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Adipose tissue–derived adipsin marks human aging in non-type 2 diabetes population

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ABSTRACT

Introduction Adipsin or complement factor D is an adipokine that augments insulin secretion, is altered in various degrees of obesity, and is involved in alternative complement pathway. However, whether adipsin has any independent association with risk factors and biomarkers in patients with type 2 diabetes (T2D) remains elusive.

Research design and methods We performed an oral glucose tolerance test on a subset of 43 patients with T2D from the community health cohort to access the role of adipsin in insulin secretion. We further cross-sectionally examined the role of adipsin in plasma, adipose tissue (AT), and secretion in a community cohort of 353 subjects and a hospital cohort of 52 subjects.

Results We found that plasma adipsin has no significant correlation with insulin secretion in people with diabetes. Among the risk factors of T2D, adipsin levels were independently associated only with age, and a positive correlation between plasma adipsin and age among subjects without T2D was lost in patients with T2D. Plasma adipsin levels, AT adipsin expression, and secretion were upregulated both in T2D and aging, with a corresponding drop in Homeostatic Model Assessment for assessing β-cell function. Adipsin expression was positively associated with other aging biomarkers, such as β-galactosidase, p21, and p16. These results also corroborated with existing plasma proteomic signatures of aging, including growth, and differentiation factor-15, which strongly correlated with adipsin.

Conclusions Our results demonstrate an increase in circulating adipsin in T2D and aging, and it scores as a candidate plasma marker for aging specifically in non-T2D population.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Adipsin (complement factor D) is a member of serine protease which catalyzes the rate-limiting step in the alternative complement pathway activation and is found to be associated with paraments of obesity and glucose metabolism.

WHAT THIS STUDY ADDS

⇒ Our research revealed that in individuals with diabetes, plasma adipsin has no notable impact on insulin secretion; however, a compensatory increase in adipsin is noted in patients with type 2 diabetes (T2D).

⇒ Our research highlights adipsin as a potential marker of adipose tissue aging, regardless of disease contexts, including T2D, and it meets all the requirements to score a candidate for aging.

⇒ Furthermore, the correlation shown between plasma adipsin and its mRNA expression and with bona fide aging marker, GDF-15, reinforces our study.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Identification of robust and reliable age biomarkers, which are physiological, biochemical, and molecular indicators of functional degeneration associated with aging, independent of disease effect, remains the basis.

⇒ Our research highlights that adipsin encompasses all the required criteria to score a candidate for aging.

INTRODUCTION

The adipokine adipsin, a member of the serine protease family, was first discovered while investigating alterations of specific mRNAs involved in preadipocyte differentiation during fat cell development.[1] It was then later identified as complement factor D (CFD), which catalyzes the rate-limiting step in the alternative complement pathway activation.[2] Adipsin is involved in an enzymatic cascade that releases anaphylatoxins such as C3a and C5a, and it has been demonstrated earlier to increase insulin synthesis in pancreatic beta cells through the activation of its cell surface receptor.[3] Implications of circulating adipsin in patients with type 2 diabetes (T2D) have been well explored. However, it remains ambiguous whether adipsin has any independent correlation with risk factors and biomarkers in patients with T2D.

Diabetes is a multifactorial disease with several risk factors including aging, body mass index (BMI), total cholesterol (TC) and triglyceride (TG), waist circumference (WC), and reduced Homeostatic Model Assessment for assessing β-cell function (HOMA-B)

as underscored by the American Diabetic Association (ADA) to identify high-risk individuals. Aging leads to functional decline of cells and tissue, resulting in metabolic diseases. Moreover, it also decreases glucose tolerance[4] and the inability of pancreatic beta cells to release insulin, leading to functional deterioration and loss of peripheral insulin signaling.[5] Diabetes with obesity also shares a well-fortified pathophysiological relationship,[6] with elevated TG and TC levels being independent risk factors for T2D.[7] Insulin resistance and pancreatic beta cell dysfunction are key markers for diabetes and pre-diabetes.[8]

Plasma adipsin levels were lower in the subset of patients with T2D with compromised beta cell function,[3] while increased adipsin levels were associated with a lower risk of future diabetes.[9] However, comparative studies on circulating adipsin concentrations in healthy and in individual with T2D showed both increasing and decreasing trends.[10] We have now re-evaluated the significance of adipsin in metabolic disorders and T2D by cross-sectional investigations of subjects of diverse ages and diabetes status. To address this gap, we recruited 353 subjects from the community cohort and 52 subjects from the hospital cohort of varying age to understand the association between adipsin and age. We sought to identify how adipsin alters with aging and its association in age-mediated diabetic complications.

RESEARCH DESIGN AND METHODS

Study populations

Population cohort

A total of 353 volunteers were recruited via a community-based metabolic health screening program called via "Food to Nutrition Security", which is run by a non-profit organization called SWANIRVAR.[11] Volunteers were recruited from six villages in two districts in the Indian state of West Bengal.

Hospital cohort

Patients undergoing laparoscopic surgery at ILS Hospitals, Kolkata, were recruited as subjects (age >18 years, with or without T2D). About 5 g of omentum adipose tissue (AT) samples was collected from the subjects for respective assays.

Anthropometric measurements and clinical history including duration of any associated disease of the patients were obtained. Type 2 diabetes mellitus was assigned to volunteers using the ADA's criteria.

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed in 43 subjects from the community cohort after an overnight fasting, followed by ingestion of 75 g of anhydrous glucose. Blood samples were taken at fasting (−5 min), 15, 20, 45, 60, 75, 90, and 120 min later in NaF/EDTA vials. The plasma was separated and stored at −80°C for long-term storage.

