Hypoglycaemia detection with non-invasive sensors

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Thesis 2019

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I am sciencing as fast as I can!

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Foreword

Since I admitted this thesis, the last paper, called "A multiparameter model for non-invasive detection of hypoglycemia" has been published in the scientific journal Physiological Measurement in an amended version.

DOI: <https://doi.org/10.1088/1361-6579/ab3676>

Acknowledgements

The work for this thesis has been done at the Department of Clinical and Medical Engineering in cooperation with the Diabetes Laboratory (clamping procedures) and the nephrology ward at Rikshospitalet (overnight stay before clamping procedures), all part of Oslo University Hospital. Financial support for the PhD period was kindly provided by the South Eastern Health authority in Norway, with additional funding for equipment by the Norwegian Diabetes Association. After the scheduled PhD period I have graciously been given the opportunity to continue the work while in a permanent position at the Department of Clinical and Medical Engineering.

Special thanks for help with the work for this thesis goes to:

The 21 participants who volunteered to be research subjects in our study. It was quite an arduous task, involving many blood tests and being confined to a bed for several hours during two different days. They had nothing to gain from it except from contributing to our research. I am very grateful for their effort and selflessness.

My colleague and supervisor Christian Tronstad for extensive help through the years. Without Christian, the papers could never have been finished. Since we share an office, he has also definitely done the most supervising, and he is always glad to help. He is a man of many talents, and has among other things done an oil painting for me. Most admirable of all is his positive attitude to everything and everyone. Also, he has been a great friend to me these years.

My main supervisor Ørjan Martinsen who was vital for securing funding for this PhD-project, and has had the overall responsibility for the project. As head of the Oslo Bioimpedance and Medical Technology Group he does an outstanding job as a leader, always being supportive and keeping people motivated. His sense of humour is also greatly appreciated.

Hanne Gulseth at the Diabetes laboratory who has been my main medical supervisor. Always positive and at times fiercely effective, her help has been invaluable for me during the clamping procedures, and she has also helped us a lot in the writing process. We have spent much time together during clamping procedures, which has always been fun and interesting. Also, I have very much enjoyed attending her annual new year's table.

Kåre Birkeland, who has also been a medical supervisor. He has helped us from the beginning with planning and facilitating of the clamping procedures as head of the Diabetes laboratory. His vast knowledge of the field of diabetes, and his kind and positive demeanor makes him a perfect supervisor, who I surely could have utilized even more than I did. His comments on the papers have been a great help to us, always insightful and concise advice. During the final part of my thesis work, he has been an incredible help for structuring the text and keeping the language on point.

Trond Jenssen, who has been a vital part of the team, and together with Christian Tronstad had the initial idea to test our sensor systems on diabetes patients. He helped us plan the study, and also facilitated the necessary overnight stay in a medical ward for our research subjects. He has also had valuable contributions in the writing of the three main papers of the thesis. With his inviting and kind demeanor, it was easy to contact him for all things related to research work.

Marit Bjørgaas, who has been involved in the project from the start, and at some point made us discuss the project with Brian M. Frier (who should also be thanked) at a meeting in Trondheim. He was adamant that we had to do euglycaemic clamping procedures as well as hypoglycaemic, and as it turns out, this was vitally important for the results and the statistics. Furthermore, she has been a formidable resource in the writing process, giving thorough (and extremely quick!) feedback. Also, she has been such a joy to communicate with, always supportive and friendly.

Kathrine Frey Frøslie for all statistical advice, encouragement, positive attitude, general advice in the writing process and for introducing us to the bootstrap method.

Åsne Halse, Gøril Vinje, Karin Eide and Gro Boezelijn are/were nurses at the Diabetes Laboratory who helped us through all the clamping procedures. Their professional, calm and friendly demeanor made experiment days much easier for both us and the patients. I can honestly say that every clamp procedure we did was enjoyable, they were truly a delight to work with. Gøril and Åsne deserve extra credit for also recruiting patients and keeping track of the paper work around this, preparing the clamp procedures and keeping track of all the different blood tests.

All doctors and nurses involved in recruiting patients both at the outpatient diabetes clinic at Aker, and at the Norwegian Diabetics centre. Special thanks to Kirubalini Moorthy for tirelessly recruiting new patients when we struggled to reach our goal. Also thanks to the Norwegian journal for diabetes patients, "Diabetes", for making a small article that helped us in the recruitment process.

All the nurses at the nephrology department's ward that helped us with blood glucose monitoring through the overnight stay before experiment days.

Kristin Godang for helping us with measurements of adrenaline and noradrenaline.

Jan Olav Høgetveit (my boss's boss), who was the person to give me a position for my civilian service many years ago, and is the reason why I work here at all. Since then he has been a good friend and colleague, and he and Christian together managed to get me back to the department in a PhD-position, and later in a permanent position, which basically means job security for life. It's like being blessed by the mafia, as long as I don't murder someone, I'm good.

Geir Teigstad, the director of Oslo Hospital Services (my boss's boss's boss) for approving my position and being very supportive of our work at the R&D department.

The current leader at my department, also a good friend and colleague, Håvard Kalvøy, who I was lucky to share an office with for a long time, and who helped us with the technical side of the clamping procedures when Christian was unavailable. He gives us the right amount of freedom to do a good job both at work and at home.

My good friend and colleague Tormod Martinsen, who has helped with many things related to graphical design, and has kept me entertained with numerous stories and crazy ideas throughout the years.

All my other good friends and colleagues at the R&D department; Fred-Johan Pettersen, Runar Strand-Amundsen and Maryam Amini. Because of you, coming in to work is always enjoyable.

My good friend and colleague André Bakken for miscellaneous help with equipment and general inquiries (and fixing a lot of stuff that is not work related too..).

All my other good friends at the Department of Clinical and Medical Engineering.

My brother Georg Elvebakk for additional advice on statistics.

Additional thanks to my other siblings w/families, my mother and my late father. To paraphrase David Sedaris; They nice, the people.

Finally, I would like to thank my beloved little family; my significant other, Linn, and my two children, Matilda and Vetle. To Matilda and Vetle; if you ever read this (you were three and one year old when I wrote this), there are so many things that are more important than degrees. Like being kind, honest and treating all people with respect. But the most important thing is to help around the house so your dad has time to watch Netflix. Now go read a book instead.

Abbreviations

ANN	Artificial Neural Network
AUROC	Area Under the Curve for the Receiver Operating Characteristic
BG	Blood Glucose
CGM	Continuous Glucose Monitor
ECG	ElectroCardioGraphy
EEG	ElectroEncephaloGraphy
EHSS	Edinburgh Hypoglycaemia Symptom Scale
FSR	Frequency of Skin conductance Responses
GH	Growth Hormone
HR	Heart Rate
IAH	Impaired Awareness of Hypoglycaemia
MARD	Mean Absolute Relative Difference
NIR	Near InfraRed
PLS	Partial Least Squares
PPV	Positive Predictive Value
QTc	corrected QT-time
SC	Skin alternating current Conductance density
SCR	Skin Conductance Response
Z	Bioimpedance

List of publications

Paper I

Tronstad C, Elvebakk O, Kalvoy H, Bjorgaas MR, Martinsen OG: Detection of sympathoadrenal discharge by parameterisation of skin conductance and ECG measurement. Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Annual Conference 2017;2017:3997-4000

Paper II

Elvebakk O, Tronstad C, Birkeland KI, Jenssen TG, Bjorgaas MR, Froslic KF, Godang K, Kalvoy H, Martinsen OG, Gulseth HL: Evaluation of Hypoglycaemia with Non-Invasive Sensors in People with Type 1 Diabetes and Impaired Awareness of Hypoglycaemia. Sci Rep 2018;8:14722

Paper III

Tronstad C, Elvebakk O, Staal OM, Kalvøy H, Høgetveit JO, Jenssen TG, Birkeland KI, Martinsen OG: Non-invasive prediction of blood glucose trends during hypoglycemia. Analytica Chimica Acta, Volume 1052, 4 April 2019, Pages 37-48. DOI: 10.1016/j.aca.2018.12.009

Paper IV

Elvebakk O, Tronstad C, Birkeland KI, Jenssen TG, Bjorgaas MR, Gulseth HL, Kalvoy H, Høgetveit JO, Martinsen OG: A multiparameter model for non-invasive detection of hypoglycemia. *As submitted to Physiological Measurement.*

1 Background

1.1 Introduction

For diabetes patients who need insulin to manage their disease (type 1 diabetes patients and some type 2 diabetes patients), there is an inherent risk of hypoglycaemic episodes.

Hypoglycaemia is a state where there is not enough glucose available in the blood, and varies from mild with little symptoms, to severe requiring assistance (table 1).

Level	Glycaemic criteria/description
Level 1	Glucose <70 mg/dL (3.9 mmol/L) and glucose $\geq$ 54 mg/dL (3.0 mmol/L)
Level 2	Glucose <54 mg/dL (3.0 mmol/L)
Level 3	A severe event characterized by altered mental and/or physical status requiring assistance

Table 1. Types of hypoglycaemia. Taken from ADA Standards of Medical Care in Diabetes 2019 (1).

While some insulin-users find it easy to control their blood glucose (BG) levels, for others it can be very demanding, and the fear of hypoglycaemia can adversely affect mental health (2; 3). There has been significant progress in diabetes technology, especially with continuous glucose monitors (CGMs), markedly so even during the latest 6-7 years since the studies in this thesis were planned. Still most diabetes patients use finger prick testing, and globally, this will probably be the case for many years. At the same time, sensor technology has leapt forward, with increasingly smaller and cheaper sensors, and more advanced algorithms. However, the quest for non-invasive measurement of BG has been somewhat of a holy grail, and many companies and competent research groups have tried (and are still trying) their hands at it, so far to no avail. Our approach was to try to make a hypoglycaemia alarm; instead of relying on glucose measurements we would try to measure other covariates to hypoglycaemia, e.g. sweating.

The idea to make a hypoglycaemia alarm is far from new (4). To make a system that is better than what is already available, some progress in sensor technology and/or data processing has to be achieved. Ideally, it should also work for patients who are most prone to severe hypoglycaemia, namely patients with impaired awareness of hypoglycaemia (IAH) (5; 6), the

idea being that non-invasive sensors might be sensitive to small changes in the physiology that patients with IAH cannot detect themselves.

1.2 Type 1 diabetes

Type 1 diabetes is characterized by loss of insulin producing beta cells in the pancreas (7) due to an autoimmune reaction. It is not known which environmental factors that triggers the autoimmunity, a lot of theories are being tested, one of them is virus infections (8). Several genes play an important role (9; 10), and in particular certain variants in the HLA-system predispose to the disease, and first degree relatives of patients with type 1 diabetes are at increased risk (11; 12). There are large variations in incidence of type 1 diabetes related to geographic localization and ethnic background (13). The incidence and prevalence of type 1 diabetes are on the rise, the mechanisms behind this is not fully understood, but are likely multifactorial (14; 15). In 2014, the NCD Risk Factor Collaboration estimated that 9 % of men and 7.9 % of women have type 1 or type 2 diabetes (16), of which type 1 make up around 10 % of the total. In Norway in 2017 the overall prevalence of diagnosed diabetes in 30-80 year old adults was 4.7 % (17). The total number of diabetes patients is expected to increase for many years to come, due to population growth and (for type 2 diabetes) increased number of elderly, sedentary lifestyle and obesity (18).

Lack of insulin results in severe metabolic aberrations, including hyperglycaemia, which is ultimately fatal without treatment. Type 1 diabetes patients are therefore completely reliant on exogenous insulin. Type 2 diabetes is caused by a combination of insulin resistance and relative insulin deficiency. A proportion of such patients are treated with insulin or oral drugs that increase endogenous insulin secretion, and may therefore also be at risk for hypoglycaemia. However, hypoglycaemia is much less of a problem in type 2 diabetes, and will not be discussed further in this thesis.

Although new treatments might be underway (19; 20), for the time being, all treatment regimens (besides islet (21) or pancreas transplantation (22)) include the administration of exogenous insulin injected subcutaneously. Traditionally, this has been done with a syringe or prefilled pen, but an increasing number of patients now use insulin pumps, sometimes coupled with a sensor performing continuous glucose measurement. The next step is a “closed loop system”, an automatic system for both measuring glucose and delivering adequate amounts of insulin, and many promising studies are already performed (23).

There are considerable costs associated with these treatments. The newer insulin pumps and continuous glucose monitors (CGMs) are only available to patients in countries with adequately funded health care, or to those who can afford it through private funds.

Unfortunately, the prevalence of diabetes is increasing rapidly in countries that do not have a well-funded health care system, like many countries in Asia with large populations (18).

Hence, there is a demand for low cost equipment to monitor BG and avoid hypoglycaemia.

Many attempts have been made to make a reliable and non-invasive BG measurement system, and while there is a continuous effort to reach this goal with some promising results, no such system exists (24).

1.3 Impaired awareness of hypoglycaemia

1.3.1 Definition

IAH is a condition where the ability to recognize emerging hypoglycaemia is reduced or lost. There is no gold standard for how to diagnose or define the condition, but typically patients have a reduced autonomic response to hypoglycaemia, also called hypoglycaemia associated autonomic failure (25; 26) and a reduced ability to recognize the symptoms of hypoglycaemia (5). Three methods for evaluating IAH with questionnaires (27-29) were compared in a paper by Geddes *et al.* (30). The authors used the Edinburgh hypoglycaemia symptom scale (EHSS) (31) and instructed patients to measure BG four times a day and to score their symptoms if BG was <3 mmol/L (the scoring sheet can be found in the appendix). They found that the questionnaires developed by Gold (27) and Clarke (28) had good concordance with an increased number of hypoglycaemic episodes, and with each other (the questionnaires can be found in the appendix). These questionnaires are frequently used, particularly the Gold questionnaire that is just a Likert-scale where the respondent scores 1 to 7 to the question “(How often) Do you know when your hypos are commencing?” A higher score signifies a low proportion of hypos recognized, and a score of ≥ 4 qualifies for the IAH diagnosis. Although such scores are not perfect, Clarke *et al.* showed that diabetes patients who say that they have problems with recognizing hypoglycaemia are generally right (28).

1.3.2 Physiological basis

A normal functioning beta cell will gradually stop insulin secretion when BG is declining. When circulating insulin levels are low, glucose uptake in insulin-sensitive tissues will stop and hepatic glucose production will increase, preventing hypoglycaemia. If BG levels continue to decrease, counterregulatory hormones will be released (32). Most important of these is glucagon. However, in subjects with type 1 diabetes, the glucagon response to hypoglycaemia often fades with time (33). Other counterregulatory hormones include adrenaline (34), growth hormone (GH) (35) and cortisol (36). In absence of glucagon, adrenaline is first to be released as hypoglycaemia progresses, usually when BG is around 3.8 mmol/L. Then follows GH at BG around 3.7 mmol/L and cortisol at BG around 3.2 mmol/L (37). As such, only adrenaline is important in the acute phase (38; 39). When too much insulin is available in circulation, as can happen after exogenous administration, adrenaline is not sufficient to prevent hypoglycaemia. If BG declines towards 3.0 mmol/L, other symptoms controlled by the autonomic nervous system occur, like hunger and sweating, and these are most recognizable for the patient (40). If no food containing glucose is ingested and the counterregulatory hormones fail to reverse hypoglycaemia, neuroglycopenia develops. Symptoms may include irritability, faintness, drowsiness, confusion, coma and even death.

The level for counterregulatory hormone reaction is affected by glucose control and previous hypoglycaemic episodes. A low average glucose level (low glycated haemoglobin, HbA1c) and frequent hypoglycaemic episodes is associated with an attenuated counterregulatory response, demonstrated both in patients and normal subjects (41-46). Autonomic symptoms are important in recognizing hypoglycaemia, and symptoms elicited by cholinergic nerves (sweat, hunger, tingling) are reported to be the most important (40). Others have found that autonomic and neuroglycopenic symptoms are found in equal measures (47; 48). In the elderly, neuroglycopenia (and thus risk of confusion and severe hypoglycaemia) can appear at higher BG levels than in young adults, and autonomic symptoms at a lower level, so that time between warning symptoms and neuroglycopenia is erased (49).

It has been reported that antecedent hypoglycaemia can blunt counterregulatory hormone responses and endogenic glucose production during exercise the next day (50), and vice versa that antecedent exercise can diminish the adrenaline counterregulatory response to hypoglycaemia the next day (51). Hence, patients who exercise often may develop IAH as defined by the Gold- and Clarke-scores.

IAH is not necessarily a permanent condition, many patients who have a constant attenuated autonomic response can actually regain symptoms to hypoglycaemia by avoiding hypoglycaemia, however, this does not regain the counterregulatory response (52; 53).

The prevalence of IAH among subjects with type 1 diabetes is reported to be between 17-20 %. It increases with longer duration of diabetes and more severe episodes of hypoglycaemia (54; 55). Despite new therapies, this prevalence does not seem to have changed (55).

1.3.3 Complications

It has been reported that IAH patients have a sixfold higher frequency of severe hypoglycaemia than patients with intact awareness (27). As such, they are more prone to serious hypoglycaemic events that can result in unconsciousness, seizures, joint dislocations, fractures, cardiac arrhythmias and death. It has been suspected that an increased number of severe hypoglycaemic episodes might have an impact on cognitive function, and recently Hansen *et al.* showed that IAH patients score lower on cognitive tests that depend on the hippocampus, which is vulnerable to hypoglycaemia (56). Quality of life is also negatively affected by IAH, in that an increased number of hypoglycaemic episodes can cause anxiety and unhappiness (2; 3).

1.3.4 Nocturnal hypoglycaemia

One much feared complication for diabetes patients is hypoglycaemia that occurs during sleep, and IAH patients are likely overrepresented among diabetes patients who pass away in their sleep (dead-in-bed-syndrome (57-59)). Over half of all episodes of severe hypoglycaemia are reported to occur during sleep (60). The body's reaction to hypoglycaemia changes during sleep, with a weaker autonomic response (61), and type 1 diabetes patients are much more likely to sleep undisturbed during hypoglycaemia compared to healthy subjects (62; 63). This makes them more vulnerable for temporary changes in heart physiology, for example prolonged QT-time, bradycardia and ectopic beats that can provoke ventricular arrhythmias and cardiac arrest (64-67). Studies using CGM has shown that only 20-33 % of noncturnal hypoglycaemic episodes are recognized by patients (68) and this is likely an important factor in the development of IAH (69). In addition, nocturnal hypoglycaemias may have an impact on quality of life with effects that extend well into the next day (70).

