Molecular phenotyping of oxidative stress in diabetes mellitus with point-of-care NMR system

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INTRODUCTION

Diabetes mellitus (DM) is one of the fastest-growing health burdens globally. Oxidative stress, which has been implicated in the pathogenesis of diabetes complication (e.g., cardiovascular event), remains poorly understood. We report a new approach to rapidly manipulate and evaluate the redox states of blood using a point-of-care NMR system. Various redox states of the hemoglobin were mapped out using the newly proposed (pseudo) two-dimensional map known as $T_1-T_2$ magnetic state diagram. We exploit the fact that oxidative stress changes the subtle molecular motion of water proton in the blood, and thus inducing a measurable shift in magnetic resonance relaxation properties. We demonstrated the clinical utilities of this technique to rapidly stratify diabetes subjects based on their oxidative status in conjunction to the traditional glycemic level to improve the patient stratification and thus the overall outcome of clinical diabetes care and management.

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Fig. 1 Functional sub-phenotyping of oxidative stress with micro MR analysis approach. a The developed bench-top sized micro MR system consists of a commercial console, detection circuit coil mounted on a micro stage, and a palm-sized 0.5 T permanent magnet. The microcapillary tube which contains a single drop of blood is slotted into the radio-frequency probe for micro MR analysis. The read-out system consists of a commercial console, detection circuit coil mounted on a micro stage, and a palm-sized 0.5 T permanent magnet. The time duration for the T2 relaxations, and T1 relaxations measurements, respectively. In order to obtain high signal-to-noise ratio under a relatively inhomogeneous magnetic environment, an array of echoes (a few thousands) within a very short echo interval (in the order of μs) were used to acquire spin-echoes from <4 μL sample volume of packed RBCs or plasma. c Redox reaction of the iron-heme in various oxidation states: Fe2+, Fe3+, Fe4+, and globin-radical Fe5+, which were chemically induced in vitro environment (Methods Online). The hemoglobins were in two possible magnetic states: diamagnetic (red) and paramagnetic state (blue). d Various redox states of hemoglobin mapped out using the proposed T1-T2 magnetic state diagram. The coordinates (in ms) were oxy-Hb (T1 = 152 ± 10, T2 = 628 ± 25), deoxy-Hb (T1 = 124 ± 12, T2 = 622 ± 15), met-Hb (T1 = 102 ± 2, T2 = 198 ± 5), ferryl-Hb (T1 = 139 ± 10, T2 = 522 ± 20), oxo ferryl-Hb (T1 = 34.2 ± 4, T2 = 95.2 ± 6), nitrosyl-Hb (T1 = 121 ± 4, T2 = 204 ± 8), and hemichrome (T1 = 108 ± 4, T2 = 603 ± 11). Three different samplings were taken from the same donor, and the results were reported as mean ± standard error measurement. The data is represented by box-plot format. e A quadrant chart of diabetic subject stratified into subgroups based on their oxidative status (e.g., antioxidant capacity (left) and oxidative stress (right), in which the oxidative stress here includes nitrosative stress and peroxidative stress), in association with their glycemic index (e.g., HbA1c). f Relationship between elevation of oxidative stress and development of DM-related complications (e.g., cardiovascular event).
are present in human circulation\(^4\). Therefore, a subtle change bonds with practically all other macromolecules (e.g., protein) that coordinate, which is unique to each redox state. Water is an attractive from the bulk water, which came into contact with macromole-

The oxidized product is much more stable the process of electron transfer, which leads to eventual formation of oxidized products. The oxidized product is much more stable and measurable using proton NMR relaxometry. 

Here, we chemically induced (Methods Online) and character-

ized various redox states of the red blood cell and represented them using \(T_1\)-\(T_2\) magnetic resonance relaxation state diagram (Fig. 1d). Each Hb species has specific oxidation states (e.g., Fe\(^{2+}\), Fe\(^{3+}\), Fe\(^{4+}\), globin-associated radical of Fe\(^{4+}\) or its' corresponding complexes) that are bound to specific neighboring proteins and dissipate energy via unique relaxations mechanism in both the longitudinal \(T_1\) and transverse \(T_2\) relaxation frames. The \(T_2\) and \(T_1\) relaxation times measurement were performed using the standard Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence and inversion recovery observed by CPMG, respectively. The pairing of both relaxation times forms a specific \(T_1\)-\(T_2\) relaxometry coordinate, which is unique to each redox state. 

