Predictors of HbA1c among adipocytokine biomarkers in African American men with varied glucose tolerance

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Research

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Abstract

Background: Adipocytokines are important in type 2 diabetes (T2D). This study explored adipocytokine associations with acute and chronic hyperglycemia in African American Men (AAM).

Methods: Fourteen adipocytokines were measured (multiplex assay) in blood samples from men with normal glucose tolerance (NGT) or T2D (drug-naïve MF(-) or using metformin MF(+)). Acute and chronic hyperglycemia were evaluated at 120-minute of OGTT and by HbA1c, respectively.

Results: AAM with T2D (N=21) compared to NGT (N=20) were significantly older (59 vs. 54 years) and had higher body mass index (BMI 35 vs. 27 kg/m²) (p<0.01 for both). Fasting and 120-minute OGTT glucose and insulin were higher in T2D than NGT, however, differences did not reach statistical significance after adjusting for age and BMI. In the fasted state, TNF-a, IL-6, PAI-1 (p<0.01 for all) and IL-13, adiponectin, adipsin, lipocalin (p<0.05 for all) were lower in T2D compared to NGT. At 120-minute post-glucose load (acute hyperglycemia) TNF-a, IL-6, IL-13, IL-8, PAI-1, adiponectin, adipsin (p<0.01 for all) and lipocalin, resistin (p<0.05 for both) were lower in T2D than in NGT group. There were no statistical differences for the other adipocytokines including GM-CSF, IL-7, IL-10, IP-10, and MCP-1. Regression analysis (adjusted for age and BMI) showed that fasting IL-8, TNF-a, adiponectin, lipocalin, resistin, adipsin, and PAI-1 were all associated with HbA1c (p<0.05 for all). Further modeling revealed that after adjusting for age, BMI, glucose tolerance status and metformin use, only adipsin remained significantly associated with HbA1c (p=0.004). The model including adipsin, TNF-a, age, BMI, and group designation (i.e. NGT, MF(-), MF(+)) explained 86% of HbA1c variability.

Conclusions: The study suggested that adipsin could be independently associated with HbA1c in AAM with varied glucose tolerance. Additional studies should corroborate these data and provide mechanistic insights for enabling adipsin-related discoveries of novel T2D treatment.

Introduction

Type 2 diabetes (T2D) is a common and emergent problem in USA and worldwide. More than 30 million Americans have diabetes (about 1 in 10), and 90% to 95% of them have T2D [1]. In the last 20 years, the number of adults diagnosed with T2D has more than doubled as the American population has aged and become more overweight or obese [1]. Worldwide the number of people with T2D has risen from 108 million in 1980 to 422 million in 2014 according to World Health Organization (WHO) [2]. In the United States and worldwide T2D is estimated to be the seventh leading cause of death [1, 2]. Worldwide in 2016, an estimated 1.6 million deaths have been attributed to diabetes and commonly death occurs before the age of 70 years [2].

T2D management remains a challenge despite of recent significant advances [3-5]. Part of this challenge includes the multifactorial metabolic confluence contributing to glucose homeostasis [6]. Among these factors, inflammation-related proteins cytokines [7-12] and adipose tissue-secreted proteins adipokines [13-16] appear to play important role. Notably, numerous studies have explored the hypothesis that
inflammation and adiposity may contribute to T2D by investigating cell signaling, animal models, and gene knockout models [7, 17, 18]. Results of these studies support the concept that T2D involves adipocytokines as mediators of low-grade subclinical inflammatory process contributing to pathogenesis of T2D at all stages, i.e. from development to progression, complications, and mortality [7-9, 19-23]. To date, however, clinical trials have failed to translate these discoveries into T2D treatment [24-26]. Recently developed new methodology enables expanding search for novel adipocytokines that can be important for advancing T2D management.

Adipokines are secreted by white adipose tissue and have been found to be active contributors to glucose homeostasis [13-16]. Among these adipokines, adipsin, resistin, and lipocalin-2 are implicated as important mediators of inflammation [11, 13, 27]. There are also data suggesting that the degree of adipose tissue inflammation, not obesity per se, is a precondition for the development of insulin resistance in T2D [28, 29]. Plasminogen activator inhibitor 1 (PAI-1) can likewise be included into adipokine family of proteins since it is secreted in substantial quantities by adipose tissue [14, 27, 30]. PAI-1, a well-known regulator of fibrinolytic system, is also implicated in inflammation-related T2D complications such as cardiovascular disease [11, 20, 31].

Cytokines are small proteins secreted by variety of cells including stromal and other cells (e.g. lymphocytes, macrophages) in adipose tissue that can act in autocrine, paracrine and hormone-like fashion to promote or reduce inflammatory processes [7-11]. Cytokines that promote inflammation include interleukins (IL) IL-7 and IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α among others [7-9, 27]. Cytokines that reduce inflammation include IL-10 and IL-13 [32, 33] while IL-6 has been implicated as pro- and anti-inflammatory mediator [34].

