Non-invasive platform to estimate fasting blood glucose levels from salivary electrochemical parameters

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Diabetes is a metabolic disorder that affects more than 400 million people worldwide. Most existing approaches for measuring fasting blood glucose levels (FBGLs) are invasive. This work presents a proof-of-concept study in which saliva is used as a proxy biofluid to estimate FBGL. Saliva collected from 175 volunteers was analysed using portable, handheld sensors to measure its electrochemical properties such as conductivity, redox potential, pH and K^+, Na^+ and Ca^{2+} ionic concentrations. These data, along with the person's gender and age, were trained and tested after casewise annotation with their true FBGL values using a set of mathematical algorithms. An accuracy of $87.4 \pm 1.7\%$ and a mean relative deviation of 14.1% ($R^2 = 0.76$) was achieved using a mathematical algorithm. All parameters except the gender were found to play a key role in the FBGL determination process. Finally, the individual electrochemical sensors were integrated into a single platform and interfaced with the authors' algorithm through a simple graphical user interface. The system was revalidated on 60 new saliva samples and gave an accuracy of $81.67 \pm 2.53\%$ ($R^2 = 0.71$). This study paves the way for rapid, efficient and painless FBGL estimation from saliva.

1. Introduction: Diabetes mellitus is a global health epidemic with as many as 415 million people suffering from its worldwide. This number is predicted to reach a staggering 642 million by 2040 [1]. If left untreated, diabetes can cause various medical complications such as heart diseases, diabetic retinopathy and kidney dysfunction [2]. Many a time, these conditions can be prevented or at least be delayed by early intervention and regular monitoring of fasting blood glucose level (FBGL), a prime indicator of diabetes using glucometers [3]. Blood is the natural choice for measuring elevated blood glucose levels (BGLs), but it poses a risk of contamination and sepsis, especially when samples are collected at point-of-care. Several recent studies have shown that alternate biofluids such as urine, saliva, tears and sweat can also be used instead of blood as they carry residual amounts of glucose [4, 5]. This has enabled the development of continuous glucose monitoring systems [6–9] that provide blood glucose information every 1–5 min. The results, however, often suffer from poor sensitivity and reproducibility owing to pH and temperature fluctuations, or electromagnetic interferences [10, 11]. In other approaches, intervening tissues such as bone or skin are used for measuring trace amounts of glucose, but again, they have had limited success due to practical limitations of sample extraction [12, 13]. Saliva is perhaps one of the simplest biofluids to retrieve and has a proven track record as a proxy biofluid (to blood) for diagnostic applications [14]. The salivary glucose has gotten raised during diabetes since the salivary glands, controlled by neural and hormonal activity, act as a filter for blood glucose and cause loss of homeostasis [15]. Thus, many groups have reported direct glucose sensing in saliva using enzymatic approaches wherein, glucose oxidase is typically immobilised on a filter paper [16] or a surface containing polymers or carbon nanotubes [17], and an outcome is obtained as an electrical or optical signal readout.

In this Letter, we explore an alternate route for blood glucose estimation based on the variation in electrochemical parameters of saliva during diabetes. Since this approach is based solely on the physical attributes of saliva, it can potentially be more cost-effective, robust and scalable than the current enzymatic methods. There is substantial evidence to suggest that the composition, pH, viscosity and buffering capacity of saliva vary significantly during diabetes [18, 19]. For instance, while the salivary pH in healthy individuals is around 7–7.5, in diabetics is typically below 7. This acidic pH is a result of higher microbial activity in the mouth caused by greater levels of glucose in saliva [13, 14]. This also makes the diabetics significantly more susceptible to oral cavities [20]. Similarly, calcium [18] and potassium [21] ionic concentrations are found to be significantly higher in the diabetic group compared to healthy individuals [22]. This increase in Ca^{2+} is attributed due to the reduction in the fluid output (oral dehydration) which in turn increases the protein concentration in the oral cavity and hence, possibly Ca^{2+} due to the alteration in ionic affinity [18]. The increase in K^+ ions is either due to increased activity of Na^+ K^+ ATPase or because of the changes in the basal membrane of the salivary gland [23]. The elevation in oxidative stress in saliva during diabetes is well observed clinically due to persistent hyperglycemia, which increases the production of free radicals, especially reactive oxygen species (ROSs), in all tissues due to glucose auto-oxidation and protein glycosylation [24]. Hyperglycemia also influences the electrical charge distribution on the surface of the cell membrane and results in increased conductivity [24].