These relaxation times reflect predominantly of proton nuclei from the bulk water, which came into contact with macromolec-

ular protein (e.g., hemoglobin, albumin)\(^4\). Water is an attractive to form hydrogen bonds with practically all other macromolecules (e.g., protein) that are present in human circulation\(^4\). Therefore, a subtle change of the molecular environment can induce a measurable change in the proton relaxation rate. Early works demonstrating proton relaxation rate is dependent on the blood oxygenation level had been carried out by Thulborn et al.\(^5\) and Gomori et al.\(^6\). These discoveries were eventually applied to measure brain activity known as functional MRI\(^5\).

Oxyhemoglobin (oxy-Hb) which has the lowest reduced ferrous (Fe\(^{2+}\)) state is the predominant Hb species in circulation. The oxy-Hb has been provisionally assigned to the center of the state diagram, which has four quadrants (i.e., Q1). Due to the semi-solid structure of RBC and oxidation process which reduces the proton relaxation time, the redox pathways of RBC were mapped out predominantly into Q1 (Fig. 1d).

Electrons in the \(d\) sub-orbital of iron hemoglobin can exist in various paired or unpaired conditions, rendering them into two possible magnetic states, i.e., diamagnetic and paramagnetic states, respectively. Hb with at least one unpaired electron, i.e., deoxygenated hemoglobin (deoxy-Hb), methemoglobin (met-Hb), nitrosyl hemoglobin (nitrosyl-Hb), and oxoferryl radical exhibit the effect of paramagnetism with much larger bulk magnetic susceptibility than its' diamagnetic counterparts i.e., oxy-Hb, ferryl Hb, and hemichrome (HC) (Fig. 1c). The magnetic relativity contributed by paramagnetic ion is highly dependent on its spin state and is directly proportional to \(S(S+1)\), where \(S\) is the spin quantum number of the total electron spin\(^6\). Each of the Hb oxidation states has a unique normalized relaxation constant \(A\)-ratio = \(T_1/T_2\) in Fig. 1c.

Nitrite-induced ferrous oxidation

Fig. 2 Nitrite-induced ferrous oxidation. Redox-titration profile of red blood cells as function of nitrite concentration in a \(T_1\) relaxation and b \(T_2\) relaxation domain. The incubation times were 10 min. The control baseline readings were \((T_{20} = 149.5, T_{10} = 621.3)\) ms, which is the readings for oxy-Hb without any nitrite exposure. The limit of detection (LOD) is about 0.0005 mM of nitrite concentration (gray bar). The data is represented by box-plot format. The corresponding concentration-dependent c A-ratio, and d \(T_1\)-\(T_2\) trajectories of the gradual inversion of Fe\(^{3+}\) subpopulation to complete formation of Fe\(^{4+}\) population. Time-dependent kinetic profile of ferrous oxidation using nitrite concentrations (500 \(\mu\)M, 4 mM, 8 mM, and 10 mM) in e \(T_1\) relaxation and f \(T_2\) relaxation domain. The corresponding g A-ratio, and h \(T_1\)-\(T_2\) trajectories in the magnetic state diagram. Three different samplings were taken from the same donor, and the results were reported as mean ± standard error measurement. For curve fitting, a general function of exp(-Ct)cos(Ct) can be used to describe the oscillatory behavior, where C is measurement.
of the RBCs, as the majority of the RBCs were able to restore to their original reduced state. Interestingly, a steep transitional oxidation zone was observed within a very narrow range of nitrite concentration; from 1 to 8 mM, which reflected the redox homeostatic responses within the concentration where the cells were biological viable despite the higher than usual chemical concentration above physiological condition. This was informative to the understanding of the functioning of RBCs at cellular and subpopulation levels (Fig. 2a–c).

Further evidence of redox homeostasis was observed in time-dependent kinetic profiles (Fig. 2e, f) over a range of nitrite concentrations (500 µM, 4 mM, 8 mM, and 10 mM). In general, the measured T₁ and T₂ readings changed in an oscillatory manner over time. This may suggest an active mechanism to regulate cellular redox homeostasis. As the RBCs aged, antioxidant capacity is reduced, thereby forming a subpopulation of cell with disproportionately low antioxidant capacity.

The amplitudes of the oscillation decreased as the nitrite concentration was increased from 500 µM to 4 mM (Fig. 2g). At much higher nitrite concentration (>10 mM), the reaction curve decayed rapidly in an exponential manner with an increasingly dampened oscillation. Similar observations were recorded using spectrophotometry (Supplementary Fig. 1). Interestingly, the corresponding kinetic profiles followed an identical path over time in the T₁–T₂ trajectories as the nitrite concentration was increased (Fig. 2h). The oxidation process drove all the trajectories toward a common coordinate (T₂ = 92.8 ms, T₁ = 190.0 ms), where all the RBCs were converted fully into met-Hb. For low nitrite concentration (e.g., 500 µM), however, the T₁–T₂ trajectory circulated around the origin and did not reach the eventual met-Hb coordinates.