Recent advances in high sensitivity bead-based multiplex assay technologies [11, 35-37] have resulted in the simultaneous measurement of adipocytokines from a single blood sample with high accuracy and reproducibility. These proteomic multiplexed immunoassays have gained widespread applications for detecting key biomarkers to provide mechanistic insight into various conditions including T2D and its complications [11, 36-38]. The multiplex methodology can improve individualized medical decision-making based on biomarker profiles in addition to specific clinical risk factors as suggested by data for cardiovascular disease [11, 36-38]. The gap of knowledge, however, remains in understanding whether individual adipocytokines are associated with acute and/or chronic hyperglycemia in human studies. We previously have observed varied response of 35 biomarkers to acute hyperglycemia in men with prediabetes [39], while other studies also observed inconsistent associations of selected adipocytokines with acute (first phase) insulin response [40] and hemoglobin A1c (HbA1c) [41] in diabetic individuals. Therefore, the primary objective of this pilot study was to explore whether the adipocytokine responses (measured by multiplex assay) to acute hyperglycemia differed by T2D status during an oral glucose tolerance test (OGTT). Secondly, we explored whether fasting adipocytokines were associated with chronic hyperglycemia measured by HbA1c (Figure 1).
Materials And Methods

Design and participants

The current analysis was performed as a sub-analysis in the cross-sectional study of Glucose Tolerance and Vitamin D insufficiency in African American Men (AAM), investigating glucose metabolism and selected biomarker interactions in AAM [42, 43]. The original study was conducted at an urban Veteran Administration Medical Center (VAMC). The intended subject populations (N=100) included those with T2D (N=50) and those without T2D (normal glucose tolerance participants, N=50) [42, 43]. The inclusion criteria were glycohemoglobin HbA1c < 5.7% for NGT or 6.5-7.4% for T2D, age 35-70 years, body mass index (BMI) 22-39.9 kg/m^2, and 25OH-vitamin D (25OHD) < 30 ng/ml. Diabetic participants were allowed to participate so long as they were non-medically treated (lifestyle modification) or using metformin alone. Exclusion criteria were chronic kidney disease (stages 3b, 4, and 5), chronic glucocorticoid use (3 months or longer), taking non-metformin antihyperglycemic medications, significant T2D complications, and health conditions requiring recent (within 6 months) hospitalization. The participants came for a single study visit after an overnight fast. The research staff reviewed past medical history and medication use. The OGTT using 75 g glucose load was performed and blood was collected for biomarker analysis as previously described [42, 43]. For this pilot study blood samples for analysis of adipocytokines, were chosen at random based on availability of samples at 0- and 120-minute of OGTT. The final sample included 41 participants, N=20 with normal glucose tolerance, and N=21 with T2D. The recruitment dates were from December 01, 2013 to April 15, 2016 (Fig. 1).

Biometrics and glycemic measures

Biometrics including weight (kg), height (m), and calculated BMI (kg/m^2) were performed using study-specific standardized techniques [42, 43]. The age-adjusted Charlson index of chronic disease was calculated as previously described [42, 43]. Glycemic control was assessed by HbA1c, as well as fasting and 2-hour (120-minute) post-glucose load glucose and insulin [42-44]. Blood glucose, insulin and HbA1c were measured in the CLIA-approved clinical laboratory applying laboratory standards of care and references [42-44].

Adipocytokine biomarkers analysis

Blood for biomarkers was collected during OGTT and serum was stored at -80°C until assayed. Adipocytokine biomarkers were measured at the Rush Biomarker development Core research laboratory, applying laboratory standards of care and references [39, 45, 46]. A total 14 serum adipocytokine biomarkers were explored in the Luminex immunobead platform using commercially available kits that were implemented according to the manufacturer's recommended protocols. All primary data points were collected on a Luminex FLEXMAP 3D® system with concentrations calculated based on 7-point standard curves using a five-parametric fit algorithm in xPONENT® v4.0.3 (Luminex Corp., Austin, TX). All data met minimum quality control thresholds defined by the kit manufacturer with percent coefficient of variation (%CV) values ≤10, all as previously defined [45, 46].
The cytokines including GM-CSF, IL-6, IL-7, IL-8, IL-10, IL-13, TNF-α, IP-10, MCP-1 were analyzed using the MILLIPLEX MAP Human High Sensitivity T Cell Panel - Immunology Multiplex Assay (catalog number HCYTOMAG-60K-01, Millipore Corporation, Billerica, MA). The adipokines including adiponectin, adipsin, lipocalin-2/NGAL, PAI-1 (Total), and resistin were analyzed using MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 - Endocrine Multiplex Assay (catalog number HADK1MAG-61K-05, Millipore Corporation, Billerica, MA). The multiplex bead technology has been confirmed to be highly reproducible and appropriately correlated with values obtained with classical Enzyme-linked immunosorbent assays [47-50].

