Human SHC-transforming protein 1 and its isoforms p66shc: A novel marker for prediabetes

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ABSTRACT
Aims: Prediabetes is a multifactorial condition. Current guidelines for diabetes screening recommend either the use of glycated hemoglobin (HbA1c), or blood glucose level (BGL). This research aimed to identify if p66shc a component of the Human SHC-Transforming Protein 1 (Shc1), a mitochondrial associated oxidative stress biomarker, is significantly altered in patients with elevated BGL. Furthermore, we evaluated if inflammatory and oxidative stress markers, such as p66shc, are a useful addition to the regularly used biomarkers to increase sensitivity for identification of prediabetes.

Methods: All participants attended the Diabetic Health Screening at Charles Sturt University (CSU), Australia. The cross-sectional clinical study collected demographic and clinical variables from 346 participants and classified into control or prediabetes based on fasting BGL. Blood and urine samples were analyzed for oxidative stress and inflammation markers. Logistic regression was used to compare multidimensional diagnostic models for prediabetes, including p66shc/Shc1, to the current HbA1c-only model in terms of sensitivity, specificity and predictive accuracy. Significance was set at \( P \leq 0.05 \).

Results: A significant decrease of p66shc/Shc1 was determined in prediabetes compared to controls (\( P \leq 0.05 \)). HbA1c testing resulted in an accuracy of 62%, while adding p66shc and triglycerides increased predictive accuracy to 88.05%. When HbA1c was omitted and Shc1 was combined with 8-hydroxy-2'-deoxyguanosine (8-OHdG) and monocyte chemo-attractant protein-1 (MCP-1), a predictive accuracy of 89.5% was achieved.

Conclusion: Our findings showed a major improvement of sensitivity to identify prediabetes by including oxidative stress and inflammatory biomarkers underlining beneficial diagnostic information, which most likely improves prevention and early treatment options in prediabetes.

INTRODUCTION
Prediabetes and type 2 diabetes mellitus are multifactorial conditions affecting multiple organ systems1–3. Current guidelines for diabetes screening recommend either impaired fasting glucose, impaired glucose tolerance or glycated hemoglobin testing4. Applying these guidelines, prediabetes can be classified by impaired fasting glucose level (IFG) of 100–125 mg/dL (5.6–7 mmol/L), impaired glucose tolerance of 140–199 mg/dL (7.8–11.0 mmol/L) and/or glycated hemoglobin (HbA1c) level between 5.7% and 6.4%5. Current clinical screening usually includes only blood glucose level (BGL) or HbA1c4,6. This has been shown to underestimate the presence of prediabetes or type 2 diabetes mellitus and only provides limited information about the complex pathophysiology associated with disease progression7. Knowledge of the presence of oxidative stress and inflammatory processes, forming a complex pattern of disease progression, is therefore an important step towards early, effective treatment8–10.

In prediabetes, an elevated BGL already leads to increased reactive oxygen species (ROS) production. The adaptor protein p66shc has recently been shown to play a role in regulating intracellular reactive oxygen species and oxidative stress11.
p66shc is an isoform of the Human SHC-Transforming Protein 1 (Shc1). Shc1 is an intracellular scaffold protein, which is involved in downstream pathways of cell surface signaling receptors, such as the Insulin signaling pathway. The interaction of p66shc with the electron transport chain in mitochondria leads to the production of reactive oxygen species (ROS), especially during high glucose loads. This mechanism can lead to oxidative stress induced cell death in prediabetes. Balancing this increased p66shc activity and oxidative stress is the oxidative stress-induced increase in SIRT1 activity which down regulates p66shc. The value of p66shc as a potential prognostic marker combined with inflammatory and oxidative stress markers in prediabetes has not been reported. Figure 1 illustrates the metabolic interactions of p66shc.