Our group has previously shown that a combination of salivary electrochemical parameters such as pH, oxidation–reduction potential (ORP), conductivity and individual cationic concentrations can be collectively used to estimate FBGLs in healthy and diabetic adults. This was done using mathematical algorithms like logistic regression, artificial neural network and support vector machine which had the limitation that the output classes were finite, i.e. 0 below a threshold FBGL value and 1 above it [25]. In this Letter, we have extended this concept to use of regression models such as kernel ridge regression (KRR) [26], neural networks regression (NNR) [27] and neuralNet boosting regression (NBR) [28] instead of classifiers to estimate the actual FBGLs in patients and healthy volunteers. Also, the relative contribution from each

input salivary electrochemical parameter is estimated using the weights of perceptron of neural network to identify the critical set of design parameters required to conceptualise an integrated electrochemical sensor having minimum feature attributes. Finally, our optimised algorithm has been implemented on a standalone platform in which all the sensors are integrated and interfaced to a single display using an embedded microprocessor and a GUI. A complete process flow diagram of our proof of concept study is shown in Fig. 1.

2. Methodology

2.1. Sample collection: The protocol for sample collection and the inclusion criteria used for volunteer selection was as described earlier [29]. Briefly, 175 volunteers comprising half diabetic and half healthy in the age range of 18–69 yr were recruited. The volunteers were divided into two groups – (i) healthy volunteers (FBGL: 80–120 mg/dl; 41 females; 46 males; age range of 18–62 yr; mean age 35 ± 11 yr), (ii) clinically diagnosed type II diabetes mellitus patients (FBGL $\geq$120 mg/dl; 47 females; 41 males; age range of 21–69 yr; mean age 47 ± 10 yr). Approximately 3 ml of saliva was collected from each volunteer and immediately analysed for conductivity (Cond.), redox potential (ORP), pH, and calcium, potassium and sodium ionic concentrations. The pH and ORP values were measured using the F-71 Laqua Lab (Japan) pH/ORP meter. The conductivity and concentration of Na^+, K^+, and Ca^{++} electrolytes were recorded using the Horiba Laqua twin series ion selective models. The venous plasma FBGL values of these volunteers were measured using an automatic biochemical analyser (COBAS INTEGRA 400 plus).

2.2. Data fitting: To find the optimal mapping between the input and output variables, we used three different mathematical regression algorithms – KRR, NNR and NBR, with successively increasing complexity. A brief introduction to these regression algorithms is provided in **SI**. All the 175 sample data points were represented as an eight-dimensional vector containing six real-valued electrochemical parameters of saliva, integer parameter of age and a Boolean parameter for gender, and mapped against their FBGL value. The nine parameter values were then linearly normalised between [0, 1] (i.e. the lowest value mapped to 0 and the highest to 1). As the FBGL values were all integers, they were approximated as real values by the range space approximation. This converted the problem of learning space transformation into a classical problem of regression. The data underwent uniform random shuffling, followed by partitioning into two parts – 70% training and 30% testing sets. The training set was further subjected to five-fold cross-validation. To deal with data complexity, non-linear regression techniques such as KRR and NRR were applied using Python 2.7 along with its numpy and scipy libraries. The learning

Fig. 1 *Process flow diagram for our study*

process was further improved by using the ideas of ensemble learning and boosting through the NBR model. Both the input and output parameters were represented as numpy arrays with shapes (175, 8) and (175, 1), respectively. The estimated FBGL values were further classified as 0 or 1 based on an assumed threshold value of 120 mg/dl (1 for FBGL $\geq$120 mg/dl) [30] and the performance of each regression model was evaluated in terms of R^2, mean relative deviation, accuracy, specificity, precision, sensitivity and $F1$ score (see SI for details).