1.3.5 Management

There is no definitive treatment for IAH, but avoidance of hypoglycaemia can improve symptomatic responses, and the improvement can last for years (53; 71). A non-blinded study has shown that the use of the long-acting insulin analogue insulin detemir improved symptoms as compared to human insulin (72), and another study reported that the combination of insulin detemir and insulin aspart reduced the number of severe hypoglycaemias in patients at risk (73). Caffeine has been suggested as a possible treatment, as it can increase intensity of symptoms and counterregulatory responses, but the doses needed are probably too large to be a practical alternative (74). Insulin pumps and CGM with either an insulin suspend function or the newer hybrid closed-loop systems are promising (75; 76), and also have the possibility of remote monitoring with a 100 % response rate to low glucose alarms (77).

1.4 Non-invasive sensors for blood glucose

How frequent a diabetes patient performs a BG measurement varies between individuals, from less than once a day to several times per hour due to different needs, personal preferences, reimbursement issues and other factors. A manual measurement involves some preparation of the device with a test strip, and a mildly painful pin prick. While many handle this situation with ease, others find it a constant nuisance, and as a result have a suboptimal BG regulation. A non-invasive sensor would be game-changing, in that it would considerably ease the situation for many patients. Considering the number of patients around the world and the considerable economic potential, many have tried to develop a sensor good enough for medical use, but so far, none have succeeded. A whole range of technologies have been tried out, but it seems extremely difficult to get a stable and reliable result. Below follows a selection of technologies, the rationale behind them, and some possible reasons why the accuracy is not good enough. It is not a comprehensive list, rather an overview over the most studied technologies.

A non-invasive technique is defined as no piercing or damage to the skin whatsoever. Ideally, no pain should be involved as well, but one exemption is made for the reverse iontophoresis method.

1.4.1 Technologies based on light

In this text, when the word light is used, it refers to the whole electromagnetic spectrum, not just visible light. Light is electromagnetic radiation that moves through a vacuum at a constant speed, a.k.a. the speed of light. It consists of one electric and one magnetic field that oscillate in synchronization, perpendicular to each other and to the direction of the wave (figure 1). The wave can be described by

Figure 1. The electromagnetic wave.

wavelength or oscillation frequency, a given wavelength will always have the same oscillation frequency. Different wavelengths of light have different characteristics and uses (see figure 2 for an overview). Electromagnetic waves are produced every time a charged particle (e.g. electron, proton, ion etc.) is accelerated, or when a particle transcends to a lower energy level. The waves are more commonly named photons, and are regarded as elementary particles. Photons are discrete packets of energy, and means that light can only be delivered in individual quanta of energy, not as a continuous wave. Famously, it has properties of both a wave and a particle, and is massless.

Figure 2. The electromagnetic spectrum. Wavelengths illustrated with different figures for scale in upper row, and examples of sources for the different wavelengths in the lower row.

Available under CC BY-SA 3.0 at https://commons.wikimedia.org/wiki/File:Electromagnetic_spectrum_with_sources.svg

Light can pass through a material (transmission), bounce off it (reflection) or give energy to it (absorption). With transmission and reflection, we can also have diffusion (also called scattering) of light. There are also instances in-between direct and diffuse transmission (or reflection) called mixed transmission (figure 3).

Medical sensors based on light use these different interactions between light and matter to create pictures or gather information from the human body.

When light is used in sensors, we must have both a source and a detector. (As a side note, one could say that an MRI machine makes the body itself (or the hydrogen atoms) emit light (radio waves) under the influence of a magnetic field, and thus do not have what we would think

Figure 3. Top row: Reflection; direct, mixed and diffuse: Bottom row: Transmission; direct, mixed and diffuse.

of as a typical light source.) A classic X-ray device, for example, has a source that accelerates electrons against a metal that gives off electromagnetic waves, and a photographic plate or film (in older devices) as a detector. Transmission of X-rays through the body, and absorption of X-rays by bodily tissues lets different amounts of X-rays reach the detector and gives the well-known black and white X-ray picture. Another much used application of light in hospitals is the pulse oximeter. The most common version is, like an X-ray device, transmissive. A thin part of the body, usually the finger or earlobe, is attached with a clip-on device with a light source on one side and a photodetector on the other. A light emitting diode (LED) sends through red light (660 nm) and infrared light (940 nm). Hemoglobin with bound oxygen absorbs more infrared light and lets red light through, and the opposite is true for deoxygenated hemoglobin. The absorption varies throughout the pulse cycle, at the peak of a pulse wave the absorption also peaks, and hemoglobin is responsible for the variation. Comparing the absorption spectra for the two wavelengths, one can calculate the ratio of oxygenated and deoxygenated hemoglobin. There are also pulse oximeters that utilize reflection, which use the same principle, but looks at reflected light instead of transmitted light.

Regarding glucose, the main problem is the low glucose concentration in blood compared to other substances. In addition, there are many molecules that are similar to glucose present in the body, like many of the molecules in the glucose metabolism. Glucose also has a tendency

to attach to proteins, so called glycosylation. Measurement of glycosylated red blood cells (HbA1c), sometimes called long-term BG, is tightly correlated to average levels of blood sugar during the last 2-3 months, and normal values in non-diabetic, healthy individuals are 21.3-46.4 mmol/mol (4.1-6.4 %). Other proteins are also glycated or glycosylated. And carbon, hydrogen and oxygen (the components of carbohydrates) appear structurally similar and are omnipresent in the body. Especially when using light for detection, this creates a lot of overlapping signals and makes the measurements hard to interpret. Also, when using light we are not measuring glucose in blood, rather in the interstitial fluid, which lags behind BG with around 10 minutes. Thus the reference measurement from blood will in most cases be different from a measurement from tissue no matter how precise the tissue measurement is. However, this has not been a problem to address so far, as the tissue measurements are not precise enough anyway.

1.4.1.1 Near-infrared spectroscopy

Many different wavelengths of light have been tested and the technologies are fundamentally the same, but the near infrared (NIR) has some properties that makes it popular for tissue measurements. The glucose molecule absorbs light in specific frequencies, and NIR spectroscopy looks at the absorption spectrum to try to pin down the glucose concentration, utilizing light with wavelengths in the range of 750-2500 nm. It can use diffusively reflected light for analysis, and thus be applied anywhere on the skin. Molar absorptivity (the ability of a substance to absorb light) in the NIR-range is quite small, therefore NIR can penetrate further than many other wavelengths and give information about deeper layers of tissue.

A molecule can absorb energy not just by excitation of an electron to a higher energy state, but also at discrete energy levels that changes the vibration between atoms. The bigger and more complex the molecule, the more different IR-frequencies can be absorbed. Thus, a molecule has specific absorbance qualities that helps identify it. However, the combination of absorbance bands seen in NIR is often very broad, and it can be difficult to filter out information of the specific particle you want to look at. Multivariate techniques are often used to achieve desired results.

Several researchers have demonstrated a correlation with NIR measurements and glucose concentration (78), but the accuracy is not good enough in real-world conditions. Too many physiological and environmental factors come into play, including hydration and humidity,

body temperature and ambient temperature, blood pressure and atmospheric pressure and so on. One device based on this technique was the Sensys Glucose Tracking SystemTM, which showed quite good results under controlled conditions where technicians prepared the skin area with the correct procedure of washing and drying (79), but had considerable worse results when tried on a patient population (80). There is no clinical device based on NIR for use with diabetes patients as of today.

1.4.1.2 Raman spectroscopy

Raman spectroscopy utilizes laser, and thus looks at only one frequency of light instead of a spectrum. The photons from the laser interact with the electron cloud of the molecules, and change direction and energy level (up or down) in doing so. This is termed inelastic scattering, in contrast to elastic scattering, where no energy is transferred in the interaction. Inelastic scattering makes up a very small fraction of the total scattering, less than one per million. The energy that is transferred to or from the molecule may change the rotation of the molecule or make it vibrate, i.e. the atoms in the molecule oscillate in relation to each other. Special for Raman spectroscopy is the fact that it depends on a molecules ability to form a temporary dipole to get a good signal. Water, which is a permanent dipole, will not give a signal and thus not interfere with measurements. This makes it a useful tool for measurements in biological tissue, where the strong signal from water often makes signal interpretation difficult.

Different molecules give specific shifts in the photons that are scattered from it, and this gives a “fingerprint” for each type of molecule. However, the signal is still very small compared to all other molecules in the tissue, probably much less than 1 percent of the inelastic scattering. Also, individual differences in skin thickness, hair, colour and so on, also interferes. A device would also need to keep the sensor in darkness, as natural light will add considerable noise. So it is still hard to filter out the right data, much as it is difficult to pick out one fingerprint if there are a hundred others superimposed on it. A thorough review of Raman spectroscopy for use in medicine has been done by E.B. Hanlon *et al.* for the interested reader (81).

While there have been promising results for Raman spectroscopy as a non-invasive glucose sensor (82), there are no devices on the market that employs this technology as of today.

1.4.1.3 Light scattering

Photons that are directed at skin will scatter and some will bounce back. This scattering effect is largest where the interstitial fluid is adjacent to cell walls, because the refractive indices are so different. If there is much glucose present, the scattering indices are more similar, decreasing the scattering. So there could be a reproducible relationship between glucose levels in tissue and the scattering index. However, many substances have this effect on the interstitial fluid, and differences in tissue hydration will have a large impact. There have been publications on this (83), but no product for BG measurement has been made.

1.4.1.4 Optical rotation

Some molecules, including glucose, have the ability to change the rotation of photons (84). Photons are polarized, meaning that they do not only have a direction, they also have an orientation relative to the direction. For example, a car also has a specific orientation relative to its direction, with its wheels on the ground (in most cases, hopefully). Photons on the other hand, can just as well be oriented upside down, or in any other direction, it has no practical significance. Polarized filters utilize this trait (commonly used on for example camera lenses), and only lets through photons that are orientated in a specific plane. Two such filters stacked on top of each other will let through varying amount of light when rotated in relation to each other, when the two planes are at 90 degrees, no light lets through. By sending light with a known polarization through tissue, the amount of rotated light (with the specific rotation of glucose) should vary in concordance with glucose levels. The concentration of glucose and the length photons travel in a medium with glucose decides the rotation. Since the glucose concentration is small, and the length light will travel in tissue is short, the rotation is very small, which makes it hard to measure. The technique has been investigated extensively by various actors (including Roche Diagnostics and Abbott) for measuring glucose in the anterior chamber of the eye (85). However, none have succeeded, and even if they did, the slow flow in the chamber (86) would probably mean that any measurement would lag about an hour compared to BG. There have also been attempts at measuring optical rotation in tissue, but because of change of polarization due to scattering it is even more difficult, and no one have achieved good results.

1.4.2 Technologies based on sound

Ultrasound

Ultrasound has been tried both for facilitating direct measurement of BG through sonophoresis, and as an indirect measurement by looking at difference in acoustic characteristics of a tissue when glucose concentrations varies. Sonophoresis works by applying ultrasound to the skin at a specific frequency and make cells temporarily more permeable and thus allow for glucose to leak out. It is believed to be a so called cavitation effect, and creates a very minor damage to the plasma membrane, which is quickly reestablished (87). It needs at least five minutes of exposure to ultrasound, and reasonable good results have been shown in pigs (88). As of today, no one has demonstrated good results in human subjects.

The acoustic characteristics of a tissue has been used together with other parameters to calculate BG. Glucose affects the hydration of a tissue by water shifts between extracellular and intracellular compartments (89). Less hydrated tissue is denser, and sound waves travel faster through them. By analyzing the tissue characteristics against different BG levels, this relationship may be used to predict BG within some limits. The Glucotrack is a commercially available device that uses this principle, together with bioimpedance and temperature readings (89; 90). Until now, its performance has been evaluated to come short of being approved for clinical use in patients with diabetes.

1.4.3 Technology based on light *and* sound

Photoacoustic spectroscopy

When materials absorb photons, they give off less energetic photons through a process called vibronic coupling. These photons heat the surrounding matter. If the light is pulsed, surrounding matter is heated and cooled in sync with the pulsed light which creates waves, or sound. When light with a given wavelength is pulsed, we get a sound intensity that is more intense if more light is absorbed. Thus we can plot the intensity of sound against wavelength, and we get an absorption spectrum. Naturally, this technique faces all the difficulties as the other techniques described above. Nonetheless, there have been some promising publications in the recent years on this method (91; 92), but there has also been earlier promising devices, like Glucon Aprise (93), that has not materialized. No clinically useful apparatus is available at the moment.

1.4.4 Technologies based on electricity

1.4.4.1 Electrocardiography (ECG)

ECG is one of the most ubiquitous non-invasive medical technologies there is, with invaluable applications for heart diagnostics. It works by measuring the electric potential difference (voltage) between different electrodes placed around the heart and on the arms and legs. During a heartbeat, electrolytes flow in and out of cells in a predictable manner that gives the recognizable ECG pattern, and a heart that is structurally different or damaged has some predictable changes in this pattern, which makes ECG such a valuable diagnostic tool. The link to diabetes goes through intermediate effects of insulin and adrenaline. Insulin works on cells through a Na/K-pump that drives potassium into cells, hence it lowers the concentration in blood. The concentration of potassium in blood affects depolarization of cells in the heart that can be observed on an ECG. Similarly, adrenaline also affects the level of potassium, but adrenaline has also been shown to have an independent effect on the corrected QT-time (QTc) on the ECG (94). None of these have a direct and reproducible link to a specific blood sugar level, hence, they are not very usable to predict BG accurately.

1.4.4.2 Electroencephalography (EEG)

The brain utilizes electricity (i.e. ion fluxes) when conveying information, and electrodes placed on the scalp can pick up these signals. A single cell would not create enough voltage amplitude to be picked up by non-invasive electrodes, but since many brain cells normally work in concordance, the potential is measureable, but is still in the microvolt range. During measurements it is common to either look at EVP (event related potentials, i.e. the brain's response to a stimulus) or the spectral content (i.e. background "noise"). It is commonly used for diagnosing a range of conditions that can affect the brain like epilepsy, sleep disorders, coma, encephalopathies, etc. For hypoglycaemia detection, one can think of hypoglycaemia as the event, and try to discern any changes. Typically, one will look for changes in neural oscillations, also known as brain waves. Neural oscillations can be seen for specific frequencies, and common frequencies have names, e.g. alpha waves with frequency 7.5-12.5 Hz. Other bands are called beta, delta, theta, low gamma and high gamma. Why these oscillations exist is not fully understood (95). It has been known for some time that hypoglycaemia causes EEG changes both when awake (96; 97) and during sleep (98), and it has been shown to have a fairly reproducible glucose threshold (99) that is common for many

different groups of diabetes patients, including IAH patients (100; 101). A Danish company called UNEEG Medical has developed a product called Hypo-Safe that uses a small implantable device behind the ear and automated algorithms for hypoglycaemia detection (102; 103). However, it is still marked as “under development” at their website. There are two clinical trials registered for this product, the first one was replaced by a second one, and the difference seems to be that the second one uses healthy subjects (104; 105). Results are posted on clinicaltrials.gov, and they show that the EEG reading is comparable to scalp EEG and that it does not cause much discomfort. A trial for testing on diabetes patients has been withdrawn (106), and there are, to our knowledge, no more trials registered at the moment (March 2019).

1.4.4.3 Sweat sensors

Sweating is mediated through the autonomic nervous system, where sympathetic fibers innervate sweat glands. In contrast to other postganglionic sympathetic fibers, the signal is transmitted through acetylcholine rather than noradrenaline. There are two types of sweat glands, eccrine and apocrine, where eccrine is by far the most common. While apocrine glands secrete an oily fluid and is restricted to certain parts of the body (e.g. axillae), eccrine glands secrete a watery fluid and are found all over the body. The primary function of eccrine sweating in humans is temperature regulation. Sweat sensors are designed to detect eccrine sweat, and does this through one of two methods; measuring water content in air over an area of the skin (107), or measuring the changes in the electrical properties of the skin during sweat excretion (108). When a sweat duct fills up in response to a nerve signal, a temporary increase in skin conductance occurs because ions can more easily traverse the skin through the fluid-filled ducts. This reaction is called a skin conductance response (SCR), and the changing activity of these responses is called electrodermal activity. The frequency of SCRs can be used to approximate the amount of sweating (109), and since the sweat ducts are innervated by the sympathetic nervous system, it is also a measurement of sympathetic output (110). How close in time these pulses from the nerve system can get is not known. Earlier works have evoked nerve pulses artificially with electric stimulation and have shown that one pulse per second (1Hz) seems to produce close to the maximum possible levels of sweating (111). Earlier observations from our own experiments suggest that one response every fourth second (0.25 Hz) is a quite strong sweat response.

Sweating can be seen as a byproduct of the sympathetic response that happens when BG gets too low, and the idea to use this for detecting hypoglycaemia has been investigated for many

years (112; 113). Hence, when BG is in the normal or high range, sweating gives no information about BG at all. When BG gets too low, the amount of sweating can be related to how low BG is, insofar that the lower BG gets, the more pronounced a sympathetic response will be (114). But the individual differences are large, especially so for IAH subjects, who may not have a reaction before BG reaches levels below 2.0 mmol/L (42). Also, diabetes patients who have little control over their BG and have high BG over extended periods of time may have the opposite tendency; a hypoglycaemic reaction when BG falls but is still in the normal range (43). So you can have no reaction when a given patient is hypoglycaemic, and for another patient you may have a reaction when she/he is euglycaemic. And of course, sweating for other reasons than hypoglycaemia will probably be much more common. All sweat data must therefore be considered carefully with respect to the individual and the situation.

1.4.4.4 Bioimpedance (Z)

Bioimpedance uses electrodes to measure passive electrical properties in the body by applying weak electrical currents. One of the aforementioned methods for detecting sweat uses bioimpedance. The measured resistances to a range of frequencies of alternating current are used to characterize different tissues. The capacitance in the tissues, i.e. the ability to store electrical charges, also has important effects on the measurements. Through mathematical analysis of the obtained signals, it is possible to gather information on the tissues the current travels through. One application is for example needles that can tell what kind of tissue the tip is in (115). Sweat measurements use so-called low frequency bioimpedance (typically <100 Hz), while frequencies typically used for BG detection is in the range >1 MHz. This high-frequency bioimpedance is thought to be able to measure BG through changes in the cell membranes of the red blood cells rather than via an autonomic response. Hayashi *et al.* have shown by in vivo experiments that the dielectric characteristics of red blood cells (RBCs) change as the concentration of glucose changes, and that this change does not happen with L-glucose (a biological inactive isomer of glucose) (116). Hence, there must be a change in the blood cells themselves that is responsible for this change. Livshits *et al.* has shown that this is connected to the production of adenosine triphosphate (ATP - the molecule that ultimately fuels all the cells in the body) and its binding to the GLUT1 glucose transporter in the cell membrane (117). This could mean that bioimpedance could be used to say something about BG levels, if there is a consistent relationship between BG levels and the share of red blood

cells that show this behavior. However, current has to traverse other types of tissue before it encounters red blood cells. It is therefore a no small undertaking to elucidate this signal among a horde of others, and so far, no one has managed to use bioimpedance alone to predict BG levels in humans. However, it has been used together with other sensors for BG prediction, but BG prediction is not good enough to replace blood sampling, at least not in real life conditions (118-120).