As a redox protein, the Shc1 isoform p66shc is implicated in mitochondrial reactive oxygen species (ROS) production and translation of oxidative signals into apoptosis, which was previously shown to occur during prolonged hyperglycemia such as in diabetes. In addition, Shc1 isoform p52shc is altered in diabetic cardiovascular disease and targets the insulin IGF-1 signaling pathways associated with insulin resistance. Moreover, Shc1 isoform p46shc, is associated with reduced adiposity and insulin sensitivity in ShcP mice.

Diabetes has now been recognized for some years as an inflammatory disease and the Insulin Resistance and Atherosclerosis Study (IRAS) studies reported that the levels of some inflammatory markers were independently associated with insulin sensitivity and linearly related to a number of the metabolic syndrome components. Similarly, oxidative stress has been reported as a strong biomarker for diabetes prognosis. However, inflammation and oxidative stress are not independent factors in the pathogenesis of diabetes. The accumulation

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**Figure 1** | Transfer pathway of p66shc into the mitochondrion. Cell, muscle, fat, or endothelial cells; 8-iso-PGF2α, 8-isoprostaglandin F2α; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CYCS, cytochrome c, somatic; GSH, reduced glutathione; GSR, glutathione-disulfide reductase; GSSG, oxidized glutathione; IL-1β, interleukin-1β; IL-6, interleukin-6; PKCβ, protein kinase c beta; p-p66shc, phosphorylated p66shc; ROS, reactive oxygen species; Shc1 gene, Human SHC-Transforming Protein 1. gene; SOD, superoxide dismutase. Blue, oxidized; Red, inhibition.
of mitochondrial ROS in prediabetes may increase interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels, as well as other inflammatory markers, such as Insulin Growth factor-1 (IGF-1), monocyte chemo-attractant protein-1 (MCP-1) and C-reactive protein (CRP), which can mediate insulin resistance in type 2 diabetes mellitus and promote disease progression.  

The current research investigated whether inflammatory and oxidative stress markers, in particular p66shc, show significant differences in patients with prediabetes compared to a control group. Furthermore, we examined if an additional use of inflammatory and oxidative stress markers improves the sensitivity of HbA1c to identify prediabetes.

**METHODS**

**Participant recruitment**

This cross-sectional study was performed at the Charles Sturt University (CSU) Diabetes Screening Clinic, Australia between July and September 2017. Demographic and clinical variables were collected from 346 participants following a local media announcement, and were classified by measuring blood glucose into control and prediabetes groups. The control group was made up of participants with a blood glucose level below 5.6 mmol/L. Inclusion and exclusion criteria were kept to a minimum to reflect routine patient presentation to general practice in a typical Australian rural population. Patients had to be over 40 years of age and were requested to have fasted overnight. The design of the study conformed to the general ethical standards, as outlined by the Helsinki Convention on ethical conduct in human research. All methods were carried out in accordance with the relevant guidelines and regulations and as approved by the Charles Sturt University Human Ethics Committee (Ethics Approval # 2006-042). Attendance at the clinic was voluntary, and all participants declared informed written consent prior to being enrolled.

**Anthropogenic measurement**

Height and weight were recorded to calculate body mass index (BMI). Blood pressure (BP) readings were obtained after five minutes rest by using a standard mercury sphygmomanometer and a cuff of appropriate size. Hypertension was defined as a SBP of 140 mmHg or higher or DBP 90 mmHg or higher in line with Australian guidelines. Waist circumference was measured with a flexible centimeter tape at the midpoint between the lowest rib and the iliac crest.

**Biochemical analyses**

Venous blood was collected and stored in either ethylenediaminetetraacetic acid (EDTA) or citric acid tubes as required for the biochemical assays. Urine samples were taken, stored at –20°C and analyzed within 2–3 days using appropriate enzyme-linked immunosorbent assay (ELISA) kits as outlined previously. ELISA kits were stored at 2–8°C until required. The assays applied a quantitative sandwich enzyme technique, and samples were measured in duplicate. All markers were averaged over two wells with samples and standards. Color change was measured by photometry. Final biomarker concentrations were analyzed by Curve Expert 1.3. (Flarebio Biotech LLC, College Park, MD, USA). The CSU Diabetes Screening Clinic analyzed several pro- and anti-inflammatory markers, such as IL-1β, IL-6, MCP-1 and CRP in blood plasma samples. In addition, venous blood samples were taken to determine oxidative stress markers, including glutathione (GSH) and glutathione disulphide (GSSG), as well as the cell proliferation related biomarker Insulin like growth factor 1 (IGF-1).