Blood collection

For community and hospital cohorts, fasting blood samples were collected and the plasma was separated and stored at −80°C for further biochemical analysis.

Biochemical measurements

TG, TC and glucose levels were measured using laboratory kits following the manufacturer's protocol (Randox, UK). Insulin levels were measured by human insulin ELISA kit (Merck, Germany) following the manufacturer's protocol. Homeostasis Model Assessment (HOMA)-Insulin Resistance (IR) was calculated using the formula [fasting plasma insulin (µIU/mL)×fasting plasma glucose (mmol/L)]/22.5; HOMA-beta cell was calculated using the formula (B)=20×fasting insulin (µIU/mL)/[fasting plasma glucose (mmol/L)−3.5].

Lipolysis and glucose uptake assay on AT explants

ATs were processed to remove blood vessels and clots, and minced into small portions. Around 20 mg of tissue was used for lipolysis and glucose uptake.

Lipolysis assay

Adipose explants were treated with or without (-)-isoproterenol (Sigma) at a concentration of 10^{-6} M for 24 hours. Glycerol content was then measured using glycerol assay kit (Sigma) to measure both basal and stimulated lipolysis.

Glucose uptake assay

Adipose explants were incubated with 2% fatty acid-free bovine serum albumin (BSA in KRH buffer for 2 hours, and the explants were stimulated with or without 100 nM insulin for 15 min followed by treatment of 200 µM 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose) (Invitrogen) for 20 min. The explants were then lysed, and the relative fluorescence was measured with a fluorimeter (excitation of 475 nm, emission of 550 nm).

RNA isolation and quantitative PCR (qPCR)

RNA was isolated from the AT. cDNA was prepared using iScript (Bio-Rad) followed by real-time PCR for the quantification of the expression of adipokine genes using SYBR Green (Bio-Rad) by a LightCycler 96 System (Roche).

Primers used for qPCR were as follows: human adiponectin forward: 5′-GGGATTGGAGACTTACG-3′, human adiponectin reverse: 5′-GACTGTGATGTGGTAGG-3′; human adipsin forward: 5′-CTACAGCTGTCGGAGAAG-3′, human adipsin reverse: 5′-CCGCGTGGTTGACTATG-3′; human leptin forward: 5′-GTCAGTCTCCTCCAAAC-3′, human leptin reverse: 5′-CATACTGGTGAGGATCTG-3′; human DPP4 forward: 5′-GTACGGGTTCCATATCC-3′, human DPP4 reverse: 5′-CATAGAAGCAGGAGCAG-3′; human IL-6 forward: 5′-CAAATTCGGTACATCCTC-3′, human IL-6 reverse: 5′-CATCTTTGGAAGGTTCAG-3′; human p16 forward: 5′-TGGACCTGGCTGAGGAG-3′, human p16

reverse: 5′-ATCTATGCGGGCATGGTTAC-3′; human p21 forward: 5′-ACTGTGATGCGCTAATG-3′, human p21 reverse: 5′-GTGTCTCGGTGACAAAG-3′; human GDF15 forward: 5′-TACGAGGACCTGCTAACCA-3′, human GDF15 reverse: 5′-GCACTTCTGGCGTGAGTAT-3′.

Immunoblotting

AT samples were lysed containing lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1% Triton X-100 along with protease and phosphatase inhibitor cocktail (Roche). Protein estimation was done using BCA assay kit (Thermo). Supernatants were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membrane, and incubated overnight with primary antibody followed by incubation with peroxidase-conjugated secondary antibodies, and the bands were visualized with a ChemiDoc MP imaging system (Bio-Rad).

Detection of adipokines

Adipsin, adiponectin, leptin, dipeptidyl peptidase 4 (DPP4), and growth differentiation factor-15 (GDF-15) levels were estimated in the plasma samples using ELISA kits according to the manufacturer's protocol (R&D Systems).

Histology and image analysis

Paraffin sections were imaged under light microscope (EVOS XL Core, Thermo) at ×20 magnification. The stained slides images were analyzed using AdipoCount (Adiposoft V.1.16). We extracted the area and number of each single adipocyte for each annotated region.

Immunohistochemistry (IHC)

Paraffin sections for IHC were baked at 80°C for 15 min and rehydrated. Antigen retrieval was done by heating the slides in a microwave with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Two different kits were used for immunohistochemical staining: VECTASTAIN ABC KIT (biotinylated horseradish peroxidase anti-rabbit IgG) and ImmPRESS duet double staining polymer kit (horseradish peroxidase anti-mouse IgG/alkaline phosphatase anti-rabbit IgG).

Human plasma proteome analysis

We reanalyzed the publicly available data of human plasma proteome from four independent cohorts from the USA and Europe (VASeattle, PRIN06, PRIN09, and GEHA)[12] to calculate the correlation coefficient for 1305 plasma samples with normalized relative fluorescence unit (RFU) of age spanning from 21 to 107 years. Volcano plot of correlation coefficient was generated with the plasma proteome samples. Heatmap was generated using Phantasus[13] by normalized RFU plasma proteome between the age groups.

β-Gal activity

Plasma β-galactosidase activity was performed by using 1 mM 4-methylumbelliferyl-β-D-galactoside (Cayman Chemical, USA) in citrate phosphate buffer pH 4.0, which was used as substrate. About 20 µL of plasma samples was incubated with 200 µL substrate solution for 1 hour at 37°C. Following which, the reaction was stopped by adding 1 mL of 0.25 M glycine carbonate buffer at pH 10.4. Then, 100 µL of the solution containing the fluorescence 4-methylumbelliferone was measured in a fluorimeter (Synergy H1 fluorimeter, BioTek), excitation maximum at 385 nm and emission at 454 nm in black 96-well plates for detection. Enzymatic activity was expressed in µM/mL/hour.