1.4.4.5 Reverse iontophoresis

Iontophoresis is the process where ions move in a medium driven by a voltage difference. Reverse iontophoresis use this to pull ions (in this case sodium and potassium) through the skin. By process of electrophoresis, glucose is also drawn through the skin and allows for measurement by a standard glucose sensor. This system has been developed and commercialized as a stand-alone BG measure device to replace pin-prick testing. The most well-known device is Glucowatch (121). Although it reached market, it had several shortcomings. The reverse iontophoresis was not completely pain-free and caused skin irritation. Also, the device needed calibration with pin-prick-testing, sweating could interfere considerably, and it had a tendency to report too high BG and gave many false alarms (122). Further development of the device stopped, and it disappeared from the marketplace. For the moment being, there is no device based on this technology available.

1.4.5 Measurement of glucose in other body fluids

There are several fluids excreted from the body that can be analyzed for glucose content. The sweet smell and taste from the urine of afflicted patients were for a long time the most effective way of diagnosis. Urine testing was still more common than BG testing until mid-20th century because it was so readily available. However, it would not make sense for the body to excrete glucose, a vital source of energy. Hence, under physiological conditions, there is no glucose excreted in saliva, sweat, urine or any other fluid. Rumors said that Google tried to construct a device similar to a contact lens to be placed on the cornea that could measure glucose in the tear film of the eye, and there was also recently a publication on a lens under development, although not shown to work on humans (123). Recent reports indicate that Google gave up the project. The potential may be limited, as glucose level in tears do not show a close correlation with blood glucose levels (124).

1.4.6 Temperature sensors

Modern temperature sensors are based on electricity. Where traditional sensors, or thermometers, are based on a material that expands with increasing temperature, modern sensors detect changes in voltage or resistance in a circuit. For example, the temperature unit used in our experiment (a Siemens Dräger 5204669 temperature probe) uses thermistor technology. A thermistor is a resistor that is made out of a material that changes its resistance depending on the temperature, typically a ceramic or polymer material. When the characteristics of the thermistor are known, the measured resistance is easily translated to a temperature reading.

Like sweat, one can imagine that temperature will vary during a hypoglycaemic reaction due to circulating adrenaline that will constrict blood flow to the skin (125), and this information can possibly be of use as a part of a multisensory system. In addition to all the challenges that also afflict sweat measurements, temperature is obviously influenced by clothing and thus difficult to interpret. However, in combination with other sensors it may give valuable information, and is for example used in the multisensory device developed by Zanon *et al.* (120). Ambient temperature can also be incorporated in a multisensory system, an example would be to correct for thermoregulatory sweating, as opposed sweating due to increased sympathetic output during hypoglycaemia.

1.4.7 Accelerometers

Obviously, accelerometers cannot measure BG by itself. However, motions can indirectly be associated with BG. Thus it can be used with other sensors like ECG, temperature and sweating to tell if it is likely that the recorded data is influenced by different forms of physical activity, if the subject is awake or sleeping, or if specific motions related to hypoglycaemia are detected. Examples of the latter may include slowed down movements due to neuroglycopenia, or trembling due to adrenaline release.

Accelerometers can be attached to an object and measure acceleration, and by integration also speed and distance. They can measure in one, two or three planes, depending on usage. For an accelerometer meant to be worn by humans, typically three planes are used, because humans have ability to move in any direction. An accelerometer made for airbags in cars for example, often has just two planes, because a sensor for vertical acceleration/deceleration is superfluous. Conceptually, an accelerometer consists of a mass that is displaced when a force

acts upon it, and this displacement is (in digital accelerometers) converted to an electric signal that can be used to compute the acceleration. One method of doing this would be to use a piezoelectric material. The piezoelectric effect is when a material accumulates an electric charge in response to mechanical stress (discovered by Pierre Curie and his somewhat less famous older brother Jaques). It is present in many materials, like DNA, but typically different crystals are used commercially. An example of a simple design for an accelerometer can be a mass placed on a cantilever with a piezoelectric crystal at its base (where compression is highest).

1.4.8 Dogs

Many diabetes patients have noticed that their dogs can alert them of hypoglycaemia (126). How they do it is not known, but dogs have excellent sense of smell, and there have been some studies showing a relationship between BG and so-called volatile organic compounds (127). Even humans can smell ketone breath when there is severe hyperglycaemia. However, dogs could just as well pick up on small behavioral changes in the owners. One study took swabs from the skin of diabetes patients during hypoglycaemia and euglycaemia and tried to learn dogs to discriminate between them with little success (128), indicating that other mechanisms are at play. Despite the lack of scientific evidence, there are organizations that work toward providing diabetes patients with dogs that are trained to detect hypo- and hyperglycaemic episodes (129; 130). Since there are considerable amounts of work that accompanies owning a dog, and the weak evidence for hypoglycaemia detection, whether a diabetes alert dog is the right choice depends on the individual patient.

1.5 A brief overview of glucose measurement and non-invasive measurement

1.5.1 Glucose measurement in blood and interstitial fluid

Most diabetes patients still rely on finger prick testing with a test strip and a small, pocket-sized BG meter. In the last 20 years however, continuous glucose monitoring systems (CGMs) that measure glucose concentration in the interstitial fluid (131) have become more and more common. While CGMs have a time delay compared to BG measurements (132), it

has been shown that CGM improves glycaemic control (133; 134), lowers glycated hemoglobin (135), and that it detects many unrecognized hypoglycaemias (136). CGM does not necessarily decrease the number of episodes with severe hypoglycaemia (137), but it apparently does for IAH patients (138), but IAH continues to be a risk factor (139). Also, if CGM is used alongside insulin pumps (so-called closed loop systems), new algorithms for predicting hypoglycaemia can suspend insulin delivery before BG enters the hypoglycaemia range (140), and can also implement glucagon (141). Recently, a system that is somewhere between CGM and self-monitoring has been introduced, where a small sensor placed on the upper arm takes a reading every time you scan it (142). An implantable device that can be used for at least 90 days before being changed has also recently shown very good results (143). However, none of these devices are non-invasive.

1.5.2 Non-invasive methods

Regarding non-invasive methods, there have been attempts at this since 1974, when the first patents on optical rotation were filed (144; 145). There have been many attempts at solving the problem using many different technologies and large sums of money. Because of the market potential and possible large reward (one can assume that every diabetes patient would rather want a non-invasive alternative), the willingness to spend money on research into this has been huge. There is not much merit in retelling the advances done in this field, because ultimately, we are not much closer to a solution now than 40 years ago. That is, there is still promising research being done with non-invasive units being tried out in real-life conditions (for example Zanon *et al.*, using a multisensory system (120)) with quite good results, but the accuracy is still not good enough. This feeling that the solution is “just over the horizon” has persisted for so many years, that the sensible approach is to say that we cannot say that we are closer to a solution. For the interested reader, a long-time actor in this field, John Smith, has written a small book on the history of non-invasive methods that is freely available online (85), where a plethora of different approaches are mentioned that this text does not cover.

1.6 Non-invasive hypoglycaemia alarm systems

Non-invasive hypoglycaemia alarm systems takes an easier approach to glucose measuring, in that they need not rely on exact glucose measurements. Rather than measure glucose, such alarms measure the body’s response to low BG originating from the autonomic nervous

system. These alarms are primarily meant to be used during sleep. The hypoglycaemia alarm systems on the market today are based on the detection of increased sweat and cold skin (“cold sweats”). A patent for using a sweat sensor for detecting hypoglycaemia was filed as early as 1957 (146). However, Diabetes Sentry was the first alarm to be marketed in the 1980’s, and is still available today (figure 4). It was shown that it sounded an alarm in about 2/3 of hypoglycaemic events during sleep, and that the ratio of false alarms to real events were about 3:1 (147; 148). Still, one can feel an added sense of security that at least some of

Figure 4. Picture from www.diabetessentry.com

the alarms may prevent possibly harmful episodes of hypoglycaemia, and in one study, parents of children with diabetes were hesitant to return the device due to this (149). Even an alarm that objectively does not perform very good, can have significant benefits for some users. How many diabetes patients use this alarm system today is not known, but the impression is that it is almost non-existent among patients in Norway, how many users there are internationally is not known. There is a patent indicating that they are continuing to develop the technology with a trending system (150), but there

are no studies available to show if it performs better than before. There is a British venture called Medpage Limited T/A Easylink UK that markets a very similar device, the HSA-01, which by its description seems to function exactly like the Diabetes Sentry. At the moment, these devices seem to be the only hypoglycaemia alarm systems commercially available.

A few years ago, an Australian company called Aimedics had a device called Hypomon, which was worn as a belt around the chest and probably used ECG signals and sweat measurements to detect hypoglycaemia. It was shown to have a sensitivity of 73 %, and a specificity of 68 % (151). It was marketed in Australia but was recalled in consultation with the Australian Department of Health’s Therapeutic Goods Administration. Supposedly, it did not alarm at the specified BG level, and also gave fewer alarms than specified in the instructions for use. Why this device was retracted probably has to do with how the devices are marketed and by which laws they are regulated by in different countries.

1.7 Impaired awareness of hypoglycaemia and hypoglycaemia detection

There are not many studies on detection of hypoglycaemia in subjects with IAH. Sejling *et al.* have done a study with EEG, where they concluded that awareness status did not affect EEG signals in the hypoglycaemic range (101). The Hypo-Safe utilizes this fact; see the paragraph on EEG above. But the only actual alarm systems on the market today (see the prior paragraph) relay primarily on sweat, and IAH patients differ in that they their BG gets lower before the sympathetic reaction kicks in (42; 152). IAH patients are much more prone to severe hypoglycaemia (6; 28), so these patients would benefit most from an alarm system. In the user manual for the Diabetes Sentry it reads (153); *“If you have “hypoglycemia unawareness” and you do not exhibit symptoms, or your symptoms are coming too late for you to take action, you should speak to your Diabetes Educator about how to get your symptoms to return”* (unawareness is sometimes used in the literature, impaired awareness is the preferred term since there can be many degrees of awareness). So the manufacturer concedes that if your symptoms come too late, the alarm cannot help you. They also touch on another important fact regarding hypoglycaemia symptoms, you can regain them (52; 53), and then maybe have symptoms strong enough to use an alarm. The opposite can also be a problem, diabetes patients who have poor regulation of their BG can get counterregulatory symptoms above the normal threshold (43; 45), and will thus set off the alarm well into the euglycaemic range.

So as of today, if you choose to use a hypoglycaemia alarm system, you have to accept that sensitivity and specificity are quite low, that it may set off an alarm when you are euglycaemic because that is when your symptoms appear, that it may stop working for you if your symptoms should weaken or disappear, or that it may not work for you at all. There is probably some room for improvement.

2 Aims

The main aim of this thesis was to explore the performance of a set of sensors to be used as a non-invasive alarm system to detect hypoglycaemia in patients with diabetes and IAH (figure 5). Specifically, we wanted

1. To study the relationship between plasma adrenaline concentration, sweating and ECG signals, and their usefulness to detect hypoglycaemia.
2. To assess if any combination of non-invasive sensors for detection of the autonomic reaction (four sweat sites, heart rate (HR), corrected QT-time (QTc) and skin temperature) could detect hypoglycaemia with blood glucose nadir around 2.5 mmol/L in subjects with IAH.
3. To assess the performance of NIR and Z spectroscopy for non-invasive prediction of glucose and glucose trends in BG during euglycaemia and hypoglycaemia, and assess the feasibility of hypoglycaemia detection based on this method.
4. To develop a mathematical model for identification of physiological responses related to hypoglycaemia in diabetes patients with IAH, and to assess the potential for non-invasive detection of hypoglycaemia in these patients by a wearable multisensory system.

Figure 5. The figure illustrates our long-term aim that was to integrate a number of different sensors into a device and develop an algorithm that could detect hypoglycaemia based on the input from the different sensors. This figure also shows sensors that might be incorporated at a later stage (gyroscope and accelerometer).
 Pictures by Servier medical art (<https://smart.servier.com>) licensed under CC BY-SA 3.0.

3 Materials and methods

All four papers in this thesis are derived from the same trial, so all the different measurements were done simultaneously. The different papers used different selections from the measurements. An overview of the papers and which measurements were used in which papers are compiled in table 2.

Table 2: Overview of study (grey boxes denote parameters that are not in use in the given paper).

		Paper I	Paper II	Paper III	Paper IV
Paper info	Design	All papers are from the same randomized, single-blinded crossover study, n=20			
	Main topic	Detection of adrenaline output with sweat sensors and ECG	Physiology responses in IAH patients during hypoglycemia and hypoglycaemia detection	How NIR and bioimpedance can contribute to hypoglycaemia detection	Detection of hypoglycaemia with non-invasive sensors
	Main techniques for data processing	Developed a synchronicity parameter, performance evaluated with ROC.	Linear interpolation and bootstrapping before using t-testing and wilcoxon signed rank for comparison between clamps. Synchronicity parameter from Paper I used for looking at hypoglycaemia detection, performance evaluated with ROC.	Multivariate calibration used to make BG and slope predictions, performance evaluated with trend agreement and MARD.	Developed a probabilistic model for detecting hypoglycaemia, also using some of the methods applied in Paper I-III. We used several methods to assess performance, F1-score was used as the metric for overall detection accuracy.
	Main findings	Detection of strong sympathoadrenal discharges in resting condition seems feasible.	A basic model for hypoglycaemia detection requires a strong sympathetic output for adequate precision. There was a distinct difference in hormone secretion between subjects who did and did not sense the hypoglycaemia themselves.	Plasma glucose prediction was inaccurate, trend predictions showed more promise and could possibly be of use in a hypoglycaemia prediction setup. NIR seems to be the main trend predictor, with bioimpedance acting as correction for confounding properties.	A probabilistic model showed promising results with good sensitivity and specificity. Sweating and QTc were the most important predictors. Using only sensors that could be integrated in a wrist band gave poorer results, but still better than what is available on the market today.
Measurements /parameters used in the different papers	EHSS				
	p-glucose				
	p-adrenaline				
	p-noradrenaline				
	p-glucagon				
	s-GH				
	s-cortisol				
	s-potassium				
	s-insulin				
	sweating				
	ECG-parameters				
	temperature				
	bioimpedance				
	NIR				

3.1 Study design

The study was approved by the Regional committees for medical and health research ethics in Norway (REC South East approval reference: REK 2013/813). Subjects were recruited from the outpatient diabetes clinic at Oslo University Hospital, from the Norwegian Diabetics Centre and through an ad in “Diabetes”, the journal of the Norwegian Diabetes Association. Twenty-one subjects with type 1 diabetes and IAH (based on Clarke- and Gold scores) were recruited (figure 6). All participants had both one hyperinsulinemic hypoglycaemic clamp and one hyperinsulinemic euglycaemic clamp performed in random order. Twenty subjects completed both days, one was excluded after one procedure because of problems with maintaining venous lines.

Figure 6. Flowchart illustrating the recruitment of study participants and setup of the trial.

3.1.1 Experimental procedures.

A standard operating procedure was developed for the hypoglycaemic and euglycaemic clamp. Subjects were requested to perform frequent self-monitoring 48 h prior to clamp-days, to avoid hypoglycaemic episodes (26; 154; 155). They were also told to abstain from alcohol and strenuous exercise. During the last 24 hours before clamp, only rapid-acting insulin was allowed. The evening before the clamp, the subjects were hospitalized and fasted from 2200 hours. BG was controlled by nurses during the night with intravenous rapid acting insulin according to an algorithm targeting euglycaemia during the night and BG 7-8 mmol/L the morning of the clamp.

Patients were randomly allocated (using sealed envelopes) in a 1:1 ratio to hypoglycaemic or euglycaemic clamp as first procedure. Based on weight, each subject had a constant infusion of insulin during the whole experiment, and BG was controlled by a variable glucose infusion.

During the euglycaemic clamp, target BG was 5.3 (5.0-5.5) mmol/L, and BG was kept stable for at least two hours. During the hypoglycaemic clamp, BG was gradually lowered to a target of 2.5 mmol/L, and after 15 minutes on this level, it was increased to above 5.0 mmol/L again. After the procedures, glucose infusion was continued while a light meal was served and BG was controlled regularly. When considered safe, glucose infusions were stopped and the participants were permitted to go home.

An overview over all measurements performed is given in table 3. More details can be found in the methods section of Paper II.

Table 3: Overview of different measurements performed during clamp. Green colour indicates that measurements were done at the indicated time.

Type of measurement	Details	Continual measurements	Planned synchronized measurements									Other
			1	2	3	4	5	6	7	8	9	
Questionnaire	EHSS											
Blood tests	p-glucose	*										
	p-drenaline											
	p-noradrenaline											
	p-glucagon											
	s-GH											
	s-cortisol											
	s-potassium											
	s-insulin											¶
Sweat	Hypothenar	†										
	Wrist	†										
	Forehead	†										
	Abdomen	†										
ECG	Heart rate	‡										
	QTc	‡										
Temperature	Shoulder	*										
Bioimpedance	Forearm	§										
Near Infrared	Upper arm	*										

* Every five minutes.

† Four times per second.

‡ Sampling 300 times per second.

§ 1 time per second.

¶ 3 times with 10 minutes between near end of experiment, only for euglycaemic clamp.

3.1.2 Non-invasive sensors and choice of equipment

See figure 7 for an overview of the setup. Prior to experiments, the whole setup was evaluated for electrical safety and found compliant with Annex VIII and X of the Medical Devices Directive 93/42/EEC by a committee at Oslo University Hospital. Some parts of the setup required external approval; this was done by the Norwegian foundation for testing and approving of electrical equipment (NEMKO).

Figure 7. Setup during the clamp-experiments.