Midflow urinary samples were analyzed at the CSU Diabetes Screening Clinic to determine the oxidative stress markers 8-OHdG and p66shc. Measurements of p66shc were performed with a Human SHC-Transforming Protein 1 ELISA kit (CUSA-BIO; Flarebio Biotech LLC) that recognizes primarily Shc1 and its isoform p66shc as reported by the developers. Photometry detection rate was between 25 and 1600 pg/mL. Sensitivity of the kit was at a concentration of 6.25 pg/mL. Avidin conjugated horseradish peroxidase was used with biotin in a color reaction, and photometry was performed to establish the concentration of the compound, as specified by the supplier, at 450 nm wavelength.

For analysis of Blood glucose level (BGL), HbA1c, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides venous blood samples were sent to the local Dorevitch Pathology Laboratory, Albury, NSW.

**Statistical analyses**

Statistical analyses were performed with IBM SPSS (V22) and R version 3.4.2 (R Core Team, 2017). Sample size was determined using G*Power (https://stats.idre.ucla.edu/other/gpower/) with effect size set at 0.6, power at 0.8 and P < 0.05, and data distribution was determined by applying the Shapiro–Wilk test. Statistical methods included A Chi-square test was used to analyse medication use in the two groups. General linear modelling (GLM) compared the means of the two groups. For further advanced investigation and modelling, logistic regression modelling was applied. A good predictive power for the model was assumed for an overall correct prediction of ≥70 percent. Significant results were set at P < 0.05. Model results are presented in terms of accuracy, sensitivity and specificity.

**RESULTS**

**General clinical data**

Participants with a BGL below 5.6 mmol/L were classified as controls (n = 242), while those with BGL between 5.6 and <7 mmol/L were categorized as prediabetic (n = 105). Besides BGL of the physiological variables measured, only supine SBP was significantly different between the two groups (Table 1).

**Comorbidities, medication use and common pathology markers**

In order to analyze common comorbidities of type 2 diabetes mellitus, we determined the occurrence of hypertension (HT)
and cardio-vascular diseases (CVD), such as coronary heart disease, cerebrovascular disease, peripheral arterial disease, in our investigated population. In addition, we determined the use of long-term medication and the levels of common pathology markers (Table 1).

For antidepressants (AD), antihypertensives (AHT), thyroid hormones (TH) and proton-pump inhibitors (PPIs) the control group presented with higher regular use, whilst the use of statins (ST) and nonsteroidal anti-inflammatory drugs (NSAIDs) was higher in the prediabetes (Table 2).

Of the common pathology markers, such as total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL), only TG was significantly higher in the prediabetes group (Figure 2).

**Inflammatory and oxidative stress markers**

Inflammatory markers were not significantly different between the two groups (Figure 3).

Glutathione was significantly decreased in the prediabetes as compared to the control group, suggesting mild oxidative stress (1643.19 ± 67.25 μM & 1838.07 ± 55.25 μM). Similarly, the GSH/GSSG ratio was significantly decreased (7.47 ± 0.41 & 5.86 ± 0.38; Figure 4). Shc1/p66shc was also significantly decreased in the prediabetes group (52.30 ± 2.87 pg/mL vs 42.25 ± 2.33 pg/mL; Figure 4, Table 1).