Statistical analysis

The current analysis explored the cross-sectional associations between adipocytokines at baseline (fasting state, 0-minute OGTT) and following the 2-hour post-glucose OGTT (i.e. acute hyperglycemia) in research participants grouped by chronic glycemic control (i.e. HbA1c). The groups were normal glucose tolerance (NGT), and T2D, either drug naïve for metformin (MF(-)), or taking metformin (MF(+)). Data were described as mean ± standard deviation (SD) or standard error (SE) and log transformation was applied as appropriate for non-normal data distribution. To explore the independent associations between chronic glycemic control and adipocytokines we used linear regression analysis, adjusting for age and BMI. We then used backward stepwise multiple linear regression analysis to explore the relationship between chronic glycemic control (HbA1c) and fasting adipocytokines. Variables were excluded from the final model if the p-value>0.1000. Post estimation collinearity was probed using the variance inflation factor (VIF), for collinearity and 1/VIF for multicollinearity. All analyses were adjusted for age and BMI. An alpha p-value of p<0.05 was considered statistically significant. All analyses were performed in STATA v.14 (College Station, TX, USA).

Results

Study population characteristics and glycemic indices

The final sample included 41 participants, 20 with NGT and 21 with T2D. Sixteen of the T2D participants were taking metformin, while 5 were metformin naïve. Men with T2D were significantly older, weighed more, and had higher BMI and HbA1c than participants with NGT (p<0.01 for all) (Table 1). Fasting and 120-minute post-glucose load glucose and insulin were higher in T2D than NGT, however, the differences were not significant after adjusting for age and BMI. Overall, participants with T2D suffered a higher burden of disease as assessed by Charlson index (<0.05) but were prescribed a similar number of medications. The results differed slightly when T2D participants were separated into taking metformin MF(+) or metformin naïve MF(-). Specifically, MF(+) participants carried a significantly higher burden of disease (Charlson index (p<0.001) while tended to be prescribed less medications (p=0.07). Because of the age and adiposity differences, we adjusted all subsequent analyses for age and BMI.

Table 1 Participants characteristics by glucose status
| Variable                  | NGT (n = 20) | T2D (n = 21) | MF(+) (n = 16) | MF(-) (n = 5) | Combined (n = 21) |
|---------------------------|--------------|--------------|----------------|--------------|------------------|
| General                   |              |              |                |              |                  |
| Age, yr                   | 54.1 ± 5.8   | 60.1 ± 3.1** | 56.6 ± 6.7     | 59.2 ± 4.3** |                  |
| Body weight, kg           | 84.7 ± 11.5  | 105.5 ± 14.2** | 106.8 ± 14.7** | 105.8 ± 13.9** |                  |
| BMI, kg/m²                | 27.4 ± 3.3   | 35.4 ± 3.2 **| 34.8 ± 2.4**   | 35.3 ± 3.0 **|                  |
| Charlson index            | 1.5          | 3.0 **       | 1.0            | 3.0*          |                  |
| Number of all meds        | 9.0          | 9.0          | 2.0            | 8.0           |                  |
| Fasting Glycemic Indices  |              |              |                |              |                  |
| HbA1c, %                  | 5.3 ± 0.3    | 6.9 ± 0.5**  | 6.8 ± 0.2**    | 6.8 ± 0.4**  |                  |
| Fasting glucose, mg/dL    | 93.6 ± 17.4  | 123.6 ± 27.8 | 119.8 ± 24.0   | 122.7 ± 26.4 |                  |
| Fasting Insulin, mIU/L    | 8.1 ± 3.9    | 35.9 ± 55.7  | 19.7 ± 5.1     | 32.1 ± 48.8  |                  |
| 120min Glycemic Indices   |              |              |                |              |                  |
| Glucose 120 min, mg/dL    | 109.6 ± 42.0 | 198.1 ± 70.1 | 187.4 ± 100.0  | 195.6 ± 75.5 |                  |
| Insulin 120 min, mIU/L    | 43.3 ± 45.1  | 104.2 ± 63.7 | 67.9 ± 25.6    | 95.6 ± 58.5  |                  |

Data are mean ± standard deviation. Glucose and insulin were Log transformed prior to analysis. All group comparisons are adjusted for age and BMI where applicable. P-value of <0.05 is used to denote statistical significance, reference group is NGT. Fasting represents 0-minute of OGTT. Abbreviations: meds, medications; NGT, normal glucose tolerance; T2D, type 2 diabetes; MF(+) taking metformin; MF(-), metformin naïve; 120 min, 120 minutes post-glucose load during OGTT; * p<0.05, ** p<0.01.