2.3. Feature contribution: The mining of feature importance was performed by weight analysis of the neural network's hidden layer. Since the output layer in regression had only one perceptron, the feature importance was calculated by the coefficients of the input features and finding their respective relative percentage values in that neural network

$$\text{Feature importance}\,(x_i) = \oint_{\forall x_j, j \neq i} \frac{\partial(\text{NeuralNet}(X))}{\partial x_i}$$

$$\text{where } X = [x_1, x_2, \ldots, x_n] \text{ and } \forall x_k, 0 \leq x_k \leq 1 \tag{1}$$

2.4. Integrated platform design for direct sensing: The commercial sensors used for our conductivity and ion-selective measurements were opened and the analog output signals from the conditioning unit (OPAMP-LMC6042 CMOS Dual Micropower) were directly extracted and connected to the input of an Arduino microcontroller (RoboKart UNO R3 board with DIP ATmega328). Similarly, the pH and ORP device signals were also connected via an Arduino-compatible kit (SKU: SEN0161) (Fig. S1). The processed output from the controller was synced with our optimised algorithm using Matlab2014a. A GUI was then developed in MATLAB that allowed user-related data entry (personal details such as ID number, age, sex etc.), system calibration, and display of FBGL and salivary electrochemical parameters on a laptop screen. The sensors were calibrated using the standard solutions provided by the vendor and showed a good correlation of 0.9 with the original sensor suggesting that our 'reverse-engineering approach' had little effect on the sensor performance (see SI for details). To revalidate the integrated system, 60 new volunteers were recruited and divided into two groups – (i) healthy (FBGL: 80–120 mg/dl; 14 females; 16 males; 20–60 yr; mean age 31 ± 7 yr) and (ii) clinically diagnosed type II diabetic (FBGL $\geq$120 mg/dl; 18 females; 12 males; 20–63 yr; mean age 45 ± 11 yr). Each volunteer's fasting saliva was collected in the same manner as described above and analysed using the integrated system. The performance of the integrated platform was determined using the Clarke error grid analysis approach that is used to assess the clinical significance of differences between the glucose measurement technique under test and the reference venous blood glucose measurements (see SI for more details).

3. Results

3.1. Data collection and normalisation: The number of samples ($n = 175$) used for our model fitting was chosen as per the Chebyshev's inequality which states that the minimum number of data points required to estimate an outcome in the bound confidence interval of δ with a confidence level of $(1 - \varepsilon)$ can be given by the following equation:

$$P(|X_{\text{sample}} - X_{\text{population}}| \geq \delta) \leq \frac{\sigma^2}{n\delta^2} = \varepsilon$$
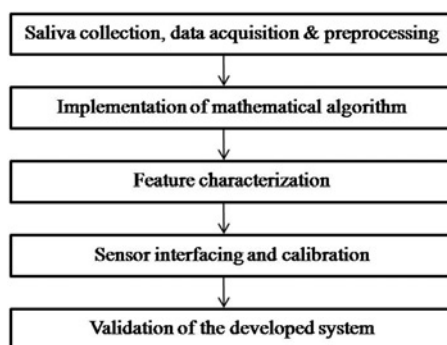
$$\text{or, } n = \frac{\sigma^2}{\varepsilon\delta^2} \tag{2}$$

where X_{sample} and $X_{population}$ are the sample and population means, respectively. By assuming (i) the total population of India to be 1.25 billion, (ii) 65% of the people to lie in the range of 20–79 yr age group (target population), (iii) 9% of the target population to have diabetes, (iv) the population follows a Bernoulli's distribution with σ^2 variance, (v) $\delta = 10\%$, and (vi) $(1 - \varepsilon) = 95\%$, we estimated that the minimum number of samples to be tested should be at least 164 in order to correctly represent the wider dataset. Therefore, a total of 175 volunteers were recruited for our study.

3.2. Mathematical analysis: A 175×8 shaped matrix was created from the data collected from the volunteers. To minimise the bias towards a particular parameter due to differences in their units of measurement, all the data were linearly normalised within their original ranges and then subjected to model fitting. In all the cases, the results were classified as per high and low FBGL values taking 120 mg/dl as the threshold. In the KRR model, a mean relative deviation was obtained to be 29.4% with a coefficient of determination, R^2 equal to 0.41 after fully optimising the α and γ space parameters (Fig. S2). The results of other performance parameters are listed in Table 1.