**Logistic regression models of p66Shc and other biomarkers**

The logistic regression model with only HbA1c as a factor was significant \( P < 0.05 \), but showed a sensitivity of only 50% and a specificity of 74.30%, with an overall accuracy of 62.1%. Accuracy was significantly improved when Shc1/p66shc was included leading to a sensitivity of 74.8%, a specificity of 75% and an overall accuracy of 74.9%. The best HbA1c-inclusive model for the prediction of prediabetes was a combination HbA1c, Shc1/p66shc and triglycerides, with an accuracy of 88.05%. All models that included HbA1c had a significance of \( P < 0.05 \) (Table 3).

Exclusion of HbA1c improved the performance of the model. The best model combined Shc1/p66shc with 8-OHdG and MCP-1 with an accuracy of 89.5%, sensitivity of 100% and specificity of 78.9% \( (P < 0.01) \). Combining Shc1/p66shc with

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**Table 1** | General clinical data

|                      | Control (mean ± SD) | Prediabetes (mean ± SD) |
|----------------------|---------------------|-------------------------|
| **Age (yrs.)**       | 62.4 ± 1.1          | 64.7 ± 1.5              |
| **Sex (m/f)**        | (96/146)            | (42/62)                 |
| **BGL (mmol/L)**     | 4.9 ± 0.4           | 4.1 ± 0.3***            |
| **p66Shc (pg/mL)**   | 52.3 ± 2.9          | 42.3 ± 2.3**            |
| **HbA1c (%)**        | 5.6 ± 0.02          | 5.7 ± 0.04              |
| **WC (cm)**          | 90.4 ± 1.1          | 93.8 ± 1.7              |
| **BMI (kg/m²)**      | 25.8 ± 0.4          | 264 ± 0.6               |
| **Lying DBP (mmHg)** | 122.3 ± 1.4         | 1283 ± 24*              |
| **Lying SBP (mmHg)** | 74.6 ± 0.7          | 772 ± 1.2               |

Values are means ± standard deviation. Significance: *P ≤ 0.05; ***P ≤ 0.01; ****P ≤ 0.001 vs control group. BGL, blood glucose level; BMI, body mass index; f, female; HbA1c, glycosylated haemoglobin; m, male; Supine DBP, supine diastolic blood pressure; Supine SBP, supine systolic blood pressure; WC, waist circumference; Yrs., year.

**Table 2** | Medication

| Medication | Control n (%) | Prediabetes n (%) |
|------------|---------------|-------------------|
| AA         | 1 (0.4)       | 2 (1.9)           |
| AC         | 6 (2.5)       | 2 (1.9)           |
| AD         | 36 (14.9)     | 10 (9.5)          |
| AHT        | 113 (46.7)    | 45 (42.9)         |
| BDZ        | 9 (3.7)       | 6 (5.7)           |
| CS         | 7 (2.9)       | 4 (3.8)           |
| GTN        | 3 (1.2)       | 3 (2.9)           |
| HRT        | 2 (0.8)       | 1 (0.9)           |
| NSAIDs     | 94 (38.8)     | 46 (43.8)         |
| ST         | 58 (24)       | 31 (29.5)         |
| TH         | 28 (11.6)     | 11 (10.5)         |
| PD         | 1 (0.4)       | 1 (0.9)           |
| PPIs       | 54 (22.3)     | 20 (19)           |
| XOI        | 7 (2.9)       | 4 (3.8)           |
| O          | 1 (0.41)      | 0 (0.00)          |
| NM         | 122 (50.41)   | 54 (51.43)        |

AA, Antiarhythmic agents; AC, anticoagulants; AD, antidepressants; AHT, antihypertensives; BDZ, benzodiazepines; CS, corticosteroids; GTN, glyceryl trinitrate; HRT, hormone replacement therapy; NM, no regularly medication use; NSAIDs, nonsteroidal anti-inflammatory drugs; O, opioids; PD, Parkinson disease medication; PPIs, proton-pump inhibitors; ST, statins; TH, thyroid hormones; XOI, xanthine oxidase inhibitor.
other common markers including BMI had an overall accuracy of 86.5%, with a sensitivity of 100% and a specificity of 73.2% \((P < 0.05)\). The combination of Shc1/p66shc, CRP and total cholesterol had a sensitivity of 73%, a specificity of 100%, and an overall correct predicate of 86.5% \((P < 0.01; \text{Table 3})\).

