160x59mm (300 x 300 DPI)

Suppl. Table 1 – Clinical characteristics according to quartiles of plasma GDF-15

	Quartiles of plasma GDF-15				
	I	**II**	**III**	**IV**	*Statistics [§]*
No diabetes					
n	123	126	123	123	
GDF-15 (Arb.Units)	450±876	655±56	883±85	1484±437	*<0.0001*
Age (years)	61±8	66±9	68±8	73±7	*<0.0001*
Women (%)	47	34	36	33	*ns*
Hypertension (%)	35	47	63	64	*0.0001*
Any CVD (%)	30	44	52	66	*<0.0001*
Smoking nev./ex/curr. (%)	53/40/7	41/51/8	43/50/7	24/57/19	*<0.0001*
Smoke load (Pack-years)	8±14	11±18	13±21	21±24	*0.0001*
BMI (kg/m^2)	26.5±3.9	27.1±3.9	27.9±4.2	27.7±3.8	*ns*
SBP (mmHg)	127±15	133±14	136±16	136±19	*<0.0001*
ACEi/AT2a (%)	26	34	45	53	*<0.0001*
Diuretics (%)	6	8	17	26	*<0.0001*
Diabetes					
n	237	236	232	238	
GDF-15 (Arb. Units)	687±162	1110±110	1566±170	2630±838	*<0.0001*
Age (years)	64±8	68±8	69±8	70±7	*<0.0001*
Women (%)	36	33	32	32	*ns*
Hypertension (%)	67	74	81	76	*0.0007*
Any CVD (%)	34	43	48	64	*<0.0001*
Smoking nev./ex/curr. (%)	40/54/5	39/50/11	34/51/15	30/59/11	*0.003*
Smoke load (pack years)	14±23	18±24	22±25	24±32	*0.0016*
Diab. duration	8±7	10±8	12±8	13±8	*<0.0001*
BMI (kg/m^2)	30.4±4.6	30.3±4.9	30.4±4.9	30.6±5.1	*ns*
SBP (mmHg)	133±18	137±18	139±18	141±20	*<0.0001*
ACEi/AT2a (%)	56	67	72	76	*<0.0001*
Diuretics (%)	20	21	34	39	*<0.0001*
Diet only (%)	30	15	8	4	*<0.0001*
Metformin (%)	48	67	75	83	*<0.0001*
SU (%)	23	21	36	35	*0.0002*
DDPIVi (%)	5	9	12	17	*0.0005*
Insulin (%)	15	24	25	31	*0.0012*

[§] Differences across quartiles of GDF-15 were investigated using either logistic (χ^2) or Wilcoxon/Kruskal-Wallis Test for frequency or continuous values, respectively; $p \leq 0.05$ was considered statistically significant. ACEi: angiotensin converting enzyme inhibitors; SU: sulphonylureas; DDPIVi: dipeptidyl peptidase IV inhibitors.

Suppl. Table 2 – Biochemical variables according to quartiles of plasma GDF-15

	I	II	III	IV	Statistics [§]
No diabetes					
Creatinine (μmol/L)	76±14	80±16	81±14	90±25	***<0.0001***
eGFR (ml/min/1.74m^2)	87±17	84±16	81±15	74±19	***<0.0001***
Total Cholesterol (mmol/L)	5.1±1.0	4.9±1.1	4.6±0.9	4.4±1.0	***<0.0001***
LDL Cholesterol (mmol/L)	3.0±0.9	2.9±0.9	2.6±0.8	2.5±0.9	***<0.0001***
HDL Cholesterol (mmol/L)	1.6±0.5	1.5±0.4	1.5±0.4	1.4±0.4	***0.0074***
Triglycerides (mmol/L)	1.3±0.7	1.4±0.7	1.3±0.7	1.4±0.6	*0.3352*
Statin Tx (%)	37	50	60	63	***0.0002***
HbA$_{1c}$ (mmol/mol)	38±4	38±4	40±4	40±4	***0.0002***
NT-proBNP (AU)	16±11	22±14	27±19	41±25	***<0.0001***
Diabetes					
Creatinine (μmol/L)	77±16	83±23	83±24	103±49	***<0.0001***
eGFR (ml/min/1.73m^2)	87±17	83±22	84±22	67±21	***<0.0001***
Total Cholesterol (mmol/L)	4.5±1.1	4.1±0.9	4.1±0.9	3.9±0.9	***<0.0001***
LDL Cholesterol (mmol/L)	2.5±0.9	2.2±0.9	2.2±0.8	2.1±0.8	***<0.0001***
HDL Cholesterol (mmol/L)	1.3±0.4	1.3±0.4	1.2±0.4	1.2±0.4	***0.0142***
Triglycerides (mmol/L)	1.6±0.9	1.5±0.7	1.8±1.0	1.7±1.0	*0.0586*
Statin Tx (%)	65	76	73	79	***0.0096***
HbA$_{1c}$ (mmol/mol)	55±12	57±13	60±14	59±14	***0.0003***
NT-proBNP (AU)	19±15	23±17	27±17	34±23	***<0.0001***

[§] Differences across quartiles of GDF-15 were investigated using either logistic (χ^2) or Wilcoxon/Kruskal-Wallis for frequency or continuous values, respectively; $p \leq 0.05$ was considered statistically significant.

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