3.1.2.1 Sweat

The Sudologger sweat sensor is developed based on an original idea by Sverre Grimnes, and developed in cooperation with Ørjan G. Martinsen and Christian Tronstad (108; 109; 156; 157). It uses commercially available ECG-patches for use on the skin (158). It is a state-of-the-art instrument for sweat measurements, and is the only one available that is capable of measuring in several channels simultaneously.

Skin alternating current conductance density (SC) was measured four times per second, and measurements were converted into frequency of skin conductance responses (FSR). The frequencies were calculated using a five-minute moving window, where the number of peaks on the graph from the SC measurements was counted. This number was then used as an indicator for sweating, which again is an indicator for sympathetic activation stemming from hypoglycaemia. Since we cannot use measurements that extend into the future, the number represents the average sweating in the five minutes before the given time.

The electrodes that were used are of the type Kendall 1051NPSM neonatal electrodes, shown to be suitable for skin conductance measurement (158).

3.1.2.2 ECG and temperature device

The ECG measurements were done using a standard ECG monitor, the SC9000XL made by Siemens Medical Systems. Data were digitized with a sample rate of 300 per seconds. HR was computed by identifying the R-peaks on the ECG, and using a five minute moving window as for sweat measurements, i.e. pulse is averaged over five minutes. QT-intervals were computed by identifying the Q-point and T-point, using HR correction and the same five minute moving window. Both for R-peak, Q-point and T-point several different mathematical curve calculations and filters were used, for a more thorough description please see the methods section of Paper IV.

The same monitor was used for temperature measurements, as it had an integrated temperature probe (Siemens Dräger 5204669). Readings were manually logged every five minutes at the same time as BG measurements; we did not have access to continuous logging.

3.1.2.3 Bioimpedance device

For bioimpedance measurements we used the VIA ECHO 2500 vector impedance analyzer (AEA Technology, USA) as it is capable of accurate measurements using high frequency alternating current. A frequency sweep measurement was performed every 5 minutes at the same time as BG measurements were taken. Based on previous research, the frequency range used was 1 MHz – 200 MHz (159; 160), for a total of 250 linearly distributed frequencies.

We used wet-gel ECG electrodes of the type BlueSensor™ Q00A (Ambu A/S, Denmark). These electrodes maintain stable contact and reduce the contribution from the stratum

corneum impedance. They were attached at least 10 minutes before data acquisition began to avoid impedance drift in the first minutes before the gel/skin contact stabilizes (158).

3.1.2.4 Near Infrared instrument

We borrowed the Spektron® NIR device through our cooperation with Prediktor AS (who have designed the instrument), and used it together with a commercially available tungsten halogen light source from Ocean Optics (LS-1 model, light range 880-2200 nm), and a custom made probe that attached the combined light source/sensor to the skin. The reflection probe used (FCR-7IR400-2-ME from Avantes) have six transmitting light fibers with a separation distance of 0.2 mm from the light emitting fibers. A frequency sweep measurement was done once every second during the whole experiment, using 256 channels linearly distributed over the whole spectrum (880-2200 nm).

3.1.3 Biochemical analysis

Glucose was measured in whole blood using the YSI 2300 STAT Plus glucose analyzer (YSI Life Sciences, Ohio, United States), precision is reported in the manual as $\pm 2\%$ of the reading or 0.2 mmol/L, whichever is larger. Full blood glucose values were subsequently converted to plasma glucose. Plasma glucagon, serum GH and serum cortisol were analyzed at the Hormone laboratory, plasma adrenaline and plasma noradrenaline at the Section of endocrinology, both Oslo University Hospital. For details, see Methods in Paper II.

3.2 Statistical analyses and data processing

3.2.1 Paper I

Linear interpolation was employed to maximize the number of data points for adrenaline measurements. A synchronicity parameter for the different sweat channels was developed. Three p-adrenaline levels were tested with different combinations of sweat channels, and each combination was tested with different thresholds for detection, creating a plot with area under the curve for the receiver operating characteristic (AUROC) for each parameter combination, and this was used as a performance measure.

3.2.2 Paper II

Linear interpolation was used to create data points for comparison between clamps. Normality testing was done using Lilliefors and Shapiro-Wilkes methods, and independent samples t-tests were used when data had a normal distribution, and Wilcoxon Rank Sum was used when data were not normally distributed. The bootstrap method (161), was used to create confidence intervals (using the matlab *bootci* function) and can be applied whether data are normally distributed or not. The Pearson product-moment correlation coefficient was used for correlation testing. For the Edinburgh hypoglycaemia symptom scale, we made a qualitative rule instead of using statistical methods to determine change. The Fisher exact test was used to see if there was a difference in number of subjects from the Reaction and Non-reaction group who had a change in EHSS. The synchronicity parameter from Paper I was tested for hypoglycaemia detection, and AUROC was used as performance parameter.

3.2.3 Paper III

Two types of multivariate regression were tested, partial least squares (PLS) and artificial neural network (ANN), of which ANN performed best. Using NIR, Z and skin temperature we made a model that tried to predict BG and the BG trends. Model parameters were tuned and validated by means of repeated double cross-validation (162). The preprocessing of the NIR data included noise reduction and normalization using several different techniques, which are standard in multivariate analysis of NIR spectroscopy. The impedance data were calculated into resistance (a material's opposition to a current passing through it) and reactance (a material's opposition to a *change* in current passing through it).

Performance of the ANN for trend predictions was evaluated for performance using a test called weighted trend error, and performance for BG prediction was evaluated using mean absolute relative difference (MARD) and the Clarke error grid (163).

3.2.4 Paper IV

With the aim of developing a method to detect hypoglycaemia, we chose to use a mathematical model with probabilistic network topology. In short, we used the information from the different sensors and let them go through several different steps where different algorithms are used, trying to optimize the information for the detection of hypoglycaemia. Data from each parameter is preprocessed (for details, see the paper) and is given a

probability between 0 and 1 for hypoglycaemia. Then they are combined in a probabilistic network which gives different weights to the parameters in the overall probability of a hypoglycaemic event termed $p(\text{hypo})$. This new parameter was then tested for how well it predicted hypoglycaemia during the hypoglycaemic and euglycaemic periods. Gathering true and false negatives and positives, we calculated sensitivity (also called recall), specificity, positive predictive value (PPV, also called precision), F1 score (also called F-score or F-measure) and AUROC. F1 score was used as the metric for overall detection accuracy; it is a type of average (the harmonic average) of PPV and sensitivity. Perfect PPV and sensitivity gives a score of 1, worst is 0.

4 Main results, summary of papers

4.1 Paper I

All in all, there were 6 parameters included in the measurement series in this paper, 4 sweat sites and two ECG features (HR and QTc). Using just SC measurements, the abdominal and wrist measurements gave best AUROC, and this could be further improved by parametrization into FSR, which uses a low-pass filter and a peak-counting algorithm during a 5 minute moving window. The combination of all FSR measurements yielded almost the same detection as either the abdomen or the wrist FSR separately. HR and QTc gave poor detection initially, but with baseline correction gave AUROC comparable to FSR, and a combination of the two gave the best detection for the 1500 pmol/L level of adrenaline. Adding all the rest of the parameters gave better AUROC for the highest adrenaline level, but the overall best performance was achieved using only FSR from the forehead and abdomen combined with HR. However, a combination of just wrist sweating and HR gave almost as good results.

4.2 Paper II

With the exception of skin temperature, all parameters showed a significant change during the hypoglycaemic clamp compared with the euglycaemic clamp. For a quick overview, see figure 8. While several of the counterregulatory hormones were elevated for some time both before and after blood glucose nadir (nadir), sweating and heart rate showed a more concentrated response just after nadir. The hypothenar sweat site and heart rate had a small period with decrease during hypoglycaemic clamp about 20 minutes after nadir.

Figure 8. The X-axis shows time in minutes before or after glucose nadir during the hypoglycaemic clamp and similarly adjusted timing of the euglycaemic clamp. The upper panel shows the mean (line) plasma glucose (PG) values and 95% CI (colored area) for the hypoglycaemic clamp (red) and euglycaemic clamp (blue). In the lower panel, red and blue boxes denote significant ($p < 0.05$) differences for the given parameter during the hypoglycaemic clamp (higher value during hypoglycaemic clamps as compared to euglycaemic - red) or in the euglycaemic clamp (higher value during euglycaemic clamps as compared to hypoglycaemic - blue). Red dotted vertical lines denote the time frame for the hypoglycaemic part (below 4 mmol/L) of the hypoglycaemic clamp, and black dotted vertical line denotes PG nadir.

After completing both the euglycaemic and the hypoglycaemic clamp, we asked all subjects if they had noticed differences between the two clamp procedures. Most said that both felt the same, but a subset of 5 subjects stated that they had felt “low”, i.e. they had typical symptoms to hypoglycaemia (one of them stated that the symptoms could mean high as well as low blood sugar), and they identified the hypoglycaemic clamp. When analyzing the data it soon became clear that these two groups showed very different responses, and that these differences were important when trying to make a composite parameter for detecting hypoglycaemia. The group that could identify hypoglycaemia was called “Reaction” ($n=5$), and the other “Non-reaction” ($n=15$). When referring to the whole group the term “all subjects” is used. When referring to all subjects, results are compared between the hypoglycaemic and euglycaemic clamps. When comparisons are made between the Reaction

and Non-reaction groups, these are all based on results from the hypoglycaemic clamp. There were no significant differences in the baseline subject characteristics between the Reaction and Non-reaction groups ($p>0.05$).

At nadir, the Reaction group had a significant increase in sweat at all sites compared to the Non-reaction group. The group showed also higher serum levels of adrenaline, GH and cortisol, while there were borderline significant differences for HR and QTc ($p = 0.07$ and 0.05 , respectively).

After testing different sensor combinations, we chose two different combinations for detection of hypoglycaemia. One with the best detection we could find (using QTc, HR, abdominal sweat and wrist sweat), and one that had almost as good detection and used sensors that potentially could be used in a design to be worn on the wrist (HR and wrist sweat). We measured their performance using AUROC. For all subjects, the best predictor gave an AUROC of 0.75. For the Reaction group, it scored 1.0, and for the Non-reaction group 0.65. Corresponding numbers for the combination of HR and wrist sweat only, were 0.71, 0.94 and 0.60.

4.3 Paper III

Overall, the best model had 12.5 % of measurements in the D+E zones of the Clarke error grid, with a MARD of 42.5 %. For prediction of glucose trends, several models had scores in the uD+uE zones below 5 %. There was a tendency for the models to not detect that BG increased again at the end of hypoglycaemic clamps and still have quite good performance measures. Due to this, a weighted trend agreement was used that gave better scores to models that could detect this change. The models that scored well both on the weighted trend agreement and on MARD/Clarke error grid was considered to perform best.

The best overall model used NIR, Z and temperature, with centering of the NIR predictors and ANN as multivariate technique.

4.4 Paper IV

Combining all sensors (except for the hypothenar sweat sensor), the maximum achievable F1 score was 88 %. The corresponding sensitivity, specificity and PPV were 95 %, 96 % and 83 %. We had one hypoglycaemic period that could not be detected, and four false alarms.

QTc was the most important single factor for hypoglycaemia detection, with a max F1 score of 63 % if left out, but the median prediction time (in relation to nadir) was better without it, -10.8 minutes vs. -2.2 minutes). HR and NIR+Z had only a small negative impact if left out separately. If all sweat channels were left out, the F1 score was 58 %. In terms of a sensor system with focus on wearability with technology that is readily accessible, a combination of abdominal and wrist sweat with ECG (HR and QTc) managed an F1 score of 81 %, sensitivity of 75 %, specificity of 98 % and PPV of 88 %.

If we used only the 15 participants in the Non-reaction group, the performance was not very much affected for the whole system with a reduction in max F1-score of just 0.01.

5 Discussion

The overall aim of this project was to investigate if we could detect hypoglycaemia in persons with IAH using only non-invasive sensors. We used several different technologies, some aimed at detecting increased sympathetic output and some aimed directly at the level of glucose in blood. Using sweat sensors and ECG, we found very good predictive power in some individuals, while the performance was poor in subjects with little autonomic responses. Further, there was a distinct difference between subjects that were subjectively able to detect the induced hypoglycaemia and those who could not (164). Using near infrared spectroscopy in combination with bioimpedance, we found that predicting actual BG level had high uncertainty, but that predicting the glucose trend was more reliable (165). By combining all sensors and using them in a probabilistic network model, we could discriminate between the reactions during the hypoglycaemic and euglycaemic experiment with high predictive value (166).

5.1 Methodological issues

5.1.1 Subjects

There is at present no gold standard for diagnosing IAH (5). Therefore, the method which we used to identify IAH patients may have been suboptimal for our results. We used the Clarke and Gold scores, which are two of the most widely recognized methods for identifying the condition. Others have used a physiological definition, e.g. that patients are not able to recognize that BG is experimentally lowered to 3.0 mmol/L (167). Performing hypoglycaemic clamps demands at least three people and a specialized lab, so to use it in such a screening process was not an option. As it turned out, 14 out of the 20 participants did not report symptoms (no increase in EHSS) at 2.5 mmol/L, out of the six remaining participants, one reported symptoms around 3.5 mmol/L, one around 3.0 mmol/L, and the rest after reaching nadir at 2.5 mmol/L. Based on this, 18 out of 20 would fulfill the physiological definition (no symptoms before BG > 3 mmol/L). Thus, using Clarke and Gold scores probably gave us comparable results to having used the physiological definition.

Of the six participants that had increased EHSS, five reported symptoms that would normally alert them of low BG during the hypoglycaemic clamp. We analyzed their results separately

from the rest in Paper II, where they were named Reaction group (n=5) and Non-reaction group (n=15). We chose this group instead of the six with increased EHSS, as one of our main goals was to test if we could detect reactions in a group that did not have clear subjective symptoms (the Non-reaction group). We only statistically compared the nadir point during the hypoglycaemic clamp for the two groups, due to the low number of individuals in the Reaction group. These analyses must be regarded as exploratory. For some of the parameters, like for example growth hormone, it may have been more appropriate to compare a different time point than nadir, since it is quite obvious that the biggest differences occur later (probably due to slower release of GH (168)). However, at least for a hypoglycaemic alarm system, the nadir point seems most suitable. Statistically, it would be hard to argue that we should measure different points for different parameters. We could make similar figures showing all time points, confidence intervals and significant differences, but decided not to because of the small sample size of one of the groups, and because of the fact the some samples fall short of the time window as explained earlier. We did however include means (or medians where applicable) of the two groups during hypoglycaemia alongside data from all subjects during both hypoglycaemia and euglycaemia (Paper II, figure 2, please see the corrected version of the figure), which gave a good visual representation of the two groups.

5.1.2 Equipment

Generally, the equipment worked well. Below follows a brief discussion of why we chose the equipment we did, and some possible limitations of our choices.

5.1.2.1 Sweat measurement device

Sweat sensors based on bioimpedance properties show responses much faster than sweat sensors based on evaporation, and is also the better choice when it comes to wearability. It is also more reliable, as it is immediately visible on the measurements if a sensor begins to detach from the skin. Also, these sensors are extremely sensitive, sensing changes already when sweat ducts begin to fill up. The alternative, an evaporative device, depends on sweat reaching the skin surface and evaporate, giving a time lag. However, an evaporative device measures “real” sweat and in some instances this may be more adequate, for example if one was to measure how much sweat that was excreted in patients with hyperhidrosis. But for our use, where the sweat glands’ reaction as part of the sympathetic nervous system was more important, the Sudollogger was considered the best choice.

5.1.2.2 ECG/temperature device

The SC9000XL ECG recorder (Siemens Medical Systems) was chosen mainly as a practical solution, since we had this equipment at hand. Any hospital-grade ECG monitor would probably suffice. Before analyzing the data, they had to be digitized, and we did this with a sample rate of 300 per second. We could have used a faster sampling rate, but we wanted to have real-time analyses so that we could identify problems fast, and computing power was limited. While faster sampling rates (500 Hz) have been recommended for accurate diagnostic performance in adults (169; 170), it has been shown that sampling rates of 250 Hz is comparable to sampling rates of 1000 Hz (171). Anyhow, in adults, most diagnostic information lie in the below 100 Hz range, and for the HR and QT-time measurements, the frequency is much lower (170). Another reason for using a custom made solution is that we wanted better control over the different calculations we used, so that we could look at the source data and monitor the performance ourselves instead of relying on a “black box”.

Since the ECG monitor had a temperature sensor as well, this was also a practical choice, but we could only do manual readings. Access to continuous logging would have been better, lessening the chance of incorrect readings, and excluding the need for interpolation between readings. We considered that the chance for transcription error to be miniscule, and interpolation errors to be negligible since temperature changes in skin are small and relatively slow in room temperature.

5.1.2.3 Bioimpedance device

The VIA ECHO 2500 vector impedance analyzer (AEA Technology, USA) is (among other things) a spectrum analyzer made for testing electronic equipment. It is capable of accurate measurements of bioimpedance using high frequency alternating current in the range we needed (1-200 MHz). It is commercially available, and it has a size that makes it easy to use in a setup with many devices, and has been used in research by others (160). As far as we know, there is no specialized equipment made for use with biological tissue that has the same qualities. Ideally we would use equipment that is better established in the scientific community, the use of this instrument was a tradeoff between suitability and availability.

5.1.2.4 Near infrared instrument

The Spektron® is a NIR device made by Prediktor AS originally for industrial applications. It is made for real time measurements and offers excellent wavelength stability and accuracy. The reflection probe (FCR-7IR400-2-ME from Avantes) have six transmitting light fibers with a separation distance of 0.2 mm from the light emitting fibers, while ideally it should be between 0.4-4.5, depending on the wavelength (172). Thus, the NIR instrument may not have had the ideal configuration for picking up the changes of interest. However, we obtained comparable results to other non-invasive systems used on actual patients with the same technology (120). Better predictions using NIR has been shown in a lab setting (78), but they are not directly comparable.

5.1.2.5 Potential for miniaturization

If all sensors are to be used in an alarm system, they will have to be miniaturized. As discussed in Paper IV, wireless ECG-patches are already available in miniaturized form; Z and NIR circuits and sensors are emerging, and our research group (Oslo Bioimpedance and Medical Technology Group) will be involved in miniaturizing sweat measuring equipment. Wearable body sensors represent a fast-growing and expanding field, and our assessment is that suitable hardware and sensors will materialize in just a few years. As such, although the equipment used in our experiments is impossible to carry around, a wearable alarm system using the same technology will in all likelihood be achievable in the future.