**DISCUSSION**

Recent studies have demonstrated that current diabetes diagnostic protocols, based only on impaired fasting glucose or HbA1c, may not be sensitive enough to identify prediabetes or type 2 diabetes mellitus because of individual variances in plasma glucose levels and the associated biochemical changes\(^{28}\).

Shc1 and its isoforms have a close relation to oxidative stress and to the pathology of diabetes. p66shc has been implicated in type 2 diabetes mellitus, potentially playing a major role in the development of retinopathy, nephropathy and cardiovascular complications\(^{29-31}\).

Combining Shc1/p66shc and BMI as predictive biomarkers had a sensitivity of 100% to identify prediabetes patients \((P < 0.05)\). In contrast, HbA1c showed a sensitivity of only 50% to identify prediabetes \((P < 0.05)\). Moreover, our best predictive models included Shc1/p66shc, oxidative stress, beta-oxidation and inflammatory biomarkers, suggesting a strong link between p66shc and the role of inflammation and oxidative stress in prediabetes.

Pagnin et al.\(^{31}\) previously assessed the p66shc mRNA in diabetes mellitus type 2. They reported that the level of p66shc mRNA was significantly higher in the DMT2 compared to the control group. Similarly, increased p66shc levels with increased glucose concentration in the renal tubules has also been reported\(^{16}\). Interestingly, our results in a prediabetes group displayed a significant decreased Shc1/p66shc level compared to control. Several physiological mechanisms may lead to this finding. Shc1 is a scaffold protein of the insulin receptor pathway, which may be permanently down regulated during the prediabetes stage\(^{17,32}\). Permanent hyperinsulinemia accrues in the metabolic stage of prediabetes, due the high glucose levels stimulating the insulin production. The down-regulation of the Shc1 by hyperinsulinemia, and phosphorylation of p66shc might cause the significant decrease of plasma p66shc found in our prediabetes group, which is possibly mediated by SIRT1 (Sir2uin1 lysine deacetylase)\(^{33-36}\).

The stage of disease progression and insulin responsiveness is an important pathophysiological factor that

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**Figure 3** Inflammatory markers. CRP, C-reactive protein; IL-10, interleukin-10; IL-1\(\beta\), interleukin-1\(\beta\); IL-6, plasma interleukin-6; MCP-1, Monocyte Chemotactic Protein-1.
determines levels of inflammation. As such, patients with a decreased acute insulin response were reported to have lower levels of inflammatory proteins compared with those with high insulin secretion\textsuperscript{22}, which was also found in the current research where several cytokines included in our model for prediabetes, such as IL-6, IL-10, IL-1β and MCP-1, had lower concentrations compared to the control group, but displayed no significant \textit{P}-values\textsuperscript{37}. This lower level of inflammatory proteins might also indicate insufficient stimulation of p66shc by inflammatory processes in our prediabetes group and explain the significant decreased of p66shc in the prediabetes group. Increased BGL may also lead to an increase in SIRT1, which suppresses p66shc prior to the full diabetic state\textsuperscript{13}.

Table 3 | Models of p66shc and other biomarkers

\begin{tabular}{|l|l|l|l|}
\hline
Model (HbA1c included) & Sensitivity (%) & Specificity (%) & Accuracy (%) \\
\hline
HbA1c* & 50 & 74.3 & 61.2 \\
HbA1c + p66shc** & 74.8 & 75 & 74.9 \\
HbA1c + p66shc + 8-iso-PGF2α** & 73.6 & 80 & 76.8 \\
HbA1c + p66shc + TC** & 75.4 & 100 & 87.7 \\
HbA1c + p66shc + TG** & 76.1 & 100 & 88.05 \\
\hline
Model (HbA1c excluded) & & & \\
\hline
p66shc + BMI** & 100 & 73.2 & 86.5 \\
p66shc + CRP + TC** & 73 & 100 & 86.5 \\
p66shc + 8-OHdG + MCP-1** & 100 & 78.9 & 89.5 \\
\hline
\end{tabular}