In the NNR model, the training data were iterated until the validation error between the actual and estimated FBGL values converged and reduced to $\sim$0.05 at around 10,000 iterations (Fig. 2a). The maximum number of cases fell in the range of ± 25 mg/dl blood glucose deviation (Fig. 2b). At this configuration, the NRR model resulted in an accuracy of $84.7 \pm 2.1\%$ with an R^2 value of 0.45 and mean relative deviation of 21.0% for estimating the FBGL values. It was also observed that there was an undesirable residual pattern between the error and the estimated value of BGL (data not shown). To overcome this issue and to learn from the trend in error to estimate the FBGL more efficiently, the NBR model was investigated next.

Different models were tried to improve the estimated accuracy of the NBR model (Fig. 3a). The estimation accuracy saturated at the fourth degree giving the best performance with the primary neural network of configurations 8, 16, 4, 1 and subordinate neural networks of configurations 1, 3, 1. At these conditions, the test accuracy was obtained as $87.4 \pm 1.7\%$ with an R^2 value of

Table 1 Comparison of the three regression models used for FBGL estimation

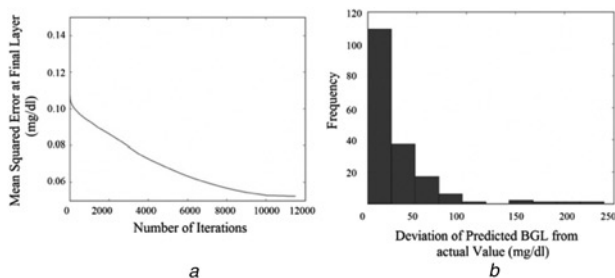| Algorithm	Accuracy	Precision	Sensitivity	$F1$ score
KRR	73.8 ± 1.9	65.7 ± 2.1	83.1 ± 0.4	73.3 ± 1.3
NRR	84.7 ± 2.1	73.7 ± 2.9	93.3 ± 1.7	83.3 ± 2.2
NBR	87.4 ± 1.7	79.1 ± 1.7	88.7 ± 2.1	83.6 ± 1.8

Fig. 2 *NNR model*
a Convergence of error at final layer with several iterations of the training dataset
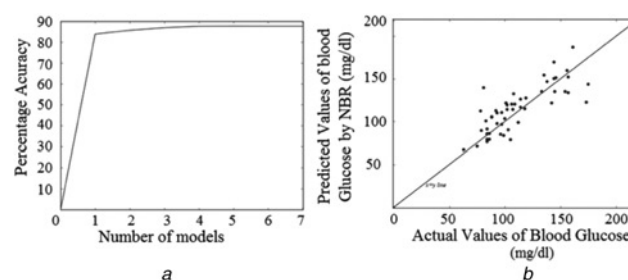*b* Frequency distribution of deviation of estimated FBGL from an actual value

Fig. 3 *NBR model*
a Accuracy of NBR with an increasing number of models
b Correlation between actual blood glucose values and those estimated by NBR for the test data

0.76 and mean relative deviation of 14.14% (Fig. 3b). An overall comparison between all the three non-linear regression models is summarised in Table 1.

3.3. Importance of parametric contribution: The best-learnt NBR model was further used for extracting the relative importance of each input feature variable for estimating the FBGL. This becomes relevant while designing the actual sensor hardware so that the most optimum system may be developed for any real application. To achieve this information, a partial differentiation was taken concerning each individual parameter and substituted with zero to determine its relative contribution in estimating the BGL value. The results obtained showed that all parameters except a person's gender played an important role in the FBGL estimation (Table 2).

3.4. Performance of the integrated sensing platform: To demonstrate proof-of-concept, we integrated all the relevant sensors into a single platform to collectively display one output using a simple GUI. For this purpose, commercial sensors were reengineered to obtain their analogue signal outputs (Fig. 4 and see SI). Analogue voltages corresponding to the standard solutions (provided by the company) were measured to understand the working principle of the sensors. The measured concentrations from our sensor setup were then correlated to the actual concentrations obtained by the ion meters (see SI). For pH and ORP, a highly compatible Arduino controller kit was employed and an Arduino UNO R3 was used for processing the sensors output. The obtained analogue signals from different sensors were then fed into the analogue pins of Arduino, which converted them into computer-compatible signals through serial communication. The processed outputs from Arduino were then synced with the algorithms in MATLAB to estimate FBGL values.