5.1.3 Comparison of data

Initially we considered doing only hypoglycaemic clamps, assuming that results from euglycaemia could be obtained both before and after hypoglycaemia. However, we were advised to include a separate day of euglycaemic clamping, and in hindsight, this turned out to be very important. Comparing directly to a euglycaemic clamp made it easier to claim that the observed changes could be attributed to differences in BG. Also, for multivariate analysis, having more data was crucial for making the algorithms to discern between hypoglycaemia and euglycaemia. And likewise, when looking at true and false alarms, having a large amount of time spent in euglycaemia where we could check for false alarms gave more credibility to our data.

Most experiments of similar design use stepped hypoglycaemia where BG typically is lowered in steps of 0.5 mmol/L at a time and maintained for a predefined time (37). As our aim was to see if we could make an alarm system, we found it more appropriate to do a continuous lowering. The exception was at the 2.5 mmol/L level, which was kept for 15 minutes. As for an experimentally induced hypoglycaemia, we think our method is closer to real life than a stepped approach.

We had close control over BG, measuring every five minutes and using a high quality method for BG measurements (YSI 2300 STAT Plus glucose analyzer). All non-invasive measurements were sampled at least as often as BG, hormones and EHSS were sampled nine times. Comparing data was not straightforward. The easy way out, at least for EHSS and hormones, would be to compare them directly, number one in the euglycaemic clamp to number one in the hypoglycaemic clamp and so on. However, the way the experiment was designed, there was no way to ascertain that the euglycaemic blood tests were drawn at the same time as the hypoglycaemic (measured from the start of each of the experiments). If we consistently had done hypoglycaemic clamp procedures first, we could time the tests in the euglycaemic clamp to be performed at the same time. But it was more important that the participants were blinded as to what kind of experiment was being done. Hence, only half of the experiment pairs were done with hypoglycaemic first, and when this was the case, we timed the euglycaemic blood tests accordingly.

For the other half of the experiment pairs (euglycaemic first), we had a time schedule for an “optimized” hypoglycaemic clamp for the blood tests. However, these could differ quite significantly from the actual hypoglycaemic clamps performed later. So e.g. blood test no. 5 in the hypoglycaemic clamp could end up, timed from the beginning of each clamp, to be much closer in time to e.g. blood test no. 8 in the euglycaemic clamp (see figure 9). The main purpose of having a euglycaemic clamp for comparison was to rule out time effects, so for an *individual participant*, euglycaemic and hypoglycaemic clamps were *aligned according to when the clamping procedures started*. However, since clamps could vary considerably in time from person to person, BG levels for the hypoglycaemic clamps *between subjects* could be very different for the same time point. We decided that the most important time point to compare between subjects was at the end of the hypoglycaemic period during the hypoglycaemic clamp. Since this was also the point with lowest BG (in most of the participants anyway), it was denoted nadir. In reality, some subjects may have measured lower BG at a point five or ten minutes before, but if so, typically just 0.1 or 0.2 mmol/L

Figure 9. How experiments were lined up when compared to each other. Red graphs are hypoglycaemic clamps, blue are euglycaemic clamps. Stipled lines show how blood tests can be shifted in time between clamping, while arrowed lines show examples of where actual comparisons are done, using interpolated values. a) Comparison of euglycaemic and hypoglycaemic clamp in a single subject, aligned from start of clamps. b) Comparison of two hypoglycaemic clamps from two different subjects, aligned from start of clamps. c) Same as b, but aligned according to nadir glucose. d) The method we used for lining up all clamps, aligning from start of clamps for euglycaemic and hypoglycaemic clamp in a single subject (as in a)), and aligning according to glucose nadir when comparing between subjects (as in c)).

below the “nadir” point, and because of the delay in the physiology, we would expect a larger counterregulatory reaction at the very end of the hypoglycaemic clamp anyhow. For these reasons, this nadir point was used as time point zero, and *all hypoglycaemic clamps were lined up* according to their individual zero when comparing *between subjects*. The euglycaemic clamps were then lined up pairwise with their individual hypoglycaemic clamp as already described. Figure 9 gives a visual representation of how data were aligned, and exemplifies the difference in lining up according to the beginning of the procedures as opposed to the nadir point.

Since measurements thus did not necessarily occur at the same time, we had to compromise and use linear interpolation to get new time points to be compared to “real” time points. There is an added insecurity in the interpolated data, but since the interpolated points are quite close in time to the “real” points, typically five minutes, we do believe that they are not far off. Other interpolations

Figure 10. Linear interpolation. Illustrates how interpolated points can at times over- and underestimate real values.

methods were tried out, but because of the spatial distribution of the measurements (typically the first one is relatively far away in time from the rest), this had some odd-looking results. Also, linear interpolation will never give a higher maximum value than the actual maximum, so there is no chance of overestimation the highest value. Other places on the graph, small overestimations and underestimations might occur (figure 10). The graphs produced from these data show a confidence interval of 95 % around the mean or median values from the measurements. We did not adjust the statistical computations due to the use of interpolation, but one might argue that we should have. But as already stated, these interpolations were for a very large part in close proximity to real measurements.

5.1.4 Mathematical modeling and statistical analysis.

In all the papers in this thesis, some mathematical modelling is involved. There is no way to be certain that the model you end up with is the best possible to solve the specific problem. Thus, the models may be further revised in the future.

The most complex modelling was done in paper III, where multivariate regression based on artificial neural networks (often used in machine learning) was employed to predict BG and BG trends. Machine learning is superior when there is a set of rules and a limited set of options that can be broken down into easy steps, but contain a very large number of possible solutions. In paper III we had several sets of data that we suspected could hold some information on BG and BG trends, and we wished to combine them and find some sort of predictive associations with the measured reference values. The different ways to combine

Figure 11. Example of overfitting (red line), the green line is a better predictor for future data points.

data were so numerous that computers would need an incredible amount of time if everything was to be checked. Thus, the amount of possible combinations had to be reduced. Different methods can be used for this “dimensionality reduction”, and are embedded in different machine learning algorithms. We tried out two of the most used techniques (PLS and ANN regression) and chose the one with best results. Having so much computing power, machines can in some instances make

complex models that fit the data almost perfectly (figure 11). However, these models tend to only fit the data that is being analyzed, and do not perform as well on new data. This problem is reduced using cross validation strategies. When used properly, machine learning is a very powerful tool, helping us to retrieve information that would otherwise be hard to obtain. It was essential for providing the results seen in paper III.

In paper IV we chose an approach where we made a probabilistic model for physiological responses to hypoglycaemia. Basically, this method translated data from the different sensors into probabilities, and combines them to a final probability for a hypoglycaemic episode. We set the probabilities based on previous knowledge about the measurements; this gave us a more understandable algorithm, instead of a “black box” solution. Also, the chance for overfitting will be drastically reduced when we set some restraints in the data based on knowledge about the physiology.

The statistical methods we used are generally quite standard. In the medical sciences though, boot-strapping is not used much. The bootstrap method is a so-called resampling method, where one sample is used repeatedly, using random sampling with replacement, creating new samples of the same size as the original sample. Having access to computers, it is more natural to use a method like bootstrapping rather than a normal curve, because we work with

actual data to create a probability distribution. Also, we need not to consider if data are normally distributed at all. In general, it is considered better to not use too many different statistical methods at the same time, and boot-strapping helped us achieve this.

Computation of real and false positive/negative alerts for hypoglycaemia was not straightforward. In paper 4, we divided both the euglycaemic and hypoglycaemic clamps into three time intervals. They were divided based on the hypoglycaemic clamp experiments, where BG was euglycaemic, and then hypoglycaemic, and then euglycaemic again. The three periods were treated as one instance each, so that several alarms in one euglycaemic period would count as just one false alarm, and the same for the hypoglycaemic period and real alarms. It could be argued that many false alarms will not be counted, but the same can be said for real alarms. This is a problem with resolution in time. When a hypoglycaemic reaction is detected, the system will trigger an alarm continuously for some time. If, for example, the alarm triggers for five minutes and the resolution is one second, this will count as 300 real positive alarms. It seems obvious that this resolution is too fine. There is no clear answer for how long the time interval should be, but at least longer than the moving window seems reasonable (30 minutes). Paper II and IV actually use two different partitions, with paper II only using one partition for the whole euglycaemic clamp. This was due to a change of minds in the time between the papers, that a time period of about 3 hours was too long to be considered as just one observation. If its benefits the statistical outcome depends on how many alerts there are during the euglycaemic clamp. If there are many alerts, counting as just one false alarm will be positive for the statistic, while a greater number of partitions will have negative impact. If there are few alerts, we get the opposite effect. Since we have relatively few alerts, the statistics are probably a little bit better in Paper IV than if we would have used the same partition as in Paper II. This was not the rationale behind the new partitioning, rather that we found that partitions of about one hour were more natural when the moving window was 30 minutes.

5.2 Discussion of main findings

5.2.1 Detection of sympathetic reactions

Paper I showed that there was a clear association between adrenaline output and both ECG changes and sweating. The hypothenar stood out as having less correlation than other sweat

sites, and more variation during the euglycaemic period hampered the specificity of the algorithm. The reason for this variation may be a more constant level of sweating, possibly facilitating a better grip (173). Different combinations of measurements had close to equal performance, and more information did not necessarily improve the combined parameter, which is perhaps surprising. However, considering that a parameter should contain information both on when something is occurring and when it is not occurring, it is likely that although it may give good information in one aspect, it might impact negatively in the other, and thus a combination will not always be for the better.

Paper II showed that the same sweat and ECG changes happened around or shortly after glucose nadir, but there was no significant change in skin temperature. Significant increases in the mean or median levels of all counterregulatory hormones were observed around the glucose nadir of the hypoglycaemic clamp, compared to the corresponding levels during the euglycaemic clamp.

However, there were large individual differences. The Reaction group (five individuals), had considerably larger reactions, and may have disproportionately impacted the data. When it came to the next step of trying to detect hypoglycaemia, it became evident that identifying sympathetic reactions on a group level did not necessarily translate to good individual detection of hypoglycaemia (see 5.2.3).

5.2.2 Predicting blood glucose levels and trends.

Inaccurate prediction of BG can be more or less dangerous depending on actual BG and if the prediction is too low or too high. To represent this, the Clarke error grid is often used (163). Inaccurate predictions give worse MARD scores, but can at the same time give quite good error grid scores, especially if the bulk of the predictions are in the euglycaemic and hyperglycaemic range (figure 12).

In Paper III, the best BG prediction

Figure 12. Fabricated example of imprecise predictions with good error grid score.

model we achieved, based on NIR, Z and temperature, gave a MARD score of 42.5 % (not very good), but we had a score >85 % in the A+B zones on the Clarke error grid, which is usually considered acceptable. But at the same, >12 % of results were in the D-zone (predicting normal BG when in fact values were in the hypoglycaemic range), which is quite unfortunate. When looking only at predictions corresponding to actual BG below 4 mmol/L, results were much worse, with >50 % of the predictions in the D+E-zones. This illustrates how easy it is to portray predictions as more promising than they really are with the Clarke error grid. When looking at just the hypoglycaemic measurements, it is clearly not good enough to be used as a non-invasive BG-meter.

Results for prediction of BG trends were generally better than for actual hypoglycaemic glucose values, with fewer instances in the potentially dangerous zones in the Rate Error Grid vs. the Clarke Error Grid (4.3 % vs. 12.5 %). NIR performed far better than Z for trend prediction, and combining the two improved results further. NIR is known to be influenced by tissue properties (174), and so are Z (175; 176), and there is a chance that the combined results reflects that input from Z helps the NIR predictions by alleviating some of the effects that tissue differences may have on the measurements. Since these tissue differences otherwise would need calibrations between individuals, and also over time in one and the same individual, this is definitely helpful for an alarm system. If this global model (applicable to everyone) actually works in more realistic settings will have to be investigated further.

Skin temperature could potentially give valuable information since lower skin temperature is associated with hypoglycaemia (177-179) and could be a possible source of error for NIR and Z measurements. The combination NIR+temperature performed almost as well as NIR+Z, hence there probably is some information in the temperature readings, although there generally was little variation in most subjects. The main difference from our experiment to others studying body/skin temperature and hypoglycaemia is that during our experiments room temperature was not tightly regulated, and subjects were allowed to put on or take off a blanket as they wished. We gathered that an approach where a person was allowed to keep a comfortable body temperature was closer to reality. This may explain why we found little (but still useful) temperature variation. Another explanation could be that the shoulder is just not a good place to measure temperature differences. Sejling *et al.* found that the nose experienced the largest temperature fall (178), although hardly a good place for a temperature sensor. It may be that larger variations exist during sleep, and provide even more information for an alarm algorithm, which potentially can be explored later.

5.2.3 Hypoglycaemia detection

In Paper II, we explored the possibility for hypoglycaemia detection based on sympathetic activation and a basic algorithm that focused on amplification of simultaneous events. In general, some of the same problems that applied for the correlation between sweating, ECG and adrenaline levels emerged also for the hypoglycaemia detection (not being able to utilize all the data). Also, when trying to detect responses on the individual level, the individual differences became obvious; for the Reaction group, detection was very good, for the Non-reaction group, detection was poor. As for the detection of adrenaline discharge, HR and wrist sweat together performed very well also for hypoglycaemia detection. While a combination of just two sensors that can be worn on the wrist is very practical when considering wearability, it may be too susceptible to other unknown factors to be an effective hypoglycaemia alarm. This may very well also be true for a system consisting of more sensors, but there is a possibility that there is a “fingerprint” for a sympathetic reaction from hypoglycaemia, and more sensors will probably make for a more secure fingerprint. But it may also transpire that there is no real difference in a sympathetic reaction to hypoglycaemia and other sympathetic reactions.

In Paper IV, 19 out of 20 hypoglycaemias could be detected with a more advanced algorithm that also utilized results from BG prediction and BG trend prediction. The improvement is based on removal of much of the between-subjects variation and focus on small changes on the individual level. The problems we had in Paper I and II with utilizing all data were overcome by employing machine learning techniques.

Out of the 19 hypoglycaemias detected, we know that 14 was not recognized by the patients (neither was the one that we could not detect), so the system managed to detect 14 out of 15 hypoglycaemic episodes not recognized by patients (and five out of five that were recognized by the participants). We managed to get the absolute number of false alarms down to five (counted as four in the statistic, for reasons discussed in 5.1.4, last paragraph) during a span of 76 hours of euglycaemia. This translates into about one alarm every 15 hours. If this is good enough probably depends on the circumstances related to the individual patient. For an IAH patient who has multiple hypoglycaemias during a day, it will probably be acceptable, for a patient that has unrecognized hypoglycaemia once a week it may be more of a nuisance. However, a hypoglycaemia alarm will only require a quick BG test to confirm or rule out hypoglycaemia, a test that most patients do multiple times a day anyhow. There will possibly

be a large population of patients who will find an alarm system with this performance useful. If it is possible to get a similar result under real life conditions remains to be seen.

With focus on wearability using current technology we found that the best sensor combination with the improved algorithm was abdomen and wrist sweat combined with ECG (HR and QTc). This combination achieved an F1 score of 81 %, sensitivity of 75 % and specificity of 98 %. If we leave out QTc and abdominal sweat (a wristband solution), we get an F1 score of 55 %, a sensitivity of 75 % and specificity of 81 %. If this is our starting point, we already have a quite large decrease in sensitivity, but it should be mentioned that the model is tuned to work best for all sensors, and that every combination with fewer sensors probably have better potential if we choose to investigate this. For the time being, one of the two sensor combinations just mentioned is probably the most likely we can achieve. The hardware that is necessary for NIR measurements is for the moment not available in a form that can be easily carried around. Hardware for Z measurements is probably more achievable, but it does not add significantly to the system without NIR. Also, keeping the NIR instrument in a stable position is crucial, and probably hard to achieve. Using the proposed configuration wrist and abdomen sweat and ECG none the less will need at least two different sensors attached to the body which also may be too much for some users. It is also technically more difficult, since there would have to be a (preferably wireless) communication between the sensor units. However, there is an evolution towards very small sensors incorporated in adhesive bandages and even tattoo-like sensors (180). If this materializes, wearing several sensors at once will probably feel less obtrusive.

If we were to develop an alarm system, it would need to have some significant improvements over other available systems. As mentioned in the introduction, there have been previous attempts, but as for now there are only two basically identical products on the market, the Diabetes Sentry in the USA and the HSA-01 in the UK, which claim to catch 2 out of 3 hypoglycaemic episodes, and the ratio of false to real alarms is reported to be 3 to 1. The Hypomon alarm in Australia was also mentioned; it was retracted due to poor performance. Our system uses considerable more sensors than the Diabetes Sentry and the Hypomon. However, a slimmed down version of the system, to be placed in a watch-like device, would not include most of the sensors. Best case scenario for such a system would be sweat measurement, HR and maybe temperature, which means that the only measurement that distinguishes it from the Diabetes Sentry is heart rate, so other improvements would be in signal processing and algorithms. If we were able to achieve the same results as in our

experiment for a one-unit system worn on the wrist, it would translate to 3 out of 4 of hypoglycaemias would be detected and the ratio of false to real alarms would be around 4 to 3, clearly an improvement over the Diabetes Sentry, especially regarding false alarms. We may improve on these results if we tune the model for a system with fewer sensors.

5.3 General discussion and limitations

5.3.1 Possible triggers for sympathetic reactions

Most of the sensors tested work primarily by detecting the body's sympathetic reactions to hypoglycaemia. As the sympathetic nervous system often is activated during normal daily life activities, responses for other reasons than hypoglycaemia will be a problem. The NIR and Z measurements do not necessarily use this response, and may rule out some of the false alarms. Due to the insecurities in the NIR and Z predictions, the algorithm does not let them suppress other measurements completely, so a strong sympathetic response can still trigger the alarm. Further testing will tell how strong the sympathetic responses during daily life activities are, and if these will trigger the alarm system. Exercise will almost certainly trigger the alarm (181), so the system might be further developed with for example motion sensors to try to rule out these responses. Psychological triggers might be even more difficult to discriminate from hypoglycaemia-induced sympathetic reactions.

As participants were confined to the bed during the experiments, avoiding reactions due to mental stress (182) was a priority. We did not impose strict silence during the experiments, however, all participants and study staff were instructed to have only calm and not too emotional conversations. However, we did see some false alarms, but we could not relate them to any disturbances in the room at the time.

It may transpire that the system only performs well during bed rest and sleep. However, this may not be a severe limitation, as detection of hypoglycaemia in IAH subjects in such situation still would be a considerable leap forward. Another advantage for a sleep-only alarm system is that improvements in placement, attachment and stability may be easier to achieve. This will be an important consideration in further experiments with a miniaturized version of the system.