Table 2 Participants characteristics by adipokine levels
| Variable | NGT (n = 20) | T2D (n = 21) | MF(+) (n = 16) | MF(-) (n = 5) | Combined (n = 21) |
|----------|--------------|--------------|----------------|---------------|------------------|
| Fasting Adipokines | | | | | |
| Adiponectin, pg/mL | 60785.0 ± 64564.3 | 13495.4 ± 15054.5* | 12227.2 ± 6467.1 | 13193.0 ± 13366.0* |
| Lipocalin, pg/mL | 896.6 ± 544.7 | 407.7 ± 203.8* | 399.3 ± 225.3* | 405.7 ± 203.3* |
| Resistin, pg/mL | 139.2 ± 88.5 | 88.6 ± 52.7 | 70.1 ± 56.3 | 84.2 ± 52.8 |
| Adipsin (pg/mL) | 8757.6 ± 3036.5 | 7524.5 ± 4096.6** | 7469.6 ± 1512.3 | 7511.5 ± 3611.7* |
| PAI-1 (total), pg/mL | 196.2 ± 101.1 | 167.6 ± 99.8** | 161.1 ± 53.3* | 166.1 ± 89.7** |
| IP-10, pg/mL | 408.5 ± 182.8 | 441.0 ± 242.8 | 286.1 ± 52.3 | 404.1 ± 222.1 |
| MCP-1, pg/mL | 434.4 ± 320.4 | 334.6 ± 146.8 | 438.4 ± 354.9 | 359.3 ± 20 |
| 120 min Adipokines | | | | | |
| Adiponectin, pg/mL | 58964.6 ± 67994.0 | 10189.7 ± 5773.9** | 9518.4 ± 1579.2** | 10029.8 ± 5827.3** |
| Lipocalin, pg/mL | 568.0 ± 282.5 | 282.5 ± 143.6 | 272.9 ± 195.9 | 280.2 ± 152.2* |
| Resistin, pg/mL | 98.5 ± 59.7 | 66.1 ± 43.5 | 44.4 ± 42.1* | 60.9 ± 43.2* |
| Adipsin, pg/mL | 7946.6 ± 2772.8 | 6745.8 ± 5154.4** | 5364.1 ± 1579.2** | 6416.9 ± 4559.4** |
| PAI-1 (total), pg/mL | 185.4 ± 110.6 | 123.7 ± 84.0** | 136.6 ± 91.9** | 324 ± 125 |
| IP-10, pg/mL | 345 ± 136 | 140.6 ± 96.5** | 123.7 ± 84.0** | 345 ± 125 |
| MCP-1, pg/mL | 383.5 ± 285.5 | 290.7 ± 131.4 | 348.0 ± 207.3 | 395.7 ± 261.5 |

Data are mean ± standard deviation. Adipocytokines were Log transformed prior to analysis. All group comparisons are adjusted for age and BMI where applicable. P-value of <0.05 is used to denote statistical significance, reference group is NGT. Fasting represents 0-minute of OGTT. Abbreviations: NGT, normal glucose tolerance; T2D, type 2 diabetes; MF(+) taking metformin; MF(-), metformin naïve; 120 min, 120 minutes post-glucose load during OGTT; * p<0.05, ** p<0.01.

**Table 3** Participants characteristics by cytokine levels
### Adipocytokines and diabetes status

To explore the effect of diabetes status on adipocytokines in both the fasted and 120-min post-glucose load we used linear regression analysis, adjusting for age and BMI (Table 2). In the fasted state, i.e. before the glucose load (0-min OGTT), cytokines TNF-a and IL-6 (p<0.01 for both) as well as IL-13 (p<0.05) were lower in T2D compared to NGT. These differences remained among MF(+), while among MF(-), the cytokines TNF-a (p<0.01) and IL-6 (p<0.05) remained significantly different. In addition, IL-8 was lower in MF(+) (p<0.01) and MF(-) (p<0.05) compared to NGT group. At 120-minute post-glucose load TNF-a, IL-6, IL-13, and IL-8 were lower in T2D compared to NGT (p<0.01 for all). In MF(+) group TNF-a, IL-8, IL-13, (p<0.01 for all) and IL-6 (p<0.05) were lower compared to NGT group. In MF(-) group some of

| Variable       | NGT (n = 20) | T2D (n = 21) |
|----------------|-------------|-------------|
|                | MF(+) (n = 16) | MF(-) (n = 5) | Combined (n = 21) |
| **Fasting Cytokines** |             |             |                |
| GM-CSF, pg/mL  | 20.3 ± 15.3 | 21.4 ± 27.1 | 26.1 ± 28.9 | 22.5 ± 26.8 |
| IL-10, pg/mL   | 7.3 ± 4.9   | 7.3 ± 6.7   | 6.4 ± 4.4   | 7.1 ± 6.1   |
| IL-13, pg/mL   | 15.3 ± 41.1 | 9.7 ± 12.0* | 11.0 ± 14.3 | 10.0 ± 12.1* |
| IL-6, pg/mL    | 6.3 ± 11.5  | 2.5 ± 2.8** | 3.7 ± 5.2*  | 2.8 ± 3.4** |
| IL-7, pg/mL    | 5.4 ± 3.2   | 6.9 ± 6.3   | 7.4 ± 6.9   | 7.0 ± 6.3   |
| IL-8, pg/mL    | 74.9 ± 91.3 | 13.4 ± 21.8** | 9.4 ± 8.0* | 12.4 ± 19.3 |
| TNF-a, pg/mL   | 14.5 ± 14.2 | 4.5 ± 2.7** | 5.0 ± 2.8** | 4.6 ± 2.7** |
| **120 min Cytokines** |             |             |                |
| GM-CSF, pg/mL  | 19.8 ± 14.8 | 20.8 ± 28.2 | 20.2 ± 21.6 | 20.6 ± 26.3 |
| IL-10, pg/mL   | 7.2 ± 4.7   | 6.9 ± 6.9   | 6.4 ± 3.6   | 6.8 ± 6.3   |
| IL-13, pg/mL   | 15.4 ± 41.5 | 9.8 ± 13.0** | 10.9 ± 9.9 | 10.0 ± 12.3** |
| IL-6, pg/mL    | 4.0 ± 9.3   | 2.7 ± 3.0*  | 2.5 ± 3.4*  | 2.6 ± 3.0** |
| IL-7, pg/mL    | 5.1 ± 3.0   | 6.4 ± 6.9   | 5.7 ± 5.7   | 6.2 ± 6.5   |
| IL-8, pg/mL    | 11.6 ± 14.9 | 5.0 ± 4.8** | 3.9 ± 1.3*  | 4.7 ± 4.2** |
| TNF-a, pg/mL   | 9.9 ± 10.3  | 4.3 ± 3.5** | 4.4 ± 2.2** | 4.3 ± 3.2** |