Antibodies

The antibodies CFD (#A8117) and β-galactosidase (#A23723) were purchased from ABclonal (Massachusetts, USA).

Statistical analysis

Clinical characteristics of the cohorts are presented as mean±SD. Shapario-Wilk test for normality was performed for continuous variables. One-way analysis of variance (ANOVA) was done to compare between the age groups for continuous variables and χ^2 test was done for categorical variables. Correlogram was performed using the *Hmisc*, *corrplot*package in R. Age, sex and BMI adjusted are presented for all variables. The independent sample two-sided Student's t-test or Mann-Whitney U test was used to compare numerical variables between groups. Multiple linear regression was done using the *lm*function in R. Analysis and graphs were generated using GraphPad Prism V.8.4.2 (679) and RStudio (V.2023.06.1+524). We considered p value=0.05 as statistically significant.

Study approval

All subjects gave written informed consent before taking part in the studies.

RESULTS

Plasma and AT adipsin are increased in T2D

To unravel how circulating adipsin is associated with insulin secretion, we performed OGTT on a subset of 43 patients with T2D from a community cohort. While glucose-stimulated insulin secretion was expectedly increased, plasma concentrations of adipsin remain unaltered over a 2-hour time period (figure 1A). Moreover, no association between insulin area under the ROC curve (AUC) and adipsin AUC (online supplemental figure 1A) or HOMA-B and circulating adipsin was observed (figure 1B), suggesting that adipsin has no noticeable impact on the glucose-induced insulin secretion in T2D. To further elucidate how circulating adipsin is associated with risk factors of T2D including beta cell function, we stratified the community cohort into subjects with and without T2D based on fasting glucose levels after adjusting for age, sex, and BMI using MatchIt function in R Studio