Study design: clinical study with subjects of DM

A cross-sectional study was carried out to stratify DM subjects based on their oxidative status. DM subjects (n = 185) who had Hba₁c measured in the outpatient clinic as part of their clinical concentration (e.g., 500–2500-fold) which may exceed and confound the biological responses or capacity of the cells to counteract the challenge.

Non-DM (n = 24) and DM subjects with poor glycemic control (n = 43) and good glycemic control (n = 29) subgroups were assessed before (black) and after nitrosative stress (colored) (Fig. 4a). The A-ratio for baseline RBCs for non-DM and DM subjects (black) were found to be in increasing trend (due to increasing HC formation), while the nitrosative stress test (colored) resulted in the A-ratio in decreasing trend (due to increasing the amount of met-Hb formation). These results were consistent with the in vivo observation (Fig. 3) and the in vitro stress test conducted (Fig. 2), respectively.

The non-DM subjects had the highest antioxidant (or anti-nitrosative) capacity as compared to DM subjects (P > 0.005), while poor glycemic control subgroup has the lowest tolerance to nitrosative stress (Fig. 4b). The actual amount of nitrosative stress (‘normalized’) were calculated by subtracting the background baseline (black, in Fig. 4a). As a result of increased glycation, Hba₁c is less stable and prone to oxidation and expectedly the poor glycemic control subgroup (AUC > 0.83) has much higher diagnostic accuracy as compared to good glycemic control subgroup (AUC < 0.76) (Fig. 4c). Significantly, the spread for DM subjects was relatively large (as compared to the non-DM controls) which suggests a large phenotypic variation (e.g., subject-to-subject variability in nitrosative susceptibility), despite being in the same glycemic subgroups (Fig. 4b).

The DM subjects were further sub-stratified into smaller subgroups based on the four quadrants (i.e., Q1–Q4), using the dual independent markers, i.e., the oxidative stress marker (in this case the nitrosative stress) proposed in this study and in conjunction with the traditional glycemic control index (e.g., Hba₁c). This approach is able to isolate a minority small subgroup in Q3 (subgroup III) consists of subjects with good glycemic control and unexpectedly (high) nitrosative stress. This dispels the conventional belief that every subject in good glycemic control subgroup was presumably healthier (e.g., lower oxidative stress) than poor glycemic control subgroup but still gave false indication about their risk factors on developing diabetes complications. Isolation of subgroup III subjects (early detection) would help to streamline clinical subjects for therapeutic purposes in a timely manner, which demonstrates the closing gap between translational science and clinical medicine.

Baseline study: oxidative status of glycated Hb in RBCs

Increased blood glucose promotes non-enzymatic glycation of plasma proteins, which include the albumin, alpha-crystalline, collagen, and low-density lipoprotein. Approximately half of the total serum protein is attributable to serum albumin. Glycation and oxidative damage cause protein modification, which affects its functionality. The micro MR analyses were performed at room temperature (26 °C). Each T₁–T₂ coordinate represents the composite redox properties of one subject’s plasma (Fig. 5a). The baseline readings of the DM subjects had, in general, much

Nitrosative stress test on glycated Hb in RBCs

To further evaluate the ability of RBCs to tolerate the nitrosative stress, we artificially challenged the RBCs with strong oxidant. We established a new protocol to evaluate the optimal concentration of oxidant (Supplementary Figs. 5–7). The concentration range must reflect the homeostatic viable range where the ability to discern the subject-to-subject variability is the largest (highest resolution). Freshly collected RBCs were incubated with 6-mM sodium nitrite for 10 min, washed three times to stop the reaction, and finally resuspended in 1x PBS for micro MR analysis (Methods Online). It worth noting, however, in order to shorten the incubation time, the concentration of the stressors used (i.e., nitrite, H₂O₂) was applied at a supra-physiological concentration (in the range of 500–2500-fold) which may exceed and confound the biological responses or capacity of the cells to counteract the challenge.

The baseline oxidative status of intact RBCs was measured and assessed before (black) and after nitrosative stress (colored) (Fig. 4a). The A-ratio for baseline RBCs for non-DM and DM subjects (black) were found to be in increasing trend (due to increasing HC formation), while the nitrosative stress test (colored) resulted in the A-ratio in decreasing trend (due to increasing the amount of met-Hb formation). These results were consistent with the in vivo observation (Fig. 3) and the in vitro stress test conducted (Fig. 2), respectively.