Data are mean ± standard deviation. All group comparisons are adjusted for age and BMI where applicable. P-value of <0.05 is used to denote statistical significance, reference group is NGT. Fasting represents 0-minute of OGTT. Abbreviations: NGT, normal glucose tolerance; T2D, type 2 diabetes; MF(+) taking metformin; MF(-), metformin naïve; 120 min, 120 minutes post-glucose load during OGTT; * p<0.05, ** p<0.01.
these cytokines remained different, TNF-α (p<0.01) and IL-6, IL-8 (p<0.05 for both). The other cytokines were not different between the groups (Table 3).

Among the adipokines (Table 2) in fasting state PAI-1 (p<0.01) as well as adiponectin, adipsin and lipocalin (p<0.05) were lower in T2D compared to NGT and remained significantly lower at 120-minute post-glucose load (p<0.01 for PAI-1, adiponectin, adipsin, and p<0.05 for lipocalin). In addition, resistin was decreased post-glucose load (p<0.05) in T2D vs. NGT. These differences remained significant for MF(+) participants in both the fasted and 120-min post glucose load, but not for lipocalin and resistin at 120-minute post-glucose load. Lastly, for MF(−) group in fasted state PAI-1 and lipocalin were lower (p<0.05) while post-glucose PAI-1, adiponectin, adipsin (p<0.01 for all) and resistin (p<0.05) were also lower compared to NGT. There were no statistical differences for the other adipokines (Table 2).

Comparison of selected adipocytokine biomarkers between NGT and T2D is shown in Figure 2.

**Adipocytokine predictors of HbA1c**

To explore the independent associations between each of the 14 adipocytokines and chronic glycemic control in the fasted state, we used linear regression analysis, adjusting for age and BMI (Table 4). All variables were log transformed because they were not normally distributed. The log of the cytokines IL-8 (p=0.032) and TNF-α (p<0.001), and the adipokines adiponectin (p=0.003), lipocalin (p=0.005), resistin (p=0.003), adipsin (0.001), and PAI-1 (p<0.001) were significantly associated with HbA1c, after adjusting for age and BMI.

**Table 4** Independent adipocytokine associations with HbA1c in the fasted state (N=41).
| Independent variable | β-coefficient [SE] | p-value |
|----------------------|--------------------|---------|
| GM-CSF               | -0.14 [0.08]       | NS      |
| IL-10                | -0.02 [0.07]       | NS      |
| IL-13                | -0.05 [0.07]       | NS      |
| IL-6                 | -0.08 [0.07]       | NS      |
| IL-7                 | -0.01 [51]         | NS      |
| IL-8                 | -0.14 [10]         | 0.032   |
| TNF-a                | -0.42 [0.09]       | <0.001  |
| Adiponectin          | -0.28 [0.09]       | 0.003   |
| Lipocalin            | -0.42 [0.14]       | 0.005   |
| Resistin             | -0.33 [32]         | 0.003   |
| Adipsin              | -0.0001 [0.00002]  | 0.001   |
| PAI-1 (total)        | -0.67 [0.14]       | <0.001  |
| IP-10                | 0.11 [43]          | NS      |
| MCP-1                | -0.02 [46]         | NS      |

Data are β-coefficient [Standard error, SE]. All variables were Log transformed prior to analysis. Each line represents a regression with HbA1c as dependent variable and an adipocytokine as an independent variable, adjusted for BMI and age.

Next backwards stepwise multiple linear regression analysis was used to develop a model for HbA1c prediction, setting the p-value for variable inclusion at p<1.000. The final model included the following log transformed variables: TNF-a, adiponectin, age, BMI, and metformin status while NGT was used as the reference group. The final model explained 86% of the variance in HbA1c (Table 5) (Figure 1). To explore any multicollinearity and tolerance effects, the variance inflation factor (VIF) and tolerance (1/VIF) analyses were performed post-estimation (Table 6). None of the VIF values exceeded 10, and therefore indicates little multicollinearity in the final model. In addition, since we found adiponectin to be a significant predictor of HbA1c, we further analyzed the association (by Pearson correlation) of adiponectin with other biomarkers. Adipsin showed significant (p<0.01) association with a few adipocytokines (Figure 3) and not with diabetes measures (body weight, BMI, glucose, and insulin).