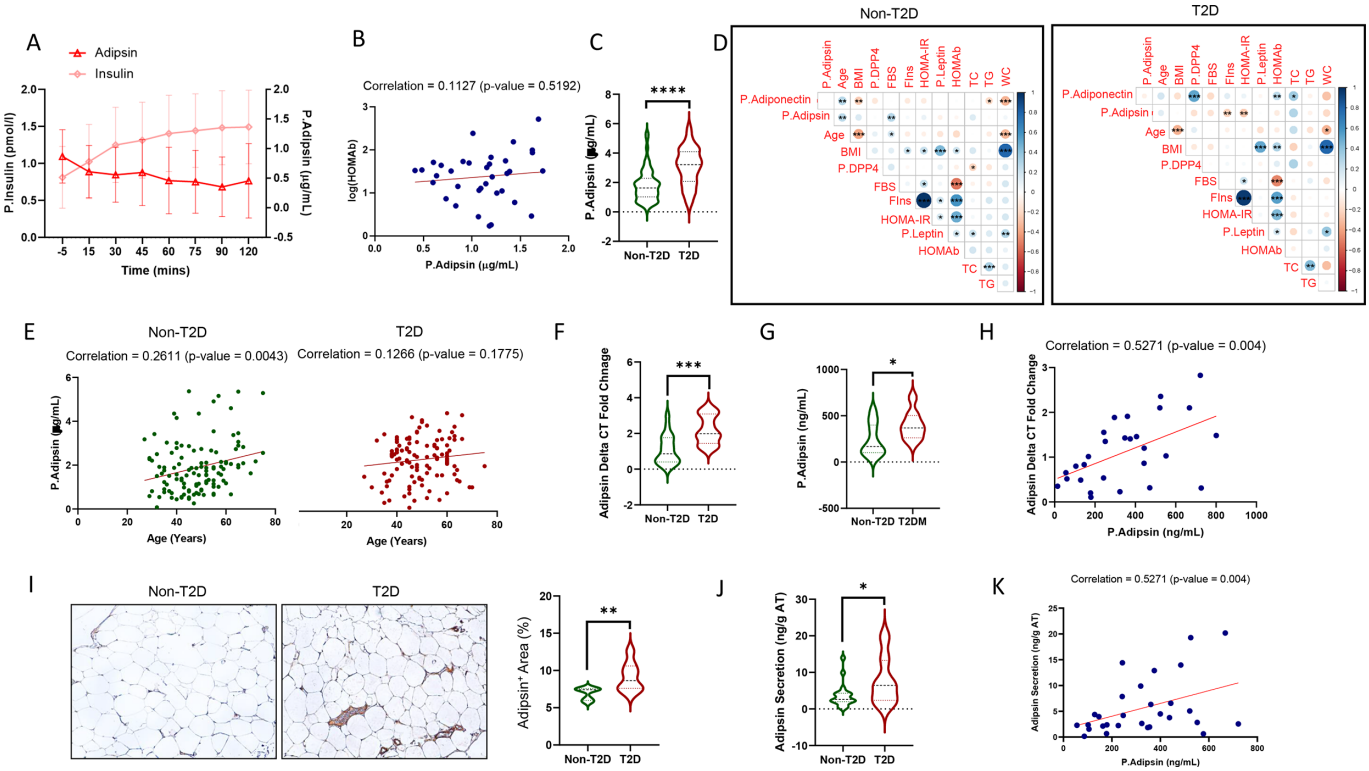

Figure 1 Adipsin mRNA expression in adipose tissue (AT), secretion from AT explants, and circulating levels of adipsin are elevated in patients with type 2 diabetes (T2D). (A) Plasma adipsin (P.Adipsin) and insulin circulating concentrations during oral glucose tolerance test in the community cohort in patients with T2D (n=43). (B) Correlation to log(HOMA-B) and P.Adipsin in the same cohort. (C) P.Adipsin levels in subjects with and without T2D with age, sex, and BMI matched in the same cohort. (D) Correlation matrix of various risk factors of T2D in subjects with and without T2D in the matched cohort. Significantly correlated variables are represented with star based on their significance value; correlation coefficient range is represented in bar. (E) Positive correlation of P.Adipsin and age in subjects without T2D in the matched cohort. (F) Differential adipsin mRNA expression in AT expressed in fold change. (G) P.Adipsin between subjects with and without T2D. (H) Positive correlation between adipsin AT mRNA expression in fold change and P.Adipsin in the hospital cohort. (I) Comparative immunohistochemistry and analysis of adipsin between subjects with and without T2D in the same cohort. (J) Differential adipsin secretion measured from AT explants between subjects with and without T2D in the same cohort. (K) Positive correlation between adipsin secretion and P.Adipsin in the same cohort. The immunohistochemistry images were quantified using ImageJ software. Values are represented in violin plots. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Statistics were calculated using Mann-Whitney test, Kruskal-Wallis one-way analysis and analysis of variance with Bonferonni post hoc test as appropriate. Pearson's correlation was performed between variables to find significant association. BMI, body mass index; FBS, fasting blood sugar; HOMA-B, Homeostatic Model Assessment for assessing β-cell function; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance; TC, total cholesterol; TG, triglyceride.

(n=121/group; table 1). It was comprised of predominantly non-obese patients with T2D with decreased HOMA-B and increased HOMA-IR with modest increase in fasting insulin. Among the plasma adipokine levels, adiponectin was expectedly low, with no change in leptin and DPP4, while mean plasma adipsin levels were more than twofold greater in subjects with T2D than in subjects without T2D (table 1 and figure 1C). To determine how plasma adipsin is associated with the diabetes risk factors including age, sex, BMI, insulin levels, adipokines, etc, correlation plots were generated for the entire cohort (online supplemental figure 1B) and separately for T2D and non-T2D groups (figure 1D). In the non-T2D group, plasma adipsin was found to be only significantly correlated with age (r=0.26, p=0.0043) and fasting blood sugar (FBS) (r=0.25 p=0.002), which were however lost in the T2D group (figure 1E). Interestingly, in the T2D group, adipsin levels had negative correlations with fasting insulin and HOMA-IR. Thus, increased plasma adipsin levels were insufficient to elicit a heightened insulin production in patients with T2D. Moreover, multivariate linear regression analysis showed an independent association of plasma adipsin with age in the non-T2D group (β=0.02, p=0.04) and not among the T2D group (online supplemental table 1).

Since AT dysfunction is common in T2D[14] and that adipsin is a bona fide adipokine,[1] we next investigated adipsin expressions in visceral AT from a hospital cohort. Glycated hemoglobin level (HbA1c) was used to categorize the subjects into non-T2D (n=33) and T2D (n=19) according to ADA criteria, and age, BMI, sex, and body fat (%) were matched. Clinical characteristics of the cohort are presented in table 2. Expectedly, subjects with T2D had higher FBS and fasting insulin levels with an increased

Table 1 Baseline patient characteristics for non-T2D and T2D with age, sex, and BMI-matched community cohort samples

	Non-diabetic	Diabetic	P value
Sample size (n)	121	121	
Age (years)	48.31 (±11.28)	48.97 (±9.98)	0.6296
BMI (kg/m^2)	23.47 (±4.06)	24.43 (±3.4)	0.0502
Sex (male/female)	42/79	42/79	1
FBS (mmol/L)	4.38 (±0.67)	8.4 (±3.24)	<2.2e-16
Fasting insulin (µIU/mL)	6.45 (±6.17)	8.75 (±16.55)	0.0991
HOMA-IR	1.27 (±1.28)	3.25 (±5.75)	7.719E-10
log(HOMA-B)	4.69 (±0.98)	3.28 (±1.05)	<2.2E-16
TG (mg/dL)	118.2 (±67.67)	155.39 (±121.12)	0.0321
TC (mg/dL)	174.33 (±43.42)	180.34 (±46.43)	0.7275
WC (cm)	83.95 (±9.54)	89.1 (±9.8)	0.003436
Plasma DPP4 (µg/mL)	2.98 (±3.98)	2.01 (±1.61)	0.4159
Plasma adiponectin (µg/mL)	5.7 (±4)	2.6 (±2.32)	3.389E-14
Plasma leptin (ng/mL)	27.16 (±28.2)	28.55 (±23.36)	0.05891
Plasma adipsin (µg/mL)	1.29 (±1.17)	3.08 (±1.88)	6.20E-12

BMI, body mass index; DPP4, dipeptidyl peptidase 4; FBS, fasting blood sugar; HOMA-B, Homeostatic Model Assessment for assessing β-cell function; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

Table 2 Baseline patient characteristics for non-T2D and T2D with age, sex, BMI, and body fat (%) matched hospital samples