5.3.2 Insulin and glucose infusions during clamp procedures

Maintaining stable BG during the night before the clamps was challenging in some cases, resulting in variable glucose levels at the start of the clamps. And sometimes BG lowering during clamps was slower or faster than expected due to individual differences in insulin sensitivity. It has been suggested that if BG decreases fast, it can by itself initiate a sympathetic response without actually reaching hypoglycaemia. Earlier works have shown that this is not the case (183-186), and thus most likely did not influence our data in any significant way.

During the experiments, glucose infusions were adjusted according to BG measurements by adjusting the infusion pump speed. While participants could not observe the infusion speed or knew that it was an infusion pump, they did observe the activities performed by the staff. We never commented on what was being done, and we could not observe any measurable reactions related to staff activity. Still remains the question if suddenly increasing levels of glucose in the bloodstream could have any effect. The largest increase in glucose infusion occurred directly after nadir when hypoglycaemia was reversed. As evident from figure 8, significant differences for all the sweat sensors happened right after nadir. This could either be because there was a delay in the reaction to hypoglycaemia, or that the sudden increase in glucose had an effect. Figure 2 in Paper II gives a better picture for how sweating changes over time (please see corrected version of the figure). It seems as sweating gradually increases before reaching a top shortly after nadir. We can conclude that if the glucose infusion has an effect, it just adds to the hypoglycaemia reaction and does not cause it by itself. Also, some of the participants have no discernible sweat reaction at all. This means that they react neither to the hypoglycaemia nor the glucose infusion. This also makes it more likely that glucose does not have an independent effect, unless the participants that do not react to hypoglycaemia also have lost a reaction to sudden glucose infusions. Since sudden glucose infusions do not normally happen in real life, one would think that it would not be possible to “blunt” this reaction, if the reaction is real. Also, the adrenaline level increases all the way until nadir is reached. Due to the fact that the next adrenaline sample was not taken until euglycaemia was reached, there may have been a further increase that was not detected, i.e. there could be a lag in the sympathetic output such that the maximum level (that might also correspond with maximum sweating) actually came a short time after nadir. This does not exclude that glucose infusion could be the culprit, but it shows that the reaction to hypoglycaemia had not peaked until or after nadir, and thus it is not surprising that maximum sweating is shortly after nadir.

All in all, it is possible, but not plausible, that glucose infusion at nadir gave a sweating response, but even if it did, it probably just added to the hypoglycaemia response that was already taking place.

5.3.3 Limitations for use in IAH patients

The alarm system depends for a large part on the sympathetic reaction in the individual patient. Others have shown that IAH patients can have very low BG before a sympathetic reaction occurs, but at some point, all of them got a reaction (42). An alarm system based on these reactions will thus very likely be able to recognize hypoglycaemia in these patients as well. Some of them will undoubtedly have so severe neuroglycopenia that they will not wake up from sleep, or be too confused to take appropriate action if an alarm goes off. But even if they cannot take action themselves, someone in the household may take action, or if they live alone; a remote alarm may be set off.

5.3.4 Possible use for patients with normal awareness

While the goal was to detect hypoglycaemia in diabetes patients with IAH, we gather that much can be inferred about the use in diabetes patients with normal awareness. More than likely, patients who are aware of their hypoglycaemic episodes, all have a strong sympathetic reaction to hypoglycaemia, as this is the primary reason for their symptoms (40). While patients with normal awareness usually have fewer problems with hypoglycaemia than IAH patients, altered states of awareness such as sleep or alcohol consumption can influence their ability to recognize it. An alarm system may be of use for such patients as they presumably have better autonomic responses.

6 Conclusion

We have shown some possible directions for improvements of non-invasive hypoglycaemia alarms. We also observed that it is challenging to detect hypoglycaemic episodes in patients with IAH based solely on data processing of sensor signals relying on sympathetic reactions. Given that such reactions does occur, the signals can easily be picked up and processed in a meaningful way. NIR and Z measurements (not relying on sympathetic reactions) do not give good BG predictions or hypoglycaemia predictions, but has some merit in prediction of BG trends. Integrating all data combined with a developed mathematical model improved hypoglycaemia detection further. Since some of the technologies used are difficult to implement in a wearable system at present, an achievable goal for the moment can be a system based on sweat and ECG, either as a wrist device or with one or two added sensors for higher accuracy. Cost/benefit when it comes to wearability and user friendliness held against actual performance of the final device(s) will be important. A system to be used during sleep is probably the most realistic option. If a miniaturized version can achieve comparable results to those shown here, the resulting device would perform better than existing hypoglycaemia alarms on the market today. The fact that these results were demonstrated on IAH patients means that the alarm system could potentially work for the majority of diabetes patients.

7 Future perspectives

We consider that the best way to move forward would be to develop a hypoglycaemia alarm that consists of a type of wrist sensor with the possibility to add more sensors for increased accuracy. A new series of experiments with hospital bound patients who are permitted to “roam free” with wearable sensors are being planned. Most likely, all sensors will be possible to miniaturize in a few years. Accelerometers should probably be incorporated for recognizing different types of activity, and there are many good miniaturized devices available. In parallel to trying to make a working miniaturized system, new algorithms that may incorporate newer machine learning techniques will be tried out. Results will show if there is any merit in going further with studies on patients outside a controlled lab environment. Further work will also have to include cooperation and feedback from user groups regarding user interface and design, and probably a commercial partner.

For the moment, CGMs are steadily improving, and the need for a hypoglycaemia alarm may decline. However, although quite unobtrusive, CGMs are invasive and they have to be changed regularly. Also, they are expensive, and may be more demanding for the user than a well-developed alarm based on a simple wrist band. Possibly, we could succeed in the development of a user friendly and low-priced hypoglycaemic alarm device available to people in low-income countries. At least from a philanthropic viewpoint, this would be a successful endpoint for our project. If we were able to use equipment that did not need frequent replacement of sensors, a small one-time investment could potentially last for years. Regrettably, it is not easy to introduce products that are specifically tailored to low-income markets with small margins for profit. One possible solution could be to license away the technology for free. Another solution would be that we could produce and sell the device with a very small profit margin.

A lot of time and research has been put into the field of diabetes technology, and we certainly would not be the first ones to fail. But working from a University Hospital, we may have more leeway to consider patients’ wellbeing over purely profitable arguments, and be able to share our knowledge.

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Appendix

Clarke Score

1) Check the category that best describes you: (check one only)

I always have symptoms when my blood sugar is low (A)

I sometimes have symptoms when my blood sugar is low (R)

I no longer have symptoms when my blood sugar is low (R)

2) Have you lost some of the symptoms that used to occur when your blood sugar was low?

yes (R) no (A)

3) In the past six months how often have you had moderate hypoglycemia episodes?

(Episodes where you might feel confused, disoriented, or lethargic and were unable to treat yourself)

Never (A) Once or twice (R) Every other month (R)

. Once a month (R) More than once a month (R)

4) In the past year how often have you had severe hypoglycemic episodes? (Episodes where you were unconscious or had a seizure and needed glucagon or intravenous glucose)

Never (A) 1 time (R) 2 times (R) 3 times (R) 4 times (R)

5 times (R) 6 times (R) 7 times (R) 8 times (R)

9 times (R) 10 times (R) 11 times (R)

12 or more times (U)

5) How often in the last month have you had readings <70 mg/dl with symptoms?

Never 1 to 3 times 1 time/week 2 to 3 times/week 4 to 5 times/week

Almost daily

6) How often in the last month have you had readings <70 mg/dl without any symptoms?

Never 1 to 3 times 1 time/week 2 to 3 times/week

4 to 5 times/week , Almost daily

(R = answer to 5 < answer to 6, A = answer to 6 > answer to 5)

7) How low does your blood sugar need to go before you feel symptoms?

60-69 mg/dl (A) 50-59 mg/dl (A) 40-49 mg/dl (R)

<40 mg/dl (R)

8) To what extent can you tell by your symptoms that your blood sugar is low?

Never (R) Rarely (R) Sometimes (R) Often (A)

Always (A)

Four or more R responses = reduced awareness; 2 or fewer R responses = aware.

Gold-score

“Do you know when your hypos are commencing?”

	Always aware					Never aware	
Awareness	1	2	3	4	5	6	7

A score of 4 or higher implies impaired awareness of hypoglycaemia.

Edinburgh Hypoglycaemia Symptom Scale

	Not at all					A great deal	
	1	2	3	4	5	6	7
Confusion	1	2	3	4	5	6	7
Sweating	1	2	3	4	5	6	7
Drowsiness	1	2	3	4	5	6	7
Weakness	1	2	3	4	5	6	7
Dizziness	1	2	3	4	5	6	7
Warmness	1	2	3	4	5	6	7
Difficulty speaking	1	2	3	4	5	6	7
Pounding heart	1	2	3	4	5	6	7
Inability to concentrate	1	2	3	4	5	6	7
Blurred vision	1	2	3	4	5	6	7
Hunger	1	2	3	4	5	6	7
Nausea	1	2	3	4	5	6	7
Anxiety	1	2	3	4	5	6	7
Tiredness	1	2	3	4	5	6	7
Tingling lips	1	2	3	4	5	6	7
Trembling	1	2	3	4	5	6	7
Headache	1	2	3	4	5	6	7

Errata

(corrections made between submitted version to printed version)

Page	Line	Original text	Amended text	Comments
5	8	Edinburgh Hypoglycaemia Symptom Score	Edinburgh Hypoglycaemia Symptom Scale	
9; 12; 12; 13; 61; 63	3; 26; 28; 16; 16; 4	..patients that..	..patients who..	Several identical corrections.
9	10	..significant progresses in..	..significant progress in..	
10	2	..pancreas (7), due to an..	..pancreas (7) due to an..	
11	14	..the Edinburgh symptom score (EHSS)..	..the Edinburgh hypoglycaemia symptom scale (EHSS)..	
12	24	..responses with endogenic..	..responses and endogenic..	
17	2	..average level of blood..	..average levels of blood..	
17	3	..and is in non-diabetic, healthy individuals 21.3-46.4 mmol/mol.	..and normal values in non-diabetic, healthy individuals are 21.3-46.4 mmol/mol.	
18	10	..changes direction..	..change direction..	
18	20	..that is scattered..	..that are scattered..	
22	16	..and is found..	..and are found..	
23	2	..information of BG..	..information about BG..	
39	1	..impedance (158). They were attached at least 10 minutes before data acquisition began to avoid impedance drift in the first minutes before the gel/skin contact stabilizes (161).	..impedance. They were attached at least 10 minutes before data acquisition began to avoid impedance drift in the first minutes before the gel/skin contact stabilizes (158).	Reference nr. 161 is incorrect and was removed. Subsequent references thus have a new reference number.
39	5	..have designed have designed..	..have designed..	
40	8	..Edinburgh symptom score..	..Edinburgh hypoglycaemia symptom scale..	
54	23	..the statistics probably is a little..	..the statistics are probably a little..	
56	31	..the larges..	..the largest..	
62	3	..also observed, that it is..	..also observed that it is..	
81	1	Edinburgh Hypoglycaemia Symptoms Score	Edinburgh Hypoglycaemia Symptom Scale	

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Author Correction: Evaluation of Hypoglycaemia with Non-Invasive Sensors in People with Type 1 Diabetes and Impaired Awareness of Hypoglycaemia

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Correction to: *Scientific Reports* <https://doi.org/10.1038/s41598-018-33189-1>, published online 03 October 2018

In Figure 2, the coloured areas representing the margins of error were omitted. In addition, in the HTML version, the black line representing the Reaction group appears blue. The correct Figure 2 appears below as Fig. 1.

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Figure 1. Hormonal and symptomatic responses during HYPO-clamp (red) and EU-clamp (blue). Red and blue lines: Medians (or means, if applicable). Colored areas: 95% CI. Black lines: medians or means for the Reaction group during HYPO-clamps. Dashed black lines: medians or means for the Non-reaction group during HYPO-clamps. Time = 0 is marked with a vertical dashed line. Dashed boxes circumscribe related graphs (blood tests, sweat measurements and ECG-data).

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Evaluation of Hypoglycaemia with Non-Invasive Sensors in People with Type 1 Diabetes and Impaired Awareness of Hypoglycaemia

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People with type 1 diabetes and impaired awareness of hypoglycaemia (IAH) are prone to severe hypoglycaemia. Previous attempts to develop non-invasive hypoglycaemia alarm systems have shown promising results, but it is not known if such alarms can detect severe hypoglycaemia in people with IAH. We aimed to explore whether a combination of non-invasive sensors could reliably evaluate hypoglycaemia (plasma glucose (PG) minimum 2.5 mmol/L) in people with IAH. Twenty participants with type 1 diabetes and IAH underwent randomly ordered, single blinded hyperinsulinemic euglycaemic and hyperinsulinemic hypoglycaemic clamps. Sweating, skin temperature, ECG, counterregulatory hormones and symptoms of hypoglycaemia were assessed. Overall, we were not able to detect clamp-induced hypoglycaemia with sufficient sensitivity and specificity for further clinical use. As a post-hoc analysis, we stratified participants according to their ability to identify hypoglycaemic symptoms during hypoglycaemic clamps. Five out of 20 participants could identify such symptoms. These participants had a significantly higher adrenaline response to hypoglycaemia ($p < 0.001$) and were reliably identified by sensors. Based on our observations, a non-invasive alarm system based on measurement of sweating responses and ECG changes during hypoglycaemia might provide an alert at a plasma glucose concentration around 2.5 mmol/L if an adequate sympatho-adrenal reaction is elicited.

Severe hypoglycaemia is one of the most feared complications among people with type 1 diabetes¹. People with impaired awareness of hypoglycaemia (IAH)² have reduced ability to recognize and respond to low plasma glucose (PG), with subsequent risk of confusion or unconsciousness³. IAH afflicts 17–28% of people with type 1 diabetes when assessing hypoglycaemia awareness status using the Clarke and Gold questionnaires, and it increases the risk of severe hypoglycaemia by sixfold^{4–8}. In a pilot study, a system to monitor the autonomic responses to variations in PG in people with type 1 diabetes with normal awareness of hypoglycaemia has shown promising results⁹. To our knowledge, no such alarm system has been tested in people with IAH. During hyperinsulinaemic hypoglycaemic clamp studies, patients with IAH have initial sympathoadrenal activation at lower PG-levels than people with normal awareness¹⁰. It is not known whether this alters the response threshold of sensors detecting the autonomic signals. Our aim was to study the physiological responses during hypoglycaemia in people with IAH by symptom scoring, hormonal responses and non-invasive sensors, and explore whether

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	All participants (n = 20)	Reaction group (n = 5)	Non-reaction group (n = 15)	p-value, difference between Reaction and Non-reaction group
Age, years	41.1 (10.9)	30.6 (11.1)	41.9 (11.1)	0.57
Diabetes duration, years	23.0 (13.8)	17.4 (10.3)	24.9 (14.6)	0.31
Sex, M/F	8/12	1/4	7/8	0.60
Height, cm	170.2 (9.1)	167.3 (8.1)	171.2 (9.5)	0.42
Weight, kg	73.1 (12.1)	71.6 (16.0)	73.6 (11.2)	0.75
BMI, kg/m ²	25.1 (2.8)	25.3 (3.6)	25.1 (2.7)	0.86
HbA _{1c} , %	6.4 (0.8)	7.0 (0.9)	6.2 (0.7)	0.07
HbA _{1c} , mmol/mol	46.7 (8.4)	52.8 (10.0)	44.7 (7.1)	
Insulin, IU/kg/day	0.64 (0.19)	0.56 (0.1)	0.67 (0.21)	0.29
Gold score ^a	5.1 (1.0)	4.8 (1.3)	5.2 (0.9)	0.61
Clarke score ^a	5.0 (1.1)	5.2 (1.1)	4.9 (1.2)	0.67

Table 1. Subject characteristics (mean (SD)). ^aA score of ≥ 4 implies IAH.

	All participants (n = 20)	Reaction group (n = 5)	Non-reaction group (n = 15)	p-value, difference between Reaction and Non-reaction group
Nadir PG ^a (mmol/L)	2.3 (0.2)	2.5 (0.1)	2.3 (0.2)	0.04
GIR ^b (mg/kg/min)	5.7 (2.0)	4.1 (0.9)	6.2 (2.0)	0.008

Table 2. Nadir plasma glucose during hypoglycaemic clamps, and glucose infusion rates at the end of euglycaemic clamps (mean (SD)). ^aPG: Plasma glucose. ^bGIR: Glucose infusion rate.

hypoglycaemia-induced autonomic responses that do not induce symptoms can be measured non-invasively in people with IAH. Study participants with type 1 diabetes and IAH underwent euglycaemic and graded hypoglycaemic hyperinsulinaemic clamps to assess if any combination of non-invasive sensor measurements (four sweat sites, heart rate (HR), corrected QT-time (QTc) and skin temperature) could detect hypoglycaemia with nadir around 2.5 mmol/L.

Results

In total, 21 participants were recruited, 20 completed both hyperinsulinemic hypoglycaemic (HYPO) and hyperinsulinemic euglycaemic (EU) clamps. One participant was excluded due to failure to maintain intravenous lines during the first clamp.

Subject characteristics, nadir plasma glucose and insulin sensitivity. Subject characteristics are presented in Table 1. Mean age was 41 (standard deviation (SD) 11) years, body mass index (BMI) 25.1 (2.8) kg/m², HbA_{1c} 6.4 (0.8) % (46.7 (8.4) mmol/mol) and diabetes duration 23 (14) years. Mean nadir PG during hypoglycaemic clamps was 2.3 (0.2) mmol/L, and mean insulin sensitivity measured from EU-clamps as glucose infusion rate (GIR) was 5.7 (2.0) mg/kg/min (Table 2). All participants had normal kidney function (estimated glomerular filtration rate (eGFR) within normal limits), and none had a history of retinopathy, autonomic neuropathy or peripheral sensori-motor neuropathy. None of the participants had any recordings of PG < 3 mmol/L during the 48 h before the clamps, but three had PG < 4 mmol/L (3.6, 3.6 and 3.7 mmol/L) the night before the EU-clamp and three had PG < 4 mmol/L (3.4, 3.4 and 3.8 mmol/L) the night before the HYPO-clamp.

In addition to presenting data for the whole group, we did a post-hoc stratification; participants were divided into two groups based on whether they were able to recognize that they had low PG during the HYPO-clamp ("Reaction group", n = 5) or not ("Non-reaction group", n = 15) to see if there were differences in their physiological response to hypoglycaemia. Median (or mean where applicable) values of measures for these groups during HYPO-clamp are presented alongside the HYPO-clamp and EU-clamp results for the whole group. Subject characteristics did not differ significantly (significance level $p < 0.05$) between the Reaction group and Non-reaction group (Table 1).