For efficient implementation of our system, a simple GUI was developed in MATLAB. The GUI had four independent operators: connect, disconnect, calibrate and test. By pressing the 'connect' tab, the system established communication between the sensors and the algorithms in a MATLAB environment. The 'calibrate' button enabled system calibration to be done manually with the different standard solutions. Once done, the 'test' tab could be pressed to run the algorithms in MATLAB which would read the respective values of all the sensors through Arduino and display them onto the GUI layout editor window within a few seconds. Accordingly, different messages were displayed if the FBGL was found lower or higher than 120 mg/dl. Finally, the sensor interface could be disconnected from the MATLAB environment by pressing the 'disconnect' button. Using this approach, the efficacy of the integrated system was tested on 60 samples by applying the NBR model. The results gave an accuracy of $81.67 \pm 2.53\%$, a mean relative deviation of 15.0% and a coefficient of determination of 0.71. The performance of the developed interface was also validated

Table 2 Relative percentage contribution of individual parameters in the estimation of the blood glucose value

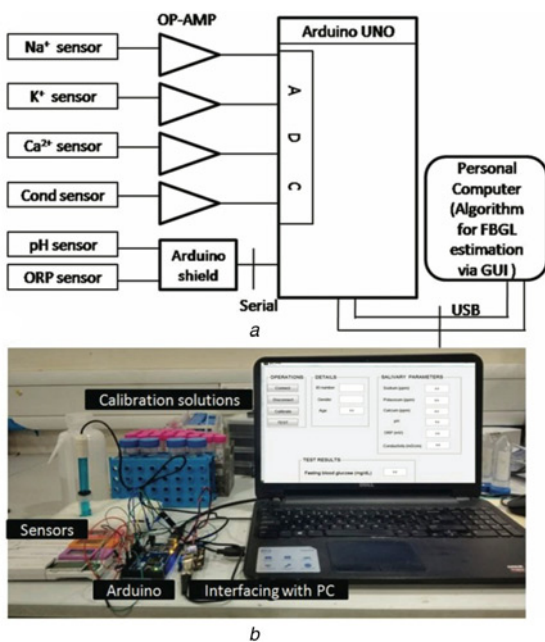| Sex	ORP	Age	Na$^+$	pH	Ca^{2+}	Cond.	K$^+$
0.5%	11.7%	11.9%	13.6%	14.2%	15.0%	16.1%	17.0%

Fig. 4 *Experimental setup to estimate FBGL*
a Block diagram of the experimental setup
b Actual integrated sensor setup with the GUI displayed on the laptop screen
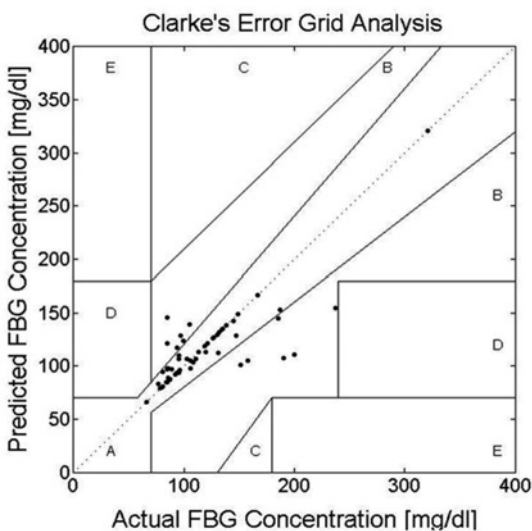

Fig. 5 *Clarke's error grid analysis of the developed interface shows that 80% of the data lie in the A zone while 20% in the B zone*

using the Clarke error grid analysis for comparison with the ISO guidelines (Fig. 5).

4. Discussion: The problem of blood glucose estimation using electrochemical parameters of saliva is a classic regression problem with high variability and non-linearity in parameters. Classically, one of the standard techniques used for model fitting is ordinary least square regression which was discarded in our case due to its underlying assumption that all the parameters of input vector X are independent of each other which may not be true. This would make the X matrix almost singular and highly prone to errors. Thus, ridge regression was chosen as the next best model by imposing a cost to the size of the coefficients. In this, a Gaussian kernel was chosen to account for the non-linearity in the data. Ridge regression showed a fair performance in terms of accuracy of estimation for critical and non-critical cases based on the estimated FBGL values. However, a low R^2 value of 0.41 meant that statistically only 41% variability between the electrochemical parameters and blood glucose was being taken into account. Neural networks allowed improving the mapping ability further by capturing the data non-linearity, non-convexity and feature extraction from the parameter space. Although this significantly improved the accuracy, the R^2 value of 0.45 obtained was still low, which indicated that the model was still not good enough to capture a considerable amount of variability in the system. The results and errors when analysed also showed a non-ideal correlation between the error of estimation and estimated value. This finally led us to choose a four-degree ensemble NBR model to train a second-order model and estimate error from the first-order estimated value of FBGL. Theoretically, any large enough order model number (n) may be chosen to eliminate the correlation between the error and estimated values.