**Table 5** Backwards stepwise multiple linear regression for HbA1c in the fasted state (N=41).
### Independent variables

| Variable      | Beta [SE] | 95% CI       | p-value |
|---------------|-----------|--------------|---------|
| TNF-a         | -0.16 [0.09] | -0.34, 0.03 | 0.088   |
| Adipsin       | -0.43 [0.14] | -0.72, -0.15 | 0.004   |
| Age (years)   | 0.03 [14]   | 0.004, 0.05  | 0.025   |
| BMI (kg/m²)   | 0.04 [52]   | 0.002, 0.08  | 0.036   |
| NGT           | Reference   | -            | -       |
| MF(-)         | 0.93 [0.26] | 0.40, 1.45   | 0.001   |
| MF(+)         | 0.86 [0.26] | 0.32, 1.10   | 0.003   |

Data are β-coefficient [Standard error, SE], and 95% Confidence intervals (CI). Adipsin and TNF-a were Log transformed. Variables with p<1.000 from Table 4 were used. Variables were removed if p>1.000. The model predicted 86% variability of HbA1c (R²-adjusted 0.86) for the whole group. Abbreviations: NGT, normal glucose tolerance; MF(-), T2D metformin naïve, MF(+) T2D using metformin.

### Table 6 Variance inflation factors (VIF) for multiple linear regression.

| Variable      | VIF   | 1/VIF  |
|---------------|-------|--------|
| TNF-a         | 1.93  | 0.519311 |
| Adipsin       | 1.22  | 0.818808 |
| Age (years)   | 1.49  | 0.670785 |
| BMI (kg/m²)   | 3.56  | 0.280930 |
| NGT           | -     | -      |
| MF(-)         | 2.72  | 0.367760 |
| MF(+)         | 6.11  | 0.163683 |

VIF of <10.0 indicates limited multicollinearity, and 1/VIF<1.0 indicates limited collinearity. Adipsin and TNF-a were Log transformed.

### Discussion

**Adipokine adipsin as a predictor of HbA1c**

The 14 measured adipocytokine biomarkers were chosen based on literature review and data including our previous data [39] showing involvement of these biomarkers in T2D development, progression and/or
complications [7-16, 19-21]. The final model accounting for the most variance in chronic glycemic control as measured by HbA1c included adipsin and TNF-α. This is striking given adipsin is one of the most abundant adipokines and a rate-limiting enzyme for the alternative complement system [53-55].

Specifically we showed that adipsin was lower in T2D compared to NGT, which was similar to majority [40, 56-58] but not all [10, 19] previously published research. In addition, this study showed that 2-hour post-glucose load adipsin was lower in T2D compared to NGT. These data were in agreement with our previous exploration of adipocytokines response to acute hyperglycemia [39]. We used randomly chosen samples from African American men with prediabetes and vitamin D insufficiency who were participating in vitamin D supplementation trial [44]. In the pilot ancillary analysis, the adipocytokines were compared between 60- vs. 0-minute of OGTT. The findings relevant to the current study were that adipsin and complement-3 (C3), a component of adipisin pathway [56], were lower at 60- vs. 0-minute during OGTT. Specifically, in 20 men with an average age 57 years, BMI 31 kg/m², and HbA1c 6.2%, adipsin was 4.26 ± 1.05 ng/ml at 60- vs. 5.62 ± 1.44 ng/ml at 0-minute (p<0.001). In addition, in the group of 19 men from the same cohort with an average age 61 years, BMI 32 kg/m², and HbA1c 6.1%, C3 was 0.35 ± 0.20 ng/ml at 60- vs. 0.46 ± 0.22 ng/ml at 0-minute (p<0.038) [39]. These data from our current and previous explorations could be interpreted as suggesting inadequate response of adipsin pathway to glucose load in T2D compared to normoglycemic participants. Although there had been no other previous studies evaluating circulating adipsin post-glucose load, our data could be interpreted as supporting previous translational studies suggesting that inadequate response of adipsin pathway contributed to inadequate insulin secretion in T2D animal model [56]. Correspondingly, adipsin role in insulin secretion was suggested by human data [40]. Specifically, adipsin positively correlated with homeostasis model assessment of β-cell function (HOMA-β), the area under the curve (AUC) of the first phase insulin secretion and acute insulin response in a group of Chinese individuals with various degrees of glucose tolerance including newly diagnosed T2D [40]. Taken together the data from previous and present research implicated failure of adipsin (and/or adipsin pathway) to rise in response to acute hyperglycemia that could be responsible at least in part for inadequate insulin increase in acute hyperglycemia in T2D.