	Non-T2D	T2D	P value
Sample size (n)	33	19	
Age (years)	42.3 (±11.8)	46.7 (±9.23)	0.07257
BMI (kg/m^2)	33.3 (±8.06)	30.8 (±9.50)	0.3088
Sex (male/female)	12/21	13/6	0.09983
HbA1c	5.34 (±0.61)	8.18 (±1.54)	1.008E-12
FBS (mg/dL)	102.79 (±17)	179.58 (±45.63)	1.85E-09
Fasting insulin (pmol/L)	23.87 (±13.8)	46.74 (±37.75)	0.03348
HOMA-IR	5.75 (±3.51)	21.61 (±19.08)	0.00000914
HOMA-B	5.19 (±0.7)	4.74 (±0.63)	0.005588
Triglycerides (mg/dL)	132.66 (±52.69)	128.19 (±38.18)	0.5957
Cholesterol (mg/dL)	149.55 (±35.55)	158.79 (±30.56)	0.3901
Adipose number	204(± 43.9)	163 (±43.3)	0.08724
Adipose area (R.U.)	11,459.26 (±3808.75)	14,614.77 (±3936.78)	0.1471
Fat mass (%)	41.8 (±11.93)	35.95 (±12.71)	0.2778
Plasma DPP4 (ng/mL)	279.6 (±181.27)	307.01 (±134.78)	0.6259
Plasma adiponectin (ng/mL)	0.43 (±0.32)	0.48 (±0.33)	0.702
Plasma leptin (ng/mL)	396.12 (±277.09)	164.64 (±114.16)	0.01224
Plasma adipsin (ng/mL)	280.35 (±220.95)	396.71 (±153.11)	0.03249

Data are represented by mean±SD.
BMI, body mass index; DPP4, dipeptidyl peptidase 4; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance.

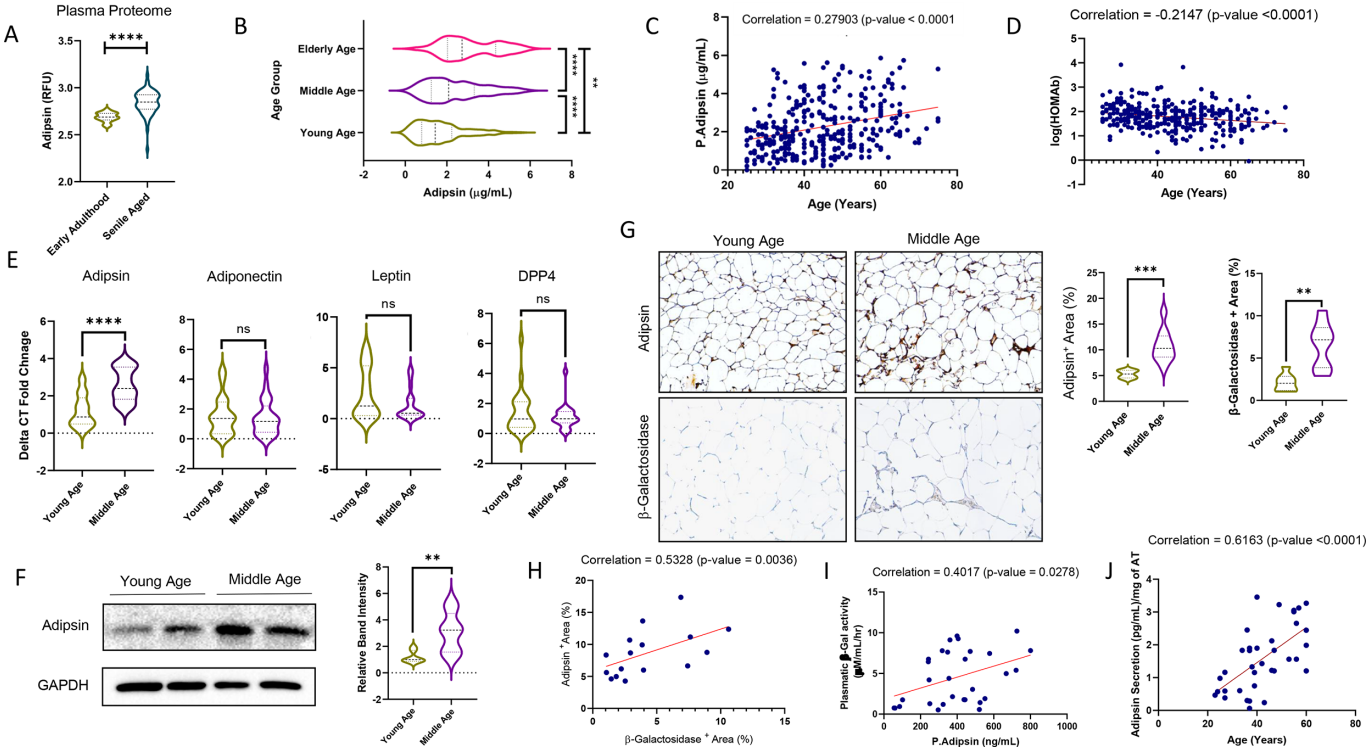

Figure 2 Adipsin mRNA expression in adipose tissue (AT), secretion from AT explants, and circulating levels of adipsin are increased across the various age groups. (A) Plasma adipsin (P.Adipsin) levels between early adulthood and senile aged in existing plasma proteome data. (B) P.Adipsin increases with age across the various age groups of young (25–44 years), middle (45–60 years), and elderly (61–75 years) age in the community cohort. (C) Positive correlation between P.Adipsin with age and (D) log(HOMA-B) with age in the same cohort. (E) Differential adipsin mRNA expression in AT expressed in fold change between young and middle age, while adiponectin, leptin, and DPP4 expression remained unvaried in the hospital cohort. (F) Representative immunoblot with analysis showing the expression of adipsin in AT between young and middle age subjects in the same cohort. (G) (Left panels) Comparative immunohistochemistry and β-galactosidase of AT of young and middle age subjects in the same cohort. (Right panels) Analysis of positively stained area between the groups. (H, I) Correlation between Adipsin$^+$ Area (%) and β-galactosidase$^+$ Area (%) of AT (H) and correlation between plasma β-galactosidase activity with plasma adipsin (I) in the same cohort. (J) Correlation between adipsin secretion from AT with age in the same cohort. The western blot and immunohistochemistry images were quantified using the ImageJ software. Values are represented in violin plots. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Statistics were calculated using Mann-Whitney test, Kruskal-Wallis one-way analysis, and analysis of variance with Bonferonni post hoc test as appropriate. Pearson's correlation was performed between variables to find significant association. DPP4, dipeptidyl peptidase 4; HOMA-B, Homeostatic Model Assessment for assessing β-cell function.