The individual contribution of input parameters suggested that gender plays little role in the estimation of FBGL. This makes our approach more inclusive. The highest contribution by K$^+$ ions is not surprising considering hyperkalemia is known to exist in diabetic patients due to the body's altered physiology [31, 32]. This happens because the lack of insulin in people with diabetes causes the breakdown of their fat cells releasing ketones into the blood, particularly in type 1. This makes the blood more acidic, a condition is known as diabetic ketoacidosis. The acidosis and high BGL work together to cause the fluid and potassium to move out of the cells into the blood circulation. Also, patients with diabetes have diminished kidney function and may not be able to excrete potassium into the urine as effectively. Dying cells during tissue destruction may further add to this condition by releasing potassium ions into the blood circulation. Our results for Ca^{2+} contribution are also in agreement with the literature that suggests that salivary Ca^{2+} increases in diabetics as compared to healthy ones [33, 34]. The reason for lower levels of pH seen in diabetics is likely due to the tendency of their oral cavity to get dry as a result of the changes in their metabolism [35]. Similarly, ORP, age and Na$^+$ ions although slightly less important, cannot simply be neglected when designing a saliva-based biosensor for FBGL estimation.

The latest ISO guidelines (15197–2013(E)) for in vitro diagnostic systems used for self-testing of diabetes mellitus require that 95% of the measured glucose values fall within ± 15 mg/dl of the average measured values of the reference measurements for glucose concentrations <100 mg/dl and within ± 15% for glucose concentrations ≥100 mg/dl [36]. Using our NBR model, we found that only 83.3% of the <100 mg/dl cases lay within the prescribed limit of ± 15 mg/dl and around 73.3% cases tested positive for ≥100 mg/dl, as shown by the Clarke error grid analysis (Fig. 5). Here, 80% of the data lay in the A zone and 20% in the B zone. The lower accuracy of our outcome may be attributed to the low number of samples used for establishing our proof of concept. Nevertheless, this approach provides a rapid, painless and effortless

way to estimate FBGL and opens new avenues for further investigations of salivary potential in diabetes. Moreover, our approach is non-enzymatic, so it overcomes the disadvantages associated with enzymatic processes that produce corrosive hydrogen peroxide as a byproduct and suffer from interferences from structurally similar organic substances like uric acid, lactate, dopamine and ascorbic acid during sensing. More samples in future may ensure better learnability of the mathematical algorithms and for choosing a more stringent confidence interval. For instance, a 5% confidence interval for 95% confidence level would require a diverse set of 720 samples. This will allow a more accurate diagnosis of diabetes using a simple, non-invasive approach.

5. Conclusion: Three non-linear regression models, namely KRR, NNR and NBR, were applied to check the correlation between a person's age, gender and salivary electrochemical parameters to their actual FBGL values. NBR showed the best-classifying accuracy of $87.4 \pm 1.7\%$ ($R^2 = 0.76$) and no correlation pattern between the error and the estimated values. Gender had little role to play in the estimation of FBGL value, whereas all other input parameters contributed significantly. K^+ ions had the maximum relative impact at 17% by the known physiology of diabetic patients. An integrated platform was developed that allowed non-invasive estimation of FBGL within a few seconds with an accuracy of $81.67 \pm 2.53\%$ using the NBR model. These results suggest that a few drops of morning saliva may be as effective as blood for measuring FBGLs. In the future, a low-cost, handheld integrated sensor with multiple ion-selective electrodes may be developed using state-of-the-art microfabrication technology. One may also study the role of salivary anions and other input variables such as body mass index to further refine the estimation model and increase its robustness and accuracy for blood glucose estimation.

8 References

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