The most unexpected finding of our study was that adipsin remained a significant predictor of HbA1c after multiple relevant adjustments in regression modeling. There were no previous studies that included multiple adipocytokines in modeling of HbA1c determinants and showing adipsin as a significant predictor of HbA1c. Previous studies measured only adipsin [58] or adipsin and IL-1β [40] and did not use HbA1c as a dependent variable in regression modeling. We preferred using HbA1c as a dependent variable in regression modeling because HbA1c represented the most clinically relevant biomarker of chronic glucose control. Contrary to our results, in regression modeling that used adipsin as a dependent variable no association was found between adipsin and HbA1c in a cohort of 137 Chinese men and women with and without T2D [40]. The discrepancy between the current and previous results could be related to differences in populations and independent variables chosen for regression modeling. The current study, however, was in agreement with previous investigation showing that circulating adipsin
was negatively associated with insulin resistance calculated from homeostasis model assessment of insulin resistance (HOMA-IR) \cite{40, 58} especially in participants with a BMI \(\geq 25 \text{ kg/m}^2\) or FPG \(\geq 100 \text{ mg/dL}\) \cite{58}.

The results of this study could be interpreted as supporting previous basic and translation research providing mechanistic insight into essential role of adipsin in T2D \cite{56, 59, 60}. Adipsin, first identified as a component of alternative complement cascade (called Factor D) secreted by monocytes and macrophages \cite{59, 61}, was later discovered as being produced by the white adipose cells \cite{56} and by the gut epithelial Paneth cells \cite{60}. Adipsin was shown to play prominent role in regulation of inflammation and glucose metabolism \cite{56, 59, 60}. Similar to the data in humans, in a mouse model adipsin was lower in T2D compared to non-diabetic mice \cite{51}, while in a mouse model of T2D development adipsin negatively correlated with glucose AUC during glucose tolerance test \cite{62}. Additionally, adipsin was shown to have a beneficial role in maintaining \(\beta\)-cell function \cite{56}. Mice genetically lacking adipsin had glucose intolerance due to fasting and glucose-stimulated insulinopenia while isolated islets from these mice had reduced glucose-stimulated insulin secretion \cite{56}. Replenishment of adipsin to diabetic mice improved glucose control by increasing insulin secretion \cite{56}. Treatment of diabetic mice with anti-diabetic agent (pioglitazone) resulted in increased gene expression for adipsin, PPAR-\(\gamma\) and Glut-4 in adipose tissue while multiple other biomarkers were not affected \cite{63}.

Further interrogation of mechanisms of adipsin action showed that adipsin activated alternative complement cascade, generating C3a from C3, a small (approximately 10KDa) cleavage fragment \cite{56}. The C3a acted directly in isolated pancreatic islets as a potent insulin secretagogue enhancing insulin secretion by 30-40% in the presence of high but not low glucose conditions and the C3a receptor was required for this beneficial effect. The action of C3a on islets was mediated by augmenting ATP levels, respiration, and cytosolic free Ca(2+) \cite{56}. It was also shown that C3a could induce both proinflammatory and anti-inflammatory responses \cite{64}. Furthermore, adipsin expression in the gut epithelial Paneth cells could be induced by \textit{Lactobacillus spp} (through TNF receptor-associated factor 2 (TRAF2) and TRAF6-mediated NF-kB signaling) and transplantation of these microbiota into germ-free mice resulted in increased adipsin in both intestinal epithelium and serum \cite{60}. Our data showing that adipsin was a significant predictor on HbA1c could be interpreted as being in agreement with these translational data showing important role of adipsin in pathophysiology of diabetes.

Overall the present study and previous human and translational research implicated adipsin pathway in linking microbiota, inflammation, and adiposity to \(\beta\)-cell physiology and T2D pathogenesis. These data, if confirmed in other populations, could enable further understanding of T2D pathophysiology and improve clinical decision-making based on specific risk profile as suggested by the data for cardiovascular disease \cite{38}.

\textbf{Cytokine TNF-\(\alpha\) as a predictor of HbA1c}
In addition to adiponectin, TNF-a was associated with HbA1c. This result was in agreement with previous cross-sectional and prospective studies suggesting association of TNF-a with T2D and risk for the incident T2D [7-9, 12, 41, 65]. Our results, however, showed that the association between TNF-a and HbA1c was no longer significant (p=0.088) after adjustment for age, BMI, and metformin use. Consistent with our observations, a cross-sectional study of adipocytokine role in T2D showed that TNF-a was higher in T2D (n=63) compared to NGT (n=304) in Mexican Americans [41]. Nonetheless, in regression analysis TNF-a was not a significant determinant of HbA1c after adjustment for age and BMI [41]. There were no other studies that investigated adipocytokines among other T2D covariates as possible determinants of HbA1c. Likewise, TNF-a was elevated in patients with incident T2D (n=192 cases) compared to non-disease-developing controls (n=384) in a nested case-control study within the prospective population-based European Prospective cohort (n=27,548) [65]. In that study, however, TNF-a was not a significant predictor of the incident T2D after adjustment for BMI [65]. There was also a weak non-significant association between TNF-a and risk of T2D in the meta-analysis of 5 prospective studies comprising a total of 10,078 participants including 2,780 cases of incident T2D [7].