Sweating. In all four sites, there were significant differences in sweating between the EU- and HYPO-clamp within 5 min. after PG nadir (Fig. 1). The hypothenar site differed from the other sites by a higher baseline sweat response during both EU- and HYPO-clamps, and a less marked response to hypoglycaemia. Average sweating levels were markedly higher in the Reaction vs. Non-reaction group at nadir (p-values: hypothenar 0.04, wrist 0.003, forehead 0.001, abdomen 0.008) (Fig. 2).

Edinburgh hypoglycaemia symptom scale (EHSS). The symptom scores increased during the HYPO-clamp, with significant differences between the EU-clamp and HYPO-clamp around glucose nadir (Fig. 1). Six of 20 participants had an increase (≥ 4 , see statistical methods) in symptom scores, and of those, four reported having a hypoglycaemic response during the HYPO-clamp, and one reported having a response that

Figure 1. Plasma glucose (PG), mean (whole line) and 95% CI (colored area) for hypoglycaemic clamp (HYPO) (red) and euglycaemic clamp (EU) (blue). All x-axes have nadir PG at zero minutes; other values are minutes relative to zero. Red and blue boxes on the time axis for the different parameters denote significantly ($p < 0.05$) higher values in the HYPO-clamp (red) or in the EU-clamp (blue). Red dotted vertical lines denote the time frame for the hypoglycaemic part (below 4 mmol/L) of the hypoglycaemic clamp, and black dotted vertical line denotes PG nadir.

could imply high as well as low PG (these five participants constitute the “Reaction group”). None of the remaining 14 participants were aware that their glucose levels were low during the hypoglycaemic clamp.

Autonomic symptoms were the most commonly reported symptoms in the EHSS^{11–13}, (see Table 3).

Counterregulatory hormones. Figure 1 displays the significant differences between the EU-clamps and the HYPO-clamps for each measurement. At PG nadir, all hormones except cortisol were elevated compared to the corresponding time in the EU-clamp. In the Reaction group vs. the Non-reaction group, adrenaline ($p < 0.001$), growth hormone (GH) ($p = 0.033$) and cortisol ($p = 0.012$) increased significantly at PG nadir (Fig. 2).

ECG and skin temperature. Heart rate (HR) during hypoglycaemia was significantly increased around PG nadir (Fig. 1). During two short periods after PG nadir, HR during HYPO-clamp was significantly slower than at the corresponding time during the EU-clamp. During the HYPO-clamp, the mean QTc increased significantly from about 5 minutes before PG nadir to 20 minutes after nadir (Fig. 1), and this QTc-increase was positively correlated with the increase in adrenaline, ($p = 0.04$, $n = 20$). During hypoglycaemia, HR and QTc increased in the Reaction group compared to the Non-reaction group (Fig. 2), but this increase was not statistically significant at nadir (HR $p = 0.07$ and QTc $p = 0.05$). There were no significant differences in skin temperature between EU- and HYPO-clamps.

Combining non-invasive sensors. We explored several different combinations of measurements for hypoglycaemia detection. We found that the combination of sweating at the wrist and abdomen together with HR and QTc provided optimal detection of hypoglycaemia: Skin temperature, forehead sweating and hypothenar sweating did not improve the performance of the predictor. Figure 3 shows this predictor applied to two participants, one from the Reaction group, and one from the Non-reaction group. We also investigated a more practical

Figure 2. Hormonal and symptomatic responses during HYPO-clamp (red) and EU-clamp (blue). Red and blue lines: Medians (or means, if applicable). Colored areas: 95% CI. Black lines: medians or means for the Reaction group during HYPO-clamps. Dashed black lines: medians or means for the Non-reaction group during HYPO-clamps. Time = 0 is marked with a vertical dashed line. Dashed boxes circumscribe related graphs (blood tests, sweat measurements and ECG-data).

Edinburgh Symptom Score	All participants (N = 20)	Reaction group (N = 5)	Non-reaction group (N = 15)	p-value, difference between Reaction and Non-reaction group
Overall	6	5	1	<0.001
Autonomic	4	4	0	0.001
Neuroglycopenic	2	2	0	0.05
Non-specific	1	1	0	0.25

Table 3. Number of participants with increase (≥ 4) in Edinburgh symptom score.

predictor based on a sensor combination of only wrist sweating and HR, possible to measure by a wrist-watch type of sensor device. We used receiver operating characteristic (ROC) curves for the two predictors, applied to three groups; all participants, the Reaction group and the Non-reaction group (Fig. 4). In our Reaction group, we observed an area under the curve for the ROC (AUROC) of 1.0 for the first predictor, and 0.94 for the second. Corresponding AUROCs for the Non-reaction group were 0.65 and 0.60, and for all participants 0.75 and 0.71, respectively.

Discussion

In this study, we have investigated symptoms and hormonal responses to insulin-induced hypoglycaemia in people with IAH and combined these data with data from non-invasive sensors, with the aim to evaluate the potential for detection of hypoglycaemia. For all participants with IAH, no combination of measurements did accurately distinguish between hypoglycaemia and euglycaemia. However, in the participants who had some remaining symptoms of hypoglycaemia (the “Reaction group”), a combination of parameters from sensors could reliably identify hypoglycaemia. The hypoglycaemia symptoms in these participants were predominantly autonomic in origin, and they had higher levels of circulating adrenaline during hypoglycaemia (as compared to the “Non-reaction group”), indicating a more pronounced autonomic response¹⁴.

Figure 3. Two examples of sensor recordings during hypoglycaemic trials, showing one case from the Reaction group (upper graph) and one case from the Non-reaction group (lower graph). PG above for comparison. Sweating activity is shown in brown (hypothénar), orange (forehead), yellow (abdomen) and green (wrist) lines. Red line: HR. Purple line: QTc. Black line: combined parameter (unitless).

We found an increase in HR and QTc during hypoglycaemia (as previously reported^{15–18}), and a sweat response in all four sites almost simultaneously. While sweat responses and HR attenuated quite fast, the increase in QTc persisted until 30 minutes after glucose nadir. As to hypothénar sweat and HR, there was an opposite effect (more sweating in the EU- clamp vs. HYPO-clamp) 20 minutes after nadir, indicating a possible increased vagal activity on top of an ongoing sympathetic response, with a possible increased risk of cardiac dysrhythmias¹⁷.

The strengths of this study include the extensive data collection during the two clamps (PG, counterregulatory hormones, symptom scores, ECG, sweating and temperature) that allowed comparison of the subjective experience of hypoglycaemia and the physiological responses over an extended period of time. The use of single blinding and randomized order of hypoglycaemic and euglycaemic clamp may imply high validity of the collected data.

Sejling *et al.*^{19,20} have previously investigated people with IAH during hypoglycaemic clamp, and compared their responses with people with normal hypoglycaemia awareness. Sweating and ECG-changes were not assessed. To our knowledge, there are no previous reports of ECG- findings during hypoglycaemic clamp in people with IAH, and the present ECG-findings are of particular importance as they may corroborate the risk of cardiac dysrhythmias during hypoglycaemia also in people with IAH^{15,17}.

We could not detect a significant change in temperature between EU- and HYPO-clamps or between the Reaction group and Non-reaction group during HYPO-clamps. Sejling *et al.* found that there is a decrement in skin temperature during hypoglycaemia, and that this decrement is less in IAH patients²⁰. Circumstances that could affect a participant's temperature include the hand/arm-warmer, placement of the sensor and the ambient

Figure 4. (a) ROC-plot for a combined parameter including HR, QTc, wrist sweat and abdominal sweat. (b) ROC-plot for a combined parameter of HR and wrist sweat.

temperature in the room. The warming unit held 37°C during the whole experiment, and room temperature was not strictly controlled. We aimed at keeping the participant comfortable, i.e. if she/he felt cold they were provided a blanket, or if she/he felt warm, we could open a window in another part of the room. Because this is how people would normally adjust their comfort temperature, it is more similar to a situation in which a sensor system would normally be used. Also, we gathered that this was the most sensible way to exclude any influence from the warming unit, as long as the participant felt that she/he had a comfortable temperature, the warming unit would not change overall body temperature. It also should be noted that the warming unit and the temperature sensor were not attached to the same arm. Since the fall in temperature during hypoglycaemia is likely adrenaline related, the fact that most of the participants did not have a very marked hypoglycaemic reaction (and a poor adrenaline response) may be an explanation to our measurements. However, we did not find a significant difference between the Reaction group and Non-Reaction group either, but as can be seen in Fig. 2 there is a downward trend in the Reaction group from nadir and onwards during HYPO-clamps. A longer clamp procedure or a more pronounced hypoglycaemia might have triggered a stronger response, or larger groups for comparison might have shown significant results.

Three participants experienced low PG the night prior to the HYPO clamp and this may have blunted their autonomic responses during the subsequent clamp. However, this mild hypoglycaemia (3.4 mmol/L at the lowest in two participants) was quickly normalized. Since the acute autonomic response to hypoglycaemia has been reported to occur at lower PG levels in people with IAH than in people with normal awareness^{10,21} and at substantially lower levels than in the present trial, we find it unlikely that a short period with PG ~ 3.5 mmol/L may have influenced the present results.

Our study participants were supine during the clamps, and as previously reported²², this may have reduced their ability to detect hypoglycaemic symptoms. But since they were aware that they hypoglycaemia could be induced, they could also have been more attentive to symptoms. Attenuation of hypoglycaemia symptoms by a high insulin level has also been proposed²³. However, the serum insulin levels in our participants ranged between 461 and 896 pmol/L during clamps, and these levels are less likely to affect symptom detection.

The IAH classification was done using two different methods, the Clarke score⁷ and the Gold score⁸, which have been validated and show good concordance in people with type 1 diabetes²⁴. However, five persons with IAH were able to identify the hypoglycaemic clamp, although the symptom scores are remarkable similar between the groups (Table 1). This may reflect the dynamic nature of the IAH syndrome²⁵, or it may be interpreted as a weakness of the self-report questionnaires, implying a possibility for misclassification. In that respect, our “Reaction group” may have been misclassified as IAH. Furthermore, avoidance of hypoglycaemia for 48 hours might have improved symptom responses²⁶.

It could be argued that PG was not lowered sufficiently to elicit responses in people with IAH. However, as our aim was to explore if we could measure small physiological responses that were not obvious to the patient, eliciting strong sympathetic responses (which would immediately be recognized by the patients) would actually be contrary to our aim. We decided that if we could detect physiological responses at 2.5 mmol/L, there would still be ample time to correct the situation. However, mean nadir PG was actually 2.3 mmol/L in our study, and the Reaction group had higher nadir PG of 2.5 mmol/L (Table 2), which excludes the possibility that the Reaction group had sympathetic activation because of lower PG nadir.

It could be questioned if we should have a group with normal awareness in our study. Ideally, a group with normal awareness should be included if the aim was to look at the differences between groups with or without a

hypoglycaemic reaction during hypoglycaemia. This was not our original objective since we did not expect typical hypoglycaemic reactions in participants with IAH at this PG level. Still, when some of them had a reaction, the differences from the Non-reaction group were so obvious that we found it appropriate to include it in our findings. The two groups were not matched, but no significant differences in group characteristics were found, and our results would therefore not be biased by baseline differences.

A hypoglycaemic clamp may be called unphysiological and not a good approximation to a hypoglycaemic episode under normal conditions. We tried to emulate a normal hypoglycaemic episode by choosing to do a clamp with steadily falling PG with a rate of decline that can be observed in CGM monitoring, as opposed to a stepped clamp. But a laboratory setting is naturally quite far from a daily life episode, but to do a clamp procedure with frequent blood sampling there is unfortunately no good alternative. Like all research in the field of non-invasive glucose measurement, the biggest hurdle is real life testing.

There have been previous attempts to develop a non-invasive hypoglycaemia alarm. The Sleep Sentry[®] is a watch-like hypoglycaemia alarm based on sweat detection that was developed in the 1980's, and is one of two commercially available alarms today (another device called HSA-01 is available in the UK, and seem to function in the same way). The now retracted HypoMon^{®27,28} was another hypoglycaemia alarm, which used several ECG-features to identify hypoglycaemia in adolescents and young adults. A pilot study from Schechter *et al.*⁹ assessed heart rate, sweating (one site only), skin temperature and tremor to detect spontaneous nocturnal hypoglycaemic episodes in 10 adolescents with type 1 diabetes. Compared to these studies, we have a larger array of measurements to assess sympathetic output. The Sleep Sentry only utilizes a single sweat sensor, the HypoMon[®] only ECG-features, and Schechter *et al.* used only one sweat site and only HR derived from the ECG. We also assessed counterregulatory hormones and hypoglycaemic symptoms in order to better detect hypoglycaemia and investigate whether our measures could detect hypoglycaemia more effectively than patients themselves.

None of the previously developed alarm systems are in widespread use. While some may find the Sleep Sentry[®] useful, it has poor sensitivity and specificity²⁹. Similarly, the HypoMon[®] failed because of low sensitivity and specificity under real world conditions. The pilot study by Schechter *et al.*⁹ reported good results (sensitivity 100%, specificity 85.7%) in adolescents with normal hypoglycaemia awareness who woke up when spontaneous hypoglycaemia occurred. We have investigated people with IAH that are more likely to not perceive hypoglycaemia, and probably would not wake up during such episodes. Our combined assessments could not detect all episodes of hypoglycaemia, but we were able to look at differences in sympathetic responses in the participants with hypoglycaemia symptoms (Reaction group) and those who could not self-detect hypoglycaemia (Non-reaction group). The present findings are important when evaluating alarm systems based on a sympathetic response, since IAH may be present in up to one in four of diabetes patients^{4–6}. Even though alarm systems such as HypoMon[®] may show good results when tested in a laboratory setting in people with normal hypoglycaemia awareness, similar alarm systems may fail as commercial products if it is not recognized that performance could vary substantially from one individual to another.

Is it impossible to construct a non-invasive sensor system for hypoglycaemia detection? According to our findings, it seems to be difficult for people with a more persistent IAH. In our Non-reaction group, the combined parameters did not perform well enough for clinical use. However, in our Reaction group, the performance of the sensors was excellent. It should be noted that this does not mean that the sensors are completely unable to detect hypoglycaemia in the Non-reaction group, but they could not detect it at this BG level (2.5 mmol/L). Most probably, all participants would have reactions at lower BG levels, but at 2.5 mmol/L it is likely that corrective action can be effected before the patient is unable to act on it due to neuroglycopenia. From Fig. 3 it is evident that setting a cut-off level for the hypoglycaemia detection combined parameter for all participants is very challenging, in light of the differences between individuals with and without a symptomatic (i.e. sympathetic) response. The Clarke and Gold scores were not different in the Reaction vs. the Non-reaction groups, precluding that such scores could be of use to identify people who might benefit from non-invasive sensors. Therefore, the use of combined measurements to detect hypoglycaemia, as in the present study, is probably most useful for people with type 1 diabetes and normal hypoglycaemia awareness, either as an added security, or in situations where they might have reduced alertness, e.g. during sleep.

Several additional challenges may arise if such sensors are to be used as an everyday hypoglycaemia alarm. Sweating, HR and QTc have physiological fluctuations during the day, e.g. during exercise. A wristband assessing sweating and HR might be the most feasible solution and has nearly as good performance as our combined parameter (AUROC 0.94 vs. 1.0 in the Reaction group).

In conclusion, in the present hypoglycaemic clamp study, several ECG parameters, sweating at four sites, counterregulatory hormones and hypoglycaemic symptoms have been simultaneously assessed in people with type 1 diabetes and IAH. We have demonstrated a difference in responses between participants who did and did not recognize hypoglycaemia. Our findings suggest that a sympathetic response with adequate adrenaline output is necessary for the detection of hypoglycaemia with ECG and sweat measurements, and that the lower PG threshold for autonomic activation in people with IAH also alters the response threshold of sensors detecting the autonomic signals. More advanced modelling along with other non-invasive sensors based on electrical or optical properties of skin to detect trends in PG may improve assessment also in people with IAH, and should be subject to further research^{30,31}.

Methods

Participants. Twenty participants (12 women) aged 18–60 years with type 1 diabetes and IAH were recruited from the outpatient population of Oslo University Hospital, the Norwegian Diabetes Centre, and the Norwegian Diabetes Association. IAH was assessed using the Clarke⁷ and Gold⁸ questionnaires. In the Clarke questionnaire eight questions characterize the participants' exposure to episodes of moderate and severe hypoglycaemia and assess the glycaemic threshold for, and symptomatic responses to, hypoglycaemia. The Gold questionnaire asks:

“Do you know when your hypos are commencing?” The respondents selected a number on a 7-point Likert scale, with 1 representing “always aware” and 7 representing “never aware”. For both tests, a score of ≥ 4 implies IAH. To be included in the study, the participant should score ≥ 3 in both tests and ≥ 4 in at least one of them.

People with heart, lung or kidney disease, hypertension, previous seizures or any condition that could increase the risk of the procedure or influence the hypoglycaemic responses were excluded from participation (see Supplemental Material, Note 1). All participants attended an information meeting and gave written informed consent prior to any investigational procedure. The study was approved by the south-eastern review board (REC South East) of the Regional committees for medical and health research ethics in Norway, and the study was performed in accordance to this approval (approval reference: REK 2013/813).

Experimental procedures. Each participant had one EU-clamp and one HYPO-clamp performed in random order, and procedures were separated by at least two weeks. Participants were requested to perform frequent self-monitoring to avoid PG values of < 4.0 mmol/L 48 hours prior to each clamp^{32–34}, and to abstain from alcohol and strenuous physical exercise. Only rapid-acting insulin was allowed 24 hours before each experiment. Participants were hospitalized the evening preceding the clamp, and fasted from 10 pm. Rapid acting insulin was infused intravenously at a variable rate during the night according to an algorithm targeting fasting PG 7–8 mmol/L. Participants were randomly allocated to EU-clamp or HYPO-clamp as first procedure, and blinded as to which type of clamp that was carried out.