HOMA-IR and lower HOMA-B and had unvaried TG and TC levels, suggesting that patients with T2D were more insulin resistant with poor glycemic control conjunct with reduced beta cell function, independent of dyslipidemia. To access AT dysfunction in T2D, we performed glucose uptake and lipolysis assay in the ex vivo explants culture. Subjects with T2D had lower glucose uptake with increased lipolysis (online supplemental figure 1C,D) underscoring AT dysfunction in diabetes.

We found that adipsin mRNA expression increased in the AT of subjects with T2D (n=52; figure 1F), while adiponectin, leptin, and DPP4 expressions remained unchanged (online supplemental figure 1E-G). Consistent with the community cohort data, plasma adipsin also showed a significant correlation with age among the non-T2D group, while such significance is lost in the T2D group (online supplemental figure 1H). Plasma adipsin was also higher in the T2D group and was positively correlated with AT expression levels (figure 1G,H). AT protein levels assayed by IHC also showed increased adipsin protein expressions (figure 1I). Consistently, secretion of adipsin from the explant culture was also enhanced in patients with T2D (figure 1J) and correlate with plasma adipsin levels (figure 1K). Taken together, increased plasma adipsin, AT adipsin expression, and secretion did not have any clinical correlate with glycemic parameters in T2D. Conversely, correlation between plasma adipsin and age in subjects without T2D is exceedingly lost in patients with diabetes.

Circulating levels and AT expressions of adipsin are increased across age groups

Aging underscores the progressive decline in AT function and results in secretion of altered adipokines which interact and communicate via diverse paracrine and autocrine mechanisms.[15] By analyzing publicly available

Table 3 Multiple linear regression analysis in hospital samples, with secreted adipsin and other clinical parameters

	β (95% CI)	P value
Age (years)	7.3 (1.64 to 12.97)	0.0147
Sex, male	0.16 (–117.56 to 117.89)	0.9977
BMI (kg/m^2)	–2.88 (–12.57 to 6.81)	0.5373
FBS (mg/dL)	0.34 (–4.74 to 5.42)	0.8893
Fasting insulin (µIU/mL)	–2.98 (–6.3 to 0.35)	0.0759
log(HOMA-B)	45.86 (–208.6 to 300.33)	0.7074
log(HOMA-IR)	78.89 (–162.02 to 319.8)	0.4975
log(plasma leptin)	51.82 (–24.39 to 128.02)	0.1687
log(plasma adiponectin)	13.37 (–56.51 to 83.24)	0.6905
log(plasma DPP4)	–10.66 (–99.56 to 78.25)	0.8027

Data are represented by mean±SD.
BMI, body mass index; DPP4, dipeptidyl peptidase 4; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; HOMA-B, Homeostatic Model Assessment for assessing β-cell function; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance.

plasma proteome data[12] of 42 adipokines across early adulthood and senile aged groups, we show that plasma levels of multiple adipokines are increased including adipsin (CFD; p<0.001) (figure 2A and online supplemental figure 2). For validating the plasma proteome data, we next stratified the community cohort (n=353) into various age groups according to WHO classification (young age 25–44 years, n=182; middle age 45–60 years, n=137; elderly age 61–75 years, n=34).[16] Clinical characteristics of the cohort are presented in online supplemental table 2. Adiposity parameters, such as BMI, WC, and glycemic parameters, such as FBS, fasting insulin, remain unaltered across the age groups. However, HOMA-B, an index for insulin secretion, decreased with aging (p<0.005), while plasma adipsin levels increased with aging (figure 2B; p<0.001). Conversely, changes in the levels of other adipokines such as leptin, DPP4, and adiponectin did not reach statistical significance. Moreover, plasma adipsin positively correlated with age (figure 2C), while HOMA-B was expectedly negatively associated with aging (figure 2D). Thus, circulating adipsin levels are increased with age with significant correlation yet fails to improve age-associated decline in insulin secretory capacity. To examine the adipsin expression in aging AT, we stratified the study subjects of the hospital cohort to young and middle age group. We found that adipsin gene expression was increased in the middle age group, while adiponectin, leptin, and DPP4 mRNA expressions remained unchanged (figure 2E). Consistently, adipsin protein levels were also increased in aging AT (figure 2F,G). An established marker of aging, β-galactosidase, was expectedly increased in AT of aged subjects (figure 2G). Interestingly, age-dependent increase in positive stain for adipsin and β-galactosidase in AT were positively correlated (figure 2H). Further, plasma β-galactosidase activity is also significantly associated with plasma adipsin levels (figure 2I). Moreover, AT-derived secreted adipsin is also significantly correlated with age (figure 2J) and multivariate linear regression analysis further revealed an independent association of adipsin secretion with age, highlighting the fact that adipsin secretion increases in an age-dependent manner (table 3).