Significance of models: *\textit{P} \leq 0.05; **\textit{P} \leq 0.01. 8-iso-PGF2α, 8-isoprostaglandin F2α; 8-OHdG, Hydroxy-2′-deoxyguanosine; GSH, glutathione; GSSH, Glutathione disulphide; p66shc-Shc1, Human SHC-Transforming Protein 1.

Figure 4 | Oxidative stress markers. 8-iso-PGF2α, 8-isoprostaglandin F2α; 8-OHdG, Hydroxy-2′-deoxyguanosine; GSH, glutathione; GSSH, Glutathione disulphide; p66shc-Shc1, Human SHC-Transforming Protein 1.
Our results indicate that accuracy in identifying prediabetes significantly improved, when Shc1/p66shc was included in the oxidative stress-inflammatory model with or without HbA1c, emphasizing the importance of the complex multifactorial signaling pathway associated with Shc1/p66shc and confirming previous results of ours and others [38–40]. The current work underlines that inflammatory factors such as MCP-1, IL-1β, IL-6 and CRP, even in a group with mild increases in BMI but with BGL levels in the prediabetes range, may contribute to disease progression [41,42]. MCP-1 was included in our best models suggesting a possible role for inflammation in prediabetes [43].

Previous studies have pointed out the high sensitivity of 8-OHdG for oxidative stress, and demonstrated the positive impact on type 2 diabetes mellitus diagnostics in combination with HbA1c [28]. The relevance of 8-OHdG to oxidative stress and prediabetes pathophysiology is emphasized by the inclusion of 8-OHdG in our model, in combination with p66shc and MCP-1. Future considerations to include are rethinking the role of oxidative stress by comparing 8-OHdG (DNA oxidation) to 8-oxo-7,8-dihydroguanosine (8-oxoGuo, RNA oxidation) as RNA oxidation, rather than DNA oxidation may be associated with type 2 diabetes mellitus using LC-MS methodology rather than ELISA [44]. However, the current study was geared to utilizing low level of equipment available especially in small, rural communities and whether significant differences in results between RNA and DNA 8-hydroxy guanine are found in clinical studies that lead to a change in treatment is debatable [45]. Similarly, the use of spot urine may have led to elevated results and a future study can use 24-h urine collection and control with creatinine. 24-h urine collection however is not a routine screening tool and has not been used in the current study. In addition, previous work has shown that unless chronic kidney disease is present the difference in results of both tests is not significant [46].

Our study was conducted in a community screening clinic, and therefore, some limitations need to be acknowledged. Recruitment limitations consisted of restriction to the geographical area where the clinic was located. Therefore, the current study focused on a clinical population, where participants attended a free community screening program from within 100 km from the CSU clinic. Other limitations consisted of not being able to include a number of pathophysiological variables, such as an oral glucose tolerance test (OGTT). Selecting a medication free group may also provide clearer results in future studies.

CONCLUSION

In conclusion, a more comprehensive assessment of prediabetes, based on its multifactorial condition and the availability of recently identified biomarkers significantly increases identification of prediabetes. Including p66shc/Shc1, 8-OHdG, CRP or MCP-1 in our diagnostic models showed a major improvement of sensitivity to identify prediabetes. Our findings underline beneficial, diagnostic information of oxidative stress and inflammatory biomarkers, which most likely improves prevention and early treatment options in prediabetes. It is recommended to include p66shc/Shc1, oxidative stress and inflammatory markers in prediabetes risk models to benefit patients by enabling earlier lifestyle and pharmacological intervention and more effective personalized, targeted treatment.

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DISCLOSURE

The authors have declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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