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Baseline study: glycation and glycoxidation of plasma

Increased blood glucose promotes non-enzymatic glycation of plasma proteins, which include the albumin, alpha-crystalline, collagen, and low-density lipoprotein. Approximately half of the total serum protein is attributable to serum albumin. Glycation and oxidative damage cause protein modification, which affects its functionality. The micro MR analyses were performed at room temperature (26 °C). Each T₁–T₂ coordinate represents the composite redox properties of one subject’s plasma (Fig. 5a). The baseline readings of the DM subjects had, in general, much
shorter $T_1$ and $T_2$ relaxation times, and it was well separated from the healthy non-DM subjects (blue). Notably, DM subjects with poor glycemic control, in particular DM subjects with more than 10% HbA1c subgroup (mean A-ratio of 2.52), saw a strong departure from the healthy controls (mean A-ratio of 2.13) (Fig. 5b).

The marked reduction in relaxation states was attributed to an increase in glycation and glycoxidation of the serum albumin, known as glucose toxicity. As a result of increased glycation (in vitro validation in Supplementary Fig. 8a) and protein oxidative damage (e.g., protein cross-linking), the mobility of bulk water proton was further restricted, leading to reduction in $T_1$ and $T_2$ relaxation times. $T_2$ relaxation however reduced much faster than $T_1$ relaxation (and hence an increase in Abaseline-ratio) (Fig. 5b). Similar trends were observed in vitro, which confirm the effect of glycation (Supplementary Fig. 8a, b) and glycoxidation (Supplementary Fig. 8c). A separate study by Cistola et al. recently found that the baseline $T_2$ of water plasma showed a strong correlation in subjects with metabolic abnormalities. Interestingly, the ROC analysis indicates plasma DM subjects with good glycemic control (AUC > 0.92) had much higher diagnostic accuracy than its’ counterpart RBCs (AUC < 0.67) (Fig. 5c), which suggest that pathological footprint of hyperglycemia is more prominent in extracellular plasma as compared to the intact RBCs. The same hypothesis is further observed and validated in DM subjects with poor glycemic control (Fig. 5d).

Peroxide induced oxidation in plasma of DM subjects

In order to evaluate the total antioxidant capacity of plasma toward oxidation, we artificially challenged the plasma with hydrogen peroxide solution (10% v/v) for an incubation time of 10 min (Methods Online, Supplementary Fig. 9). The micro MR analyses were performed before (black) and after peroxide treatment for subjects with poor glycemic control, good glycemic control, and non-DM (red, green, and blue, respectively).

The peroxidative stress test revealed that a large spread of phenotypic variations in DM subjects as compared to non-DM controls (Fig. 6a). The A-ratio for baseline measurements of oxidative stress in RBCs. a In vivo redox oxidation produces denatured Hb, known as the low-spin hemichrome (HC). HC can be chemically induced using sodium salicylate (SLS) in vitro environment. b The $T_1$–$T_2$ relaxometry coordinates of RBCs baseline readings of non-DM subjects (blue, n = 23) and subjects with poor glycemic control (red, n = 68). c Baseline readings of RBCs samples with A-ratio index of subjects with poor glycemic controls (n = 62) and good glycemic control (n = 50) subgroup as compared to healthy non-DM subjects (n = 20). The subjects with poor glycemic controls were further subdivided into >8% HbA1c (n = 47) and >10% HbA1c (n = 15) subgroups. The A-ratio for the subgroups were in increasing trends for non-DM to DM due to increasing formation of HC (Supplementary Fig. 4). The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance). The data is represented by box-plot format.
The normalized means ($A_{\text{baseline}} - A_{\text{stress}}$) were 0.26, 0.43, and 0.46 for non-DM, good glycemic, and poor glycemic control subgroup, respectively. This is due to the effect of glycoxidation which is markedly increased in DM subjects as compared to non-DM controls (Fig. 6b), in good agreement with in vitro validation (Supplementary Fig. 8b).

The proposed peroxidative susceptibility measurement (independent of $\text{HbA}_1c$) can be used to further sub-stratify the DM subjects into smaller subgroups (Fig. 6b), in a similar fashion to those for non-DM subjects (Fig. 4b). Exposure to peroxyl compound leads to an increased formation of disulfide bonds in albumin and human non-mercaptalbumin, which was also observed in several other pathological states.

**Performance analysis of proposed stress marker**

We demonstrate the clinical utilities and the sensitivity of the proposed technique by conducting observational, cross-sectional study. This study is designed to understand and evaluate the performance of the proposed biomarkers (oxidative status in RBCs) against the gold standard (oxidative stress in urine $F_2$-Isoprostanes (urinary $F_2$-IsoP)).