Plasma adipsin is a biomarker of aging

Aging is a heterogeneous process with significant variability across various population.[17] Identification of definitive biomarkers of age is critical for accurate risk stratification. This necessitates investigating different cohorts of individuals, with each cohort falling within a particular age range. Since plasma adipsin is independently associated with aging, we surmise whether circulating adipsin could serve as a biomarker of aging. To this end, we first analyzed the publicly available plasma proteome data represented by a volcano plot of correlation coefficient and significance value of 1306 number of proteins with age. Among the positively correlated proteins, adipsin/CFD level was found to be significantly upregulated (r=0.56, p<0.001), while GDF-15 had the highest significance (adjusted p value=5.6817E-23) (figure 3A). Additionally, we also found that adipsin levels significantly correlated with age in two independent European and USA cohorts, as classified in the published work[12] (figure 3B–D). Other adipokines, such as adiponectin leptin and DPP4, did not reveal any association with age in our cohort (online supplemental figure 3A-C). We next determined the gene expressions of known senescence markers, such as GDF-15, p21, p16, and IL-6, in the ATs. Expressions of GDF-15, p21, and p16 were upregulated in the middle aged subjects, while we did not find any such increase in the IL-6 expression (figure 3E and online supplemental figure 4A). Interestingly, mRNA expression of adipsin was significantly associated with GDF-15, p21, p16 and not with IL-6 (figure 3F–H and online supplemental figure 4B).

As GDF-15 is an established marker of aging,[18 19] we next asked whether adipsin could also qualify for the same. To this end, we first determined the plasma levels of GDF-15 and adipsin in the hospital cohort. As shown in figure 3E,F, circulating levels of both GDF-15 and adipsin were increased in the middle-aged group. Expectedly, plasma GDF-15 and adipsin both correlated with age (figure 3G,H). Similar to adipsin, plasma GDF-15 levels remained significantly correlated in the non-T2D group, while the significance was lost in the T2D group (online supplemental figure 4C). Of note, plasma levels and AT-secreted adipsin also had a positive correlation with GDF-15 (figure 3I,J). Thus, not only plasma and AT secretion of adipsin are enhanced with age, these parameters are also in sync with the established aging marker, GDF-15.