Of interest, two studies evaluated effect of anti-TNF-a medications on glucose metabolism. A randomized double-blind trial showed no effect of a recombinant-engineered human TNF-a-neutralizing antibody (CDP571) compared to placebo on glucose homeostasis in obese T2D patients after 4 weeks [66]. A recent retrospective study analyzed the influence of TNF-a inhibitors (TNFi) and an anti-IL-6 receptor antibody tocilizumab (TCZ) on glucose metabolism in 221 patients with rheumatoid arthritis including 109 patients with T2D [67]. TNFi were infliximab, etanercept and adalimumab. After 3 months of observation HbA1c improved regardless of the presence of T2D. In a multivariate logistic regression analysis use of TCZ but not TNFi remained a significant predictor of HbA1c reduction after adjustment for BMI, diabetes medications and other relevant factors [67].

Mechanisms of TNF-a association with T2D likely involved at least in part direct stimulation by hyperglycemia TNF-a production and activity in adipose tissue [68-70]. This mechanistic insight was provided by human and animal studies. Hyperglycemic clamps in normal subjects, in whom endogenous insulin was suppressed by concomitant administration of somatostatin, induced a rise of circulating TNF-a and IL-6 [71]. In animal models and in obese humans, circulating TNF-a and TNF-a gene expression in adipose tissue were increased and associated with insulin resistance [68-70].

In summary, TNF-a, consistently considered a significant component of inflammation, appears to be one of multiple factors contributing to T2D, and its role in complexities of T2D pathogenesis compels further elucidation.

The study had several strengths and limitations. Limitations were that study involved a small group of homogenous participants that decreased statistical power and generalizability to other populations, cross-sectional design that precluded causal assessment, non-inclusion of some clinical characteristics (e.g. dietary assessment, sleep pattern, etc.), and non-inclusion of other adipocytokines with potential importance in T2D (e.g. leptin). Strengths included use of multiplex assays allowing exploration of
multiple biomarkers, involvement of participants with variable glucose tolerance, and investigation of associations of adipocytokines with acute as well as chronic hyperglycemia.

Conclusions

This study showed for the first time that adipsin could be an independent predictor of HbA1c in African American men with varied glucose tolerance. Together with other relevant clinical characteristics and pathogenetic mediators including age, BMI, use of metformin, and circulating TNF-a, the model explained 86% of HbA1c variability, suggesting significant role of adipsin in T2D. Additional studies would be required for corroborating these observations and for providing mechanistic insights and enabling adipsin pathway-related discoveries of novel T2D treatment.

Abbreviations

T2D: type 2 diabetes mellitus; AAM: African American men; NGT: normal glucose tolerance; BMI: body mass index; WHO: World Health Organization; PAI-1: plasminogen activator inhibitor 1; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; IP-10: interferon-inducible protein-10; MCP: monocyte chemoattractant protein; TNF: tumor necrosis factor; HbA1c: hemoglobin A1c; OGTT: oral glucose tolerance test; VAMC: Veteran Administration Medical Center; BMI: body mass index; 25OHD: 25OHD-vitamin D; %CV: percent coefficient of variation; SE: standard deviation; SD: standard error; VIF: variance inflation factor; TRAF2: TNF receptor-associated factor 2; TNFi: TNF-a inhibitors; TCZ: tocilizumab.

Declarations

Ethics approval and consent to participate

The Jesse Brown VAMC Institutional Review Board approved the study and every subject signed informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Authors’ contributions

EB made substantial contributions to the conception and design of the work. EB, AA, YE contributed in data collection. EB and AA responsible for the research execution. MP, JAB, CLF and LRD analyzed the data. EB, LRD, and MS contributed in visualization of the data. All authors contributed to cowriting, reviewing and editing the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

Study design and main results. Abbreviations: AAM, African American men; BMI, body mass index; MF, metformin; NGT, normal glucose tolerance; T2D, type 2 diabetes.
Figure 2

Serum concentration of adipocytokine biomarkers. Data are median ± standard deviation. Luminex immunobead platform multiplex assay shows concentration for NGT (n=20) and T2D (n=21) of (A) Adipsin (pg/ml), (B) PAI-total (pg/ml), (C) Adiponectin (pg/ml), (D) TNF-\(\text{a}\) (pg/ml), and (E) IL-6 (pg/ml) at 0- and 120-minutes of OGTT. All adipocytokines were Log transformed prior to analysis. All group comparisons were adjusted for age and BMI where applicable. P-value of <0.05 is used to denote statistical significance, reference group is NGT, groups are compared separately at 0-min and at 120-min post-glucose load during OGTT. * p<0.05, ** p<0.01.
Figure 3

Result of Pearson correlation analysis between adipsin (x-axis) and adipocytokines (y-axis) for all participant (n=41). Scatter plot represented for Pearson correlation coefficients with p<0.01.