Subjects’ recruitment was screened randomly and two subgroups were formed (demographic in Table 1) based on a range of selection criteria (e.g., insulin sensitivity, BMI) and exclusion criteria (e.g., smoking, on medication). This subgroup consists of subjects who are insulin-resistant obese and insulin-sensitive lean (Fig. 7). The subjects were recruited with prior written informed consent approved by the Institutional Review Board of National University Hospital. Fasting blood and urine sample were collected from the subjects. Functional stress analysis was performed on the RBCs using point-of-care technology (PoCT) NMR, and the urinary $F_2$-IsoP analysis was performed using LC/MS (details in Method Online).

It appears that the oxidative stress in RBCs and urine were much higher in obese subgroup than lean subgroup (Fig. 8a). The level oxidative stress in RBCs ($P < 0.0002$) was, however, statistically far more significant than in urinary $F_2$-IsoP ($P < 0.45$). The fasting blood glucose, expectedly, was significantly ($P < 0.005$) higher in obese subgroup than lean subgroup. Interestingly, the ROC for oxidative stress in RBCs (AUC > 0.84) was much higher than fasting blood glucose (AUC > 0.74) (Fig. 8b). A poor diagnostic accuracy (AUC < 0.58) was recorded for urinary $F_2$-IsoP (Supplementary Table 2). This implies that the proposed marker has much higher predictive factor as compared to existing markers (e.g., fasting blood glucose), which is considered as one of the gold standard in the diagnosis of DM. However, extensive biomarker validation with population studies (large N) will further confirm and reveal many aspects of the biomarker, beyond the coverage of this paper.

**Fig. 4 Nitrosative stress test on glycated Hb in RBCs.**

a The A-ratio plot for RBCs in baseline (black) and pretreated sodium nitrite (red, green, blue) for subjects with non-DM subjects ($n = 24$), and DM subjects with their glycemic index in good glycemic control ($n = 43$) subgroups. The means and changes in each subgroups were indicated above the box-plots. The data is represented by box-plot format. b A quadrant chart diagram subjects stratified into subgroups based on their normalized oxidative status (nitrosative stress) in association with their glycemic levels (e.g., HbA$_{1c}$). The threshold of $A_{\text{nitrosative}}$ was at 75th percentile that of DM subjects with good glycemic control, and comes with the flexibility of adjusting based on clinical needs (e.g., population genetics). Note that the Y-axis (nitrosative stress) is analogous with Y-axis (peroxidative stress) in the quadrant shown in Fig. 6b. The details of quadrant representations are shown in Fig. 1e. The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance). The data is represented by box-plot format. c The diagnostic accuracies were evaluated using receiver operating characteristic (ROC) for non-DM subjects against DM subjects with good glycemic control (blue) and poor glycemic control (red) subgroups. The number of subjects ($n$) is indicated in parentheses.
**DISCUSSION**

We have developed a highly sensitive approach to accurately detect and quantify the redox (and hence oxidative/nitrosative) state and the subtle molecular motion changes of blood samples inferred based on the relaxation measurements. This is the first demonstration of the unique magnetic resonance relaxation properties of the various hemoglobin states, which were mapped out using the proposed magnetic state diagram. The measurement of redox properties in plasma/erythrocytes can provide a useful parameter for functional phenotyping of many biological pathways to better understand disease pathophysiology. This technology has vast potential to be applied for clinical disease diagnosis, prognosis, and monitoring, given that the specificity of the oxidative stress in association with the disease state can be further improved in the near future.

The platform presented here has several innovative features and is readily adaptable for clinical use (Supplementary Fig. 10). First, the miniaturized platform\(^3\) developed here is portable, and the proposed assays require minimal processing steps, low-cost, robust and can therefore be performed by a minimally trained operator. The high sensitivity can be attributed to the micron-sized detection coil and optimized ultra-short echo time implemented in this work. Only a minute amount of blood sample volume (<10 µL) is needed for each test, which enables the collection of sample using minimally invasive techniques such as finger prick a standard procedure in patient care.

Second, we exploited the non-destructive nature of magnetic resonance and introduced a number of in vitro functional assays that yielded parameters about the oxidative status of an individual, which may be clinically useful. It probes the primary redox event as compared to the current gold-standard biomarker, urinary F\(_2\)-IsoP, which is a downstream marker and may be susceptible to confounding factors (Table 2). Oxidative stress may affect the levels F\(_2\)-IsoP directly but the downstream urinary F\(_2\)-IsoP level may be modified indirectly by a series of events (or confounding factors) including the kidney function and hypertension (Fig. 9).