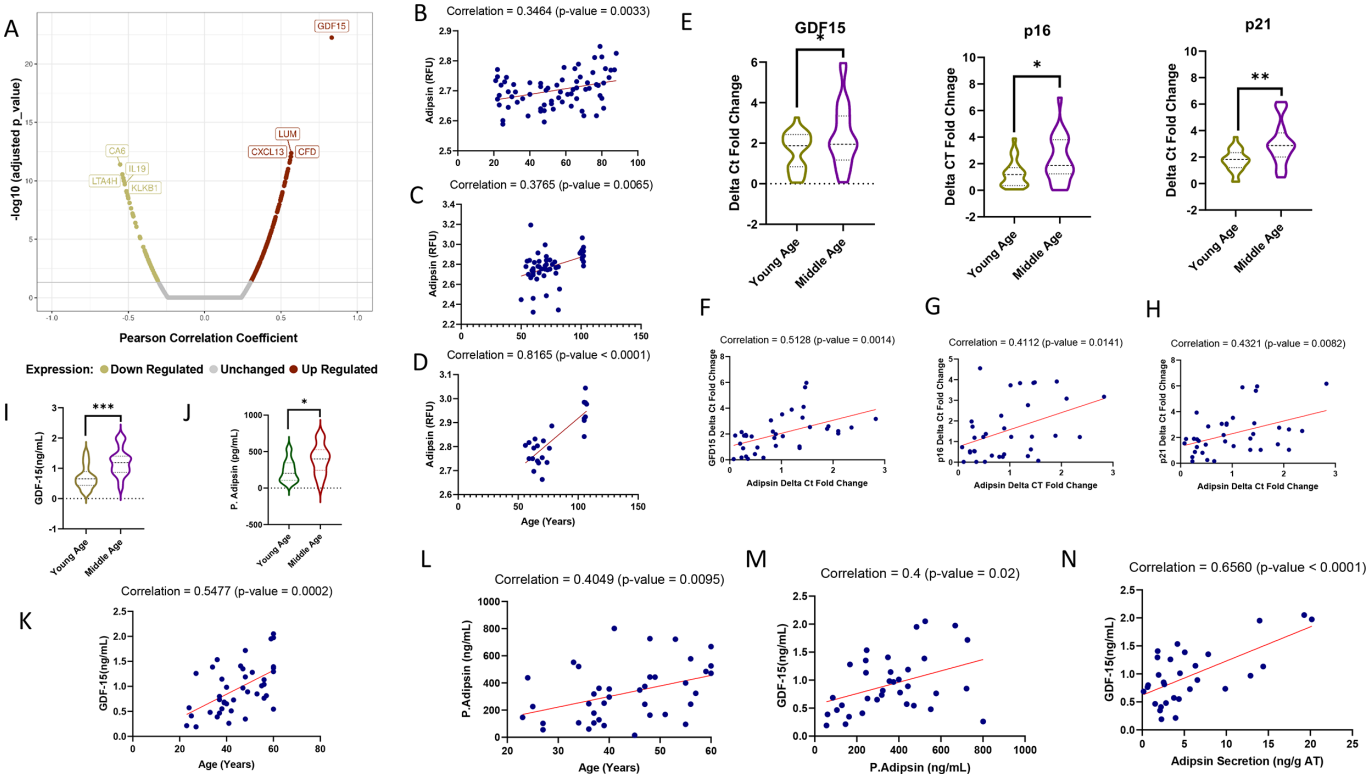

Figure 3 Adipsin increases with age and serves as a biomarker of aging. (A) Volcano plot representing the correlation of various plasma proteome which are upregulated, downregulated, or unchanged with age. Adipsin/CFD is remarkably upregulated with age. (B–D) Plasma adipsin (P.Adipsin) correlated with age in three independent cohorts of plasma proteome data from (B) Seattle and (C, D) Europe. (E) Differential GFD-15, p16, and p21 mRNA expression in the adipose tissue (AT) expressed in fold change between young and middle-aged groups. (F–H) Correlation between adipsin delta Ct fold change with GDF-15 (F), p16 (G), and p21 (H) in the same cohort. (I, J) Differential plasma GDF-15 (I) and adipsin levels (J) between young and middle age in the hospital cohort. (K, L) Correlation between plasma GDF-15 and age (K) and between plasma adipsin and age in the same cohort. (M, N) Correlation between plasma GDF-15 and adipsin (M) and between plasma GDF-15 and adipsin secretion from AT (N). Values are represented in violin plots. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Statistics were calculated using Mann-Whitney test, Kruskal-Wallis one-way analysis, and analysis of variance with Bonferonni post hoc test as appropriate. Pearson's correlation was performed between variables to find significant association. CFD, complement factor D; GDF-15, growth differentiation factor-15.

DISCUSSION

Categorizing the patient cohorts either by glycemic control or by age, we consistently found an increase in plasma adipsin levels in both classifications. Beta cell secretory function measured by HOMA-B was reciprocally low both in T2D and aging, suggesting that an increase in plasma adipsin fails to restore beta cell function in these conditions. Interestingly, we found that circulatory adipsin level correlates with age in different cohorts in Europe, the USA, and India, and such association was independent of other clinical parameters. Our study thus proposes adipsin to be a biomarker candidate for aging. Association of plasma and adipose-secreted adipsin with other senescence markers, such as β-galactosidase, p16 and p21, and bona fide aging marker, GDF-15, further ratifies our claim.

Adipsin has been reported to improve beta cell function, resulting in increased insulin secretion, and restoring adipsin in T2D mice not only reduced hyperglycemia but also preserved beta cell mass by increasing beta cell survival and preserving transcriptional identity.[9] Additionally, higher adipsin levels were associated with lower risk of future T2D incidence. However, the role of adipsin in insulin secretion in patients previously diagnosed with T2D remains elusive. Patients with T2D are shown to have higher levels of adipsin than subjects without T2D, even after adjusting for age, sex, and BMI. Our results are consistent with those of Lo *et al*, who noted increased adipsin levels in the early phases of the metabolic syndrome.[3] Furthermore, higher blood levels of adipsin in those with impaired glucose tolerence (IGT) provide support for the notion that the body may be using an internal mechanism to make up for reduced insulin secretion.[3] Increased adipsin synthesis is a sign of T2D in its early stages and acts as a sort of compensation in an organism's attempt to re-establish normal glucose and lipid metabolism. As T2D advances in the context of AT dysfunction, adipsin levels may further increase, which, in turn, could become insufficient to rescue beta cell failure. Our OGTT data, however, revealed that adipsin has no impact on the glucose-stimulated insulin secretion, further suggesting that enhanced levels of

circulating and adipose-derived adipsin cannot elicit adequate insulin secretion in hyperglycemia. As adipsin induces insulin secretion through alternative complement pathway by cleaving factor B when it is in complex with C3b, possibly through the action of C3a, and that other regulatory loops could potentially hinder it in T2D, impact of adipsin may further be attenuated. For example, serum carboxypeptidases rapidly transform C3a to the inactive form C3a-desArg, and circulating DPP4, which is often increased in T2D,[20] might inhibit C3a.

Limited investigations on the effects of adipsin on human subjects have been conducted, and the results have been inconsistent and diverse.[21–23] Plasma adipsin levels were incongruous with patients with T2D[23–26] and had heterogeneous association with HOMA-B possibly due to other confounding factors such as age, BMI, and sex, which aid in T2D pathogenesis and might be stronger determinants of plasma adipsin. Thus, the relevance of adipsin in age-associated T2D and T2D subtypes[27 28] needs further investigations. Our study additionally indicates that AT might not be the only tissue that produces adipsin; to some extent, it is also released from other tissues. Along with the subcutaneous tissue and visceral AT, the tibial nerve, coronary arteries, liver, and female breasts additionally express adipsin, according to Genotype-Tissue Expression (GTEx) repository.[29] According to the Human Protein Atlas, human CFD is most abundant in the AT where it is mostly expressed by mesenchymal origin cells like fibroblasts, followed by immune cells like macrophages and monocytes, and adipocytes. Such expression pattern is consistent with our IHC data. We investigated the relationship between adipsin mRNA expression from AT and plasma adipsin since the latter is derived from a variety of other tissue depots and found significant positive association. To determine how tissues other than AT contribute to the amounts of circulating adipsin needs to be further investigated.

Our study underscores adipsin to be a candidate marker for aging based on the American Federation for Ageing Research[30 31] criteria for aging biomarkers. Their recommendations state that a biomarker of age should have the following qualities: (1) better performance for predicting age and age-associated outcomes; (2) monitoring the aging process in systems rather than disease's effects; and (3) the ability to be repeatedly tested in a safe manner, both in humans and laboratory animals. Adipsin encompasses all the required criteria to score a candidate for aging, since adipsin increases and correlates with aging independent of disease status, including T2D. Additionally, our study revealed the association of adipsin with age in three independent cohorts. Furthermore, adipsin levels could be easily monitored in both human and rodents. We have also showed that other adipokines, such as adiponectin, leptin, and DPP4, remained unaltered in aging, suggesting specificity of adipsin in the context of aging. Our study is further strengthened by association of plasma adipsin and its mRNA expression with bona fide aging marker, GDF-15. Together, our data implicate adipsin as an important candidate for aging. Adipsin could thus also be a potential AT-specific aging marker, which however needs further study.

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