The use of isoprostanec as biomarker of oxidative status for correlation with disease outcome has so far yielded conflicting results in cross-sectional versus longitudinal studies\(^6\)\(^8\)\(^9\)\(^6\)\(^8\)\(^9\). Furthermore, they are static biomarkers that provide snapshots of the oxidative status of biological samples representing the in vivo condition of the subject at the point of collection. To accurately measure these molecules, laborious technique such as gas- or liquid-chromatography–mass spectrometry has to be employed, limiting its’ utility as diagnostic tools.

In summary, a new methodology for rapid manipulation and evaluation of the redox states in biological fluids (e.g., RBCs, plasma) using PoCT NMR is proposed. We demonstrated the

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**Fig. 5** Baseline measurements of oxidative stress in plasma. a The \(T_1\)-\(T_2\) relaxometry coordinates of plasma baseline taken from healthy non-DM subjects (blue, \(n=24\)), subjects with good glycaemic control (green, \(n=55\)) and subjects with poor glycaemic control (red, \(n=40\)). The corresponding A-ratio against the subjects with poor glycaemic control and good glycaemic control subgroups, as compared to healthy non-DM subjects. The subjects with poor glycaemic controls were further subdivided into >8% HbA\(_1c\) (\(n=15\)) and >10% HbA\(_1c\) (\(n=25\)) subgroups. The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance). The data is represented by box-plot format. The diagnostic accuracy of RBCs (blue) and plasma (red) taken from non-DM subjects and DM subjects, where their glycaemic subgroups were c good glycaemic control and d poor glycaemic control subgroups. The number of subjects \((n)\) is indicated in parentheses.
clinical utilities of this technique to rapidly sub-stratify diabetes subjects based on their oxidative status in conjunction with their standard glycemic level. Through the use of the proposed methodology, we learnt new biological insights to the disease mechanism of DM, and discussed how sub-stratification of oxidative status may facilitate the early detection (or warning) of diabetes complications (e.g., cardiovascular event). We hope that the rapid and high-throughput analysis will fill up the gap between translational science and clinical medicine, and thus improving the overall outcome of clinical diabetes care and management.

**METHODS**

Magnetic resonance relaxation measurement and detection

The proton nuclear magnetic resonance (NMR) measurements of predominantly the bulk water of red blood cells (RBCs) and plasma were carried out at the resonance frequency of 21.57 MHz using a bench-top type console (Kea Magritek, New Zealand) and portable permanent magnet (Metrolab Instruments, Switzerland), $B_0 = 0.5$ T. A homebuilt temperature controller was constructed to regulate the temperature at $26 ^\circ C$ inside the measurement chamber. This helped maintain the stability of the magnetic field and biological sample under measurement. Single nucleation pulse sequences observed by CPMG train pulses (inter echo time: 200 $\mu$s) consisting of 2000 echoes. A total of 12 scans were typically acquired for signal averaging until the signal level reached a level of 0.5 T.

### Table 1. Demographic of subject for recruited for the study. The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance).

| Age (years) | Lean ($n=21$) | Obese ($n=21$) | T-test |
|------------|---------------|----------------|--------|
|            | Mean (sem)    | Mean (sem)     | $P$-value |
| 23.3       | 0.16          | 28.6 0.64      | <0.0005 |
| 21.9       | 0.12          | 30.6 0.49      | <0.0005 |
| 66.6       | 1.24          | 87.1 2.12      | <0.0005 |
| 174.1      | 1.77          | 168.7 1.87     | 0.04000 |
| 79.7       | 0.37          | 101.0 0.65     | <0.0005 |
| 110.0      | 2.40          | 120.0 1.44     | 0.001  |
| 58.4       | 0.15          | 72.9 1.99      | <0.0005 |
| 4.8        | 0.26          | 19.3 0.85      | <0.0005 |
| 0.9        | 0.05          | 4.1 0.21       | <0.0005 |

Fig. 6  Peroxidative stress test on plasma. a The $A_{\text{peroxidative}}$-ratio plot for plasma in baseline (black) and plasma pretreated with hydrogen peroxide (red, green, blue) for non-DM subjects ($n=20$), and DM subjects with their glycemic index in good glycemic control ($n=20$), and poor glycemic control ($n=52$) subgroups. The means and changes in each subgroups were indicated above the box-plots. b A quadrant chart of diabetic subjects stratified into subgroups based on their peroxidative status (normalized $A_{\text{baseline}}-A_{\text{stress}}$) in association with their glycemic levels (e.g., HbA1c). The threshold of $A_{\text{peroxidative}}$-ratio was set at 75th percentile that of DM subjects with good glycemic control, and adjusting based on clinical needs (e.g., population genetics). Note that the Y-axis (peroxidative stress) is in analogous with Y-axis (nitrosative stress) in quadrant shown in Fig. 4b. The details of quadrant representations are shown in Fig. 1e. The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance). c The diagnostic accuracies were evaluated using Receiver Operating Characteristic (ROC) for non-DM subjects against DM subjects with good glycemic control (blue) and poor glycemic control (red) subgroups. The number of subjects ($n$) was indicated on the parentheses.
nutation frequency of 41.6 kHz. The delay between each pulse (recycle delay) was set at 1 and 4 s for RBCs and plasma, respectively.

Ethics and blood collection
This study received approval from the local Institutional Review Board of the National Healthcare Group, and all participants provided informed consent. The EDTA-anticoagulated whole blood samples were collected using standard phlebotomy procedures. Fresh samples were used unless mentioned otherwise. For other non-critical analysis all blood samples were kept at ≤4 °C within two hours of collection and were kept refrigerated until analysis.

Healthy subjects
Subjects without a past history of diabetes mellitus (DM) and had normal oral glucose tolerance test according to the American Diabetes Association criteria (fasting glucose <5.6 mmol/L; 2 h post oral glucose tolerance test glucose of <7.8 mmol/L) were recruited into this study following provision of informed consent. They were Chinese males aged between 21 and 40 years, with a body mass index below 23.5 kg/m².

Subjects with diabetes mellitus
Anonymized residual samples collected from DM patients at the outpatient clinic for measurement of glycated hemoglobin (HbA1c) as part of their clinical care, were included in this study. The HbA1c was measured using the Bio-Rad Variant II analyzer. This National Glycohemoglobin Standardization Program (NGSP) certified instrument has an analytical coefficient of variation of <2% at HbA1c concentration of 4 and 16%. Our laboratory is NGSP level 1-certified.

Fig. 7 Study design: performance analysis of the proposed markers. A cross-sectional, observational study was conducted to evaluate the proposed oxidative stress biomarker against the gold standard (urinary F₂-IsoP). Subjects were recruited based on a number of selection (e.g., insulin sensitivity) and exclusion (e.g., smoker) criterion (details in “Methods”). Two distinctive group of subjects established were the insulin-resistant obese subjects (n = 21), and insulin-sensitive lean subjects (n = 21) with demographic as shown in Table 1. The biomarkers evaluated were oxidative stress in red blood cells (the proposed technique), oxidative stress in urine F₂-IsoP, and fasting blood glucose.

Fig. 8 Performance analysis of the proposed markers. a The normalized concentration of oxidative stress (OS) in red blood cells, oxidative stress in urine F₂-IsoP, and fasting blood glucose for obese subgroup (red) and lean subgroup (black). The oxidative stress in urine were corrected for urine creatinine concentration (F₂-IsoP/Cr ratio). The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance). The data is represented by box-plot format. b The receiver operating characteristic (ROC) of the respective biomarkers; PoCT NMR (black), fasting glucose (gray), and urine F₂-IsoP (red). Area under the curve (AUC) of larger than 0.8, 0.6, and 0.4 was considered as good, medium, and poor.
Sample preparation and micro MR analysis
Fresh RBCs were washed three times with 1x PBS solution and resuspended at 10% hematocrit with PBS. The selected chemical was then mixed into the prepared blood at the desired concentration (see Biochemical Assays details below). The final concentrations were recalculated based on the entire volume. Other lower concentrations were prepared according to appropriate dilution. A horizontal shaker was used to homogeneously mix (at 200 rpm) for all the chemically treated samples at room temperature. The blood was incubated between a few minutes to a few hours, as indicated in text. The blood was then washed three times to remove the chemical residual. A heparinized microcapillary tube is used to transfer 40 µL volume of blood via capillarity action. In order to obtain packed RBCs for micro MR analysis, the microcapillary tubes were spun down at 3000 × g for 1 min.

Details on biochemical assays
Sodium nitrite treated RBCs. Twenty microliters of the desired concentration (in the range 500 µM to 100 mM) of sodium nitrite were then mixed into 180 µL of the prepared blood. Hydrogen peroxide treated RBCs. Twenty microliters of 3% hydrogen peroxide stock solution (~0.9 M), which was then mixed into 180 µL of the prepared blood. Sodium salicylate treated RBCs. Twenty microliters of natrium hydrosulfit (final concentration, Sigma-Aldrich) were then mixed into 180 µL of prepared blood. Oxoferryl Hb was prepared in two steps. The RBCs were first treated with sodium nitrite (similar to the protocol described above) to convert the RBCs into met-Hb. Hydrogen peroxide was then added into the met-Hb using the same protocol as described above. Preparation of nitrosyl-Hb. Twenty microliters of natrium hydrosulfit, Na2S2O4 (10 mM final concentration, Sigma-Aldrich) were then mixed into 180 µL of prepared blood and mix homogenously (at 200 rpm) for 10 min with a horizontal shaker. The UV-Visible spectrum was recorded immediately to confirm the presence of deoxygenated hemoglobin. Pure gas N2 was continuously purged into an airtight chamber in order to maintain the deoxygenated condition. The UV-VIS absorbance was used to confirm the presence of deoxygenated Hb by its distinct peak at 543 nm. Hydrogen peroxide treated plasma. The fresh whole blood collected was centrifuged at 14,000 × g for 5 min to separate the plasma from the packed RBCs. Ten microliters of hydrogen peroxide solution was then mixed into 90 µL of prepared plasma, and other lower concentrations were prepared according to appropriate dilutions.

Table 2. Comparison of salient features between the proposed method (PoCT NMR) benchmarking against the gold standard, urinary F2-IsoP

| Proposed method | Gold standard |
|-----------------|---------------|
| Biological liquid | RBC | Urine (isoprostane molecules) |
| Mechanism | Glutathione depletion | Lipid peroxidation |
| Method | Direct | Indirect |
| Functional test | Yes | No |
| Instrumentation | POCT NMR | LC/MS |
| Portability | Yes | No |
| Analysis time | Rapid (min) | Hours |

Performance analysis of proposed oxidative stress
Study design. Open-labeled, randomized, observational were carried out on insulin-resistant obese (n = 21) subjects and insulin-sensitive lean (n = 21) subjects. Fasting blood and urine sample were collected from the subjects. Oxidative stress analysis was performed on the RBCs using PoCT NMR, and the urine was frozen down before sending out for urinary F2-IsoP analysis with LC/MS.

Subject recruitment. We recruited 42 normoglycemic Chinese men (21–40 years; lean insulin-sensitive, n = 21 and obese insulin-resistant, n = 21). Exclusion criteria were history of smoking, thyroid disorder, malignancy, recent hospitalization, or surgery, first-degree relative with T2D, dyslipidemia and its treatment, corticosteroids usage over the past 3 months, alcohol consumption (>3 units a day), moderate-to-high intensity physical activity (>5 h a week), or change in weight over the past 3 months (>5%). The modified-WHO definition for obesity in Asians was used to define lean (18.5 ≤ BMI < 23 kg/m²) and obese (BMI ≥ 27.5 kg/m²) subjects in this study. A Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) score of <1.2 was employed for identification of insulin-sensitive lean subjects, and ≥2.5 for insulin-resistant obese subjects (Table 1).

Ethics approval
Singapore’s National Healthcare Group Domain Specific Review Board (DSRB Ref. No: C/2013/00902) approved the study protocol, and Singapore
Good Clinical Practice guideline and the principles of the 2013 Declaration of Helsinki were duly followed in performing all study procedures. Written consent was obtained from each subject before participation in this study.

Oxidative stress F2-isoprostanes measurements

Urinary free F2-isoprostanes were processed by anionic solid-phase extraction. Creatinine levels were measured to standardize the dilution of urine Photometric Analyzer (Roche Diagnostic GmbH, Germany). Samples were then measured by gas chromatography–mass spectrometry set at negative chemical ionization mode (Agilent Technologies, CA), with Triple-Isoprostanes with that of the relevant deuterated internal standard.

Statistical analysis

Unless otherwise noted, all statistical analyses were performed using OriginPro (OriginLab Corporation, USA). For statistical analysis, t-tests were used. All error bars represent the standard deviation (s.d.) or standard error measurements (s.e.m.) of means and the statistical results were stated as P-values. The statistical significance was calculated using the Student’s T-test (two-tailed, unequal variance).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author (wengkun@inj.int) upon reasonable request.

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AUTHOR CONTRIBUTIONS
W.K.P. and T.P.L. conceived the original idea and analyzed the results, and wrote the first draft of the paper together. W.K.P. designed the experiments/protocols, proposed the magnetic state diagram, and spearhead the entire hardware development. L.C. assists in hardware development and performed most of the micro MR analyses and related assays. J.H. and B.O.B. provided input regarding translational medicine applications. All the authors checked through the manuscript and analyzed the data.

COMPETING INTERESTS
The authors declare no competing financial interests and non-financial interest except that one technology disclosure related to this technology was filed.

ADDITIONAL INFORMATION
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