A fixed dose of insulin (NovoRapid, NovoNordisk, Denmark) 1.5 mU/kg/min was infused, and glucose 200 mg/mL was adjusted every 5 minutes to maintain PG around 5.3 mmol/L (EU-clamp) or to lower PG gradually to 2.5 mmol/L (HYPO-clamp). The goal was to keep PG at 2.5 ± 0.2 mmol/L for 15 minutes before more glucose was administered to raise PG to euglycaemia. For ethical reasons regarding the safety of our participants, we did not want to reduce PG further. At predefined time intervals (EU-clamp) or predefined PG levels (HYPO-clamp) arterialized venous blood samples³⁵ were obtained for measurement of adrenaline, noradrenaline, glucagon, GH, cortisol and potassium. For additional details see Supplemental Material Note 2. At the same predefined time intervals participants scored hypoglycaemic symptoms on a Likert scale from 1 (‘not present’) to 7 (‘present a great deal’) using an expanded version of the EHSS, which has been translated into Norwegian^{4,11,12}. Insulin sensitivity was measured as GIR (mg/kg/min) during the last 30 min of the EU-clamp.

Non-invasive monitoring of physiological measurements. A basic figure of the setup can be found in online Supplemental Material Fig. 1. Sweating on the palm (hypothenar eminence), on the palmar side of the wrist, forehead and abdomen (level of T9 dermatome 2 cm above and to the right of the umbilicus) were assessed with a Sudologger (Biogauge AS, Stabekk, Norway) measuring skin alternating conductance. These measurements were converted into the frequency of skin conductance responses (FSR) reflecting the activation level of the sympathetic nervous system on the specific dermatome³⁶. HR and QT-interval were assessed by continuous recording of 3-lead ECG in the lead II configuration, as a substitute for the mean QT-interval from a 12-lead recording³⁷. Bazett’s correction formula was used to standardize QT-interval in relation to HR (QTc)³⁸. The ECG was recorded using a SC9000XL ECG monitor (Siemens Medical Systems, Danvers, Ma, USA), digitized at 300 samples per second using a NI USB 6009 data acquisition device (National Instruments, Austin, Tx, USA) and processed in real time by a custom-made LabVIEW program (National Instruments).

Skin temperature was assessed every five minutes with the SC9000XL using a Siemens Drager 5204669 temperature probe taped to the left shoulder, not covered by clothing. The setup was found compliant with Annex VIII and X of the Medical Devices Directive 93/42/EEC by a committee at Oslo University Hospital, and approved by the Norwegian foundation for testing and approving of electrical equipment (NEMKO).

Analytical methods. Glucose was measured in whole blood every five minutes using an YSI 2300 STAT Plus glucose analyzer (YSI Life Sciences, Ohio, United States), and was converted to PG by multiplying with a conversion factor based on each participant’s hematocrit level. Blood samples for catecholamine analyses were collected in vacutainer tubes treated with ethylene glycol tetraacetic acid and glutathione from Sigma–Aldrich (St Louis, MO, USA) and placed on ice. Plasma was separated by centrifugation (3000 rpm, 15 min, 4 °C) and frozen at -80 °C until assayed. Analyses were done using HPLC (Agilent Technologies, Santa Clara, CA, USA) with a reversed-phase C-18 column (Chromsystems, München, Germany) and electrochemical detection (Antec, Leyden Deacade II SCC, Zoeterwoude, The Netherlands) using a commercial kit from Chromsystems. The intra- and inter-assay coefficient of variation (CV) were 3.9% and 10.8% respectively^{39,40}. All samples from an individual participant were measured in one run to minimize the interassay variability. Glucagon was analyzed with radioimmunoassay (RIA), CV 7–10% (GL-32K, Millipore corporation, Billerica, Ma, USA)³. Due to a change in analytical methods at the Hormone Laboratory, Oslo, Norway, GH of the first five participants were analyzed using immunofluorometric assays (DELFI) from Perkin Elmer Life Sciences (Wallac Oy, Turku, Finland), and the remaining 15 were analyzed using a non-competitive immunolumonometric assay, CV 6–7% (Immulite 2000, Siemens Healthcare, Llanberis, UK). Cortisol was analysed using a luminescence immunoassay, CV 7% (Immulite 2000, Siemens Healthcare, Llanberis, UK). Potassium was analyzed using an ion selective electrode principle, CV 1–2% (MODULAR, Roche Diagnostics, Indianapolis, USA).

Statistical methods. For sufficient power, 20 participants were recruited for the study (for details, see online Supplemental Material, Note 3).

The duration of individual clamps varied. We chose a common time span for both clamps based on the HYPO-clamps, spanning from 90 minutes before and 50 minutes after the individual nadirs, hence aligning all participants so that all individual nadirs occurred at time = 0. When EU-clamps were performed after

HYPO-clamps, measurements were taken at the same time points. In participants who underwent EU-clamp before HYPO-clamp, blood sampling was not always performed at similar clamp duration, and linear interpolation was used to obtain intermediate values between measurements. Then, individual EU-clamps were aligned with corresponding HYPO-clamps so that the starting time was the same, and time points could be compared directly.

For each of the 13 physiological measurements and the symptom score (EHSS), the 20 curve pairs (one EU and one HYPO) were compared pointwise (using interpolated points where needed) over the time range, using paired t-tests for normally distributed differences and the Wilcoxon Signed Rank test upon violation of the normality assumption. The bootstrap method was used to determine corresponding means or medians with pointwise confidence intervals, using the *bootci* function (using the bias corrected and accelerated percentiles method) in Matlab R2016b.

A significant symptomatic response to hypoglycaemia was defined as a score ≥ 4 higher than the highest score during euglycaemia, (including the two first scores during HYPO-clamp). This would change the score for one symptom from 1 (no symptom) to 5 (in the upper half of the score), or increase the score for two or more symptoms concurrently. For the difference between groups in number of participants with a significant symptomatic response, the Fisher exact test was used (Table 3).

When comparing the Reaction group and the Non-reaction group, we used time point zero of the HYPO-clamp (nadir) only, and tested for difference in all physiological measurements and symptom score. We used the independent samples t-test under the normality assumption and the Wilcoxon Rank Sum test upon violation of the normality assumption. The same tests were used for subject characteristics.

The associations between QTc and adrenaline were assessed by the Pearson product-moment correlation coefficient.

In order to test the ability to detect hypoglycaemia, a composite parameter for the combination of non-invasive sensor measurements was calculated based on a method we have reported previously³⁶. ROC analyses were done for the composite parameter value with a variable detection threshold and the corresponding PG level (above or below 4.0 mmol/L) for different combinations of sensor measurements, comparing their detection ability by AUROC. The recordings were split into three euglycaemic periods, (the two periods before and after hypoglycaemia during HYPO-clamp, and one period for the EU-clamp), and one hypoglycaemic period (PG < 4.0 mmol/L in the HYPO-clamp). Detection criteria were defined as follows: A true positive event was registered if at least one (composite parameter) value above the detection threshold within the hypoglycaemic period. A true negative was registered if all values were below the detection threshold for each of the euglycaemic periods. A false positive was registered if at least one value was above the detection threshold in the EU-clamp, and for at least one unique response (not extending into or coming out of the hypoglycaemic period) above the detection threshold during the euglycaemic periods of the HYPO-clamp. A false negative was registered for no values above the detection threshold during the hypoglycaemic period.

Data Availability

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

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Acknowledgements

All the individuals who gave of their time and endured through two lengthy investigational days. Åse Halsne, Gøril Vinje, Karin Eide and Gro Boezelijn, nurses at the Diabetes laboratory, Oslo University Hospital, for assistance during clamp procedures. The nursing staff at the Department of medicine, Rikshospitalet, Oslo University Hospital, for taking care of participants in the study and monitoring their plasma glucose levels during the night before trials. Doctors and nurses at the Outpatient clinic for endocrinology, Oslo University Hospital, and in particular Kirubalini J. Moorthi for help with recruiting patients. Doctors and nurses at the Norwegian Diabetics Centre for help with recruiting patients. Georg Elvebakk, associate professor at Department for mathematics and statistics, Faculty for Science and Technology, The Arctic University of Norway, for additional advice on statistics.

Author Contributions

Ole Elvebakk. Contributed to planning of the study. Responsibility for recruiting and follow-up of study participants. Oversaw all procedures and collected some of the data. Researched and interpreted data. Wrote the paper. Approved final manuscript. Christian Tronstad. Contributed to planning of the study. Responsibility for sensors and collecting of data from sensors during clamps. Researched and interpreted data. Reviewed and edited manuscript. Approved final manuscript. Kåre I. Birkeland. Contributed to planning of the study. Interpreted data. Reviewed and edited manuscript. Approved final manuscript. Trond G. Jenssen. Contributed to planning of the study. Interpreted data. Reviewed and edited manuscript. Approved final manuscript. Marit R.R. Bjorgaas. Contributed to planning of the study. Interpreted data. Reviewed and edited manuscript. Approved final manuscript. Kathrine F. Frøslie. Statistical supervision. Interpreted data. Reviewed and edited manuscript. Approved final manuscript. Kristin Godang. Analysed and interpreted results regarding adrenaline and noradrenaline tests. Approved final manuscript. Håvard Kalvøy. Contributed to planning of the study. Contributed to collection of data. Reviewed and edited manuscript. Approved final manuscript. Ørjan G. Martinsen. Contributed to planning of the study. Interpreted data. Reviewed and edited manuscript. Approved

final manuscript. Hanne L. Gulseth. Contributed to planning of the study. Main medical responsibility during clamps. Interpreted data. Reviewed and edited manuscript. Approved final manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-33189-1>.

Competing Interests: Ø. Martinsen, C. Tronstad, O. Elvebakk, H. Kalvøy, M. Bjørgaas, T. Jenssen, K. Birkeland and H. Gulseth are participating in a project with Prediktor Medical Inc. aiming to develop a commercial non-invasive glucose sensor. For Kathrine F. Frøslie and Kristin Godang no competing interest exists.

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Non-invasive prediction of blood glucose trends during hypoglycemia

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HIGHLIGHTS

- First study to assess NIR + bioimpedance for prediction of hypoglycemic glucose trends.
- Prediction of trends could be useful in hypoglycemia detection.
- Glucose level prediction was not sufficiently accurate for clinical use.
- Neural network performed better than partial least squares as prediction model.
- Bioimpedance seems to correct for individual variations in NIR-glucose relation.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:

Received 29 May 2018

Received in revised form

30 November 2018

Accepted 7 December 2018

Available online 15 December 2018

Keywords:

Non invasive
Glucose
Hypoglycemia
Multisensor
Machine learning
Neural network

ABSTRACT

Over the last four decades, there has been a pursuit for a non-invasive solution for glucose measurement, but there is not yet any viable product released. Of the many sensor modalities tried, the combination of electrical and optical measurement is among the most promising for continuous measurements. Although non-invasive prediction of exact glucose levels may seem futile, prediction of their trends may be useful for certain applications. Hypoglycemia is the most serious of the acute complications in type-1 diabetes highlighting the need for a reliable alarm, but little is known about the performance of this technology in predicting hypoglycemic glucose levels and associated trends. We aimed to assess such performance on the way to develop a multisensor system for detection of hypoglycemia, based on near-infrared (NIR), bioimpedance and skin temperature measurements taken during hypoglycemic and euglycemic glucose clamps in 20 subjects with type-1 diabetes. Performance of blood glucose prediction was assessed by global partial least squares and neural network regression models using repeated double cross-validation. Best trend prediction was obtained by including all measurements in a neural network model. Prediction of glucose level was inaccurate for threshold-based detection of hypoglycemia, but the trend predictions may provide useful information in a multisensor system. Comparing NIR and

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bioimpedance measurements, NIR seems to be the main predictor of blood glucose while bioimpedance may act as correction for individual confounding properties.

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1. Introduction

Since there is yet no cure for type 1 diabetes, good glycemic control is essential for reducing the risk of serious long term complications such as cardiovascular diseases, stroke, blindness, kidney failure, nervous system diseases and amputations. In striving for tight glycemic control, an important and dangerous acute complication is severe hypoglycemia (when a patient needs assistance to correct their blood glucose), which can lead to unconsciousness and even provoke myocardial ischemia or cardiac arrhythmia [11]. Normally, blood glucose fluctuates between 4.0 and 11.0 mmol/L (euglycemia), while values above or below these levels are defined as hyper- or hypo-glycemic respectively. Monitoring of blood or tissue glucose may prevent this, and the technological developments now provide small, fast and accurate devices for point-measurements, and also well-performing continuous glucose measurement systems allowing tracking and trend estimates throughout the day. These technologies are however still invasive, requiring either a small blood sample from finger piercing or the insertion of a small sensor subcutaneously. Hence, a lot of effort has been put into research and development in non-invasive prediction of blood glucose over the last decades, but a successful device is yet to be developed.

Although accurate non-invasive measurements still seem out of reach, published results indicate that decent prediction of trends seems feasible [8,9], which could be of value to detect impending hypoglycemia. However, little is known about the accuracy in predicting blood glucose trends during hypoglycemia by the use of non-invasive measurement. Until non-invasive glucose prediction is possible with clinically necessary accuracy, the more feasible concept of only detecting hypoglycemia may be of great value to patients with poor glucose regulation and impaired awareness of hypoglycemia.

This study is part of a project where the goal is to develop a multi-sensor-based system for non-invasive detection of hypoglycemia in patients with Type-1 diabetes and impaired awareness of hypoglycemia. As opposed to most other non-invasive methods, our approach is not a direct prediction of the blood glucose value, but detection of hypoglycemia by a combination of sensors picking up related physiological responses. These responses may still be detectable by sensors although the responses are attenuated in these patients to the extent that they are usually not noticed as symptoms. The physiological responses to hypoglycemia, such as sweating and increased heart rate [23] are not specific enough alone to serve as warnings of hypoglycemia. Thus, such measurements may be highly sensitive, but not very specific. Prediction of trends in blood glucose based on non-invasive methods such as NIR and bioimpedance spectroscopy may complement the hypoglycemia detection by providing an independent source of relevant information, possibly improving the specificity of detection. Based on previous studies, it is known that obtaining accurate non-invasive predictions of blood glucose is challenging and few studies have focused on non-invasive predictions in the hypoglycemic range. It seems however that a good prediction of trends (as opposed to the exact level of blood glucose) is feasible, and is likely beneficial. First, the performance of the non-invasive prediction of blood glucose trends during hypoglycemia must be

evaluated distinctly, which is a short-term goal of the project and the aim of this study.

Spectroscopic techniques and multivariate chemometric analysis have been widely used in medical and biological research and applications such as breast cancer risk forecasting [6], coronary artery disease [31], malignant melanoma [43] and metabolomics [33]. Among the types of spectroscopic techniques most widely used are nuclear magnetic resonance (NMR) spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), Raman spectroscopy, near-infrared (NIR) spectroscopy and bioimpedance spectroscopy, utilizing different physical mechanisms (i.e. nuclear spin in NMR, molecular absorption of light in NIR, ionic admittance in bioimpedance) in order to extract relevant information from the biological sample. With respect to diabetes and blood glucose, many methods involving different physical properties have been investigated for non-invasive blood glucose prediction over the last four decades. These include optical methods such as light absorption spectroscopy in the visible to NIR range, Raman spectroscopy, ultrasound, bioimpedance and reverse iontophoresis among others. For a comprehensive review, see Refs. [35,41], [37]. Reviewing the literature, it seems that the most tested and promising methods rely on the NIR spectroscopy [16] especially) and electrical bioimpedance [9,34], and these methods were therefore selected for this study. The rationale behind using NIR spectroscopy is based on the direct absorption of light at specific wavelengths by the glucose molecule due to its molecular structure. Catching the glucose signal in the spectrum is however not easy, as the signal variation due to the change in glucose concentration is very small compared to background absorption by water, other constituents in the blood overlapping in the absorption spectrum, and the scattering of light when traveling through the skin. Impressive results have been reported in small laboratory studies (see for example [16], but in spite of almost 20 years of research, a reliable and accurate non-invasive NIR glucose monitoring device is yet to be developed [41]. Much less is known about the relation between bioimpedance and blood glucose, except that investigators have reported an association both in vitro [21,28], and that bioimpedance has been used as a predictor together with optical measurement in vivo [7,19,42], improving the performance in comparison to using optical measurement alone [7]. It is thought that the glucose-related changes in bioimpedance is due to variations in the Na⁺ and K⁺ concentrations with plasma glucose concentration, which changes the membrane potential of the red blood cells and thereby the dielectric characteristics of blood [4,41]. Another reason for the improvement in prediction could be that bioimpedance measurement provides information on individual confounding tissue properties which influence the optical measurement, such as tissue perfusion or skin hydration, thereby producing a better global prediction by including bioimpedance in the model.

The aim of this study was to assess the performance of NIR and bioimpedance spectroscopy for non-invasive prediction of trends in blood glucose during hypoglycemia, and assess the feasibility of hypoglycemia detection based on this method. To the knowledge of the authors, this is the first study to assess the performance of this sensor combination in prediction of blood glucose levels and trends during hypoglycemia.

2. Methods

Twenty subjects with type 1 diabetes in the age group 18–60 years with impaired awareness of hypoglycemia [18] were recruited by their treating physicians at Oslo University Hospital. Subjects with other serious, chronic illnesses, or who used medication that could influence blood tests or other measurements were excluded from the study. The regional ethics committee approved the study (REK 2013/813).

Subjects were instructed to avoid hypoglycemic periods 48 h before the studies, and were hospitalized the evening before each study day for optimal glucose regulation and monitoring through the night. They fasted from 10 p.m. During tests, blood glucose (BG) was controlled using a constant insulin infusion based on weight, and a variable glucose infusion to clamp blood glucose on a preset level. BG was measured every 5 min from arterialized venous full blood using a YSI 2300 STAT Plus glucose analyzer (YSI Life Sciences, Ohio, United States) and converted to plasma glucose values, based on individual hematocrit levels. Each subject participated at two test days, one with a hypoglycemic and one with a euglycemic clamp, with approximately two weeks interval and in random order. Subjects were not told which clamp procedure that should be performed until the end of the second test day. During the euglycemic clamps, BG was clamped at 5.3 mmol/L. During hypoglycemic clamps, BG was gradually lowered to 2.5 mmol/L and then increased back to the euglycemic range. This experimental setup is illustrated in Fig. 1. Non-invasive sensors (described below) were attached to the body at least 5 min (median 20 min) before the start of the clamp.

2.1. Bioimpedance measurement

Based on previous research [19], bioimpedance was measured in the MHz range (1–200 MHz) using a VIA Echo 2500 vector impedance analyzer (AEA Technology, USA). Using a two-electrode configuration, bioimpedance was measured at the palmar side of the forearm with a spacing of approximately 8 cm between the center of the electrodes. Commercial wet-gel ECG electrodes of the type BlueSensor™ Q00A (Ambu A/S, Denmark) were used in order to maintain stable electrode contact and reduced contribution from the stratum corneum impedance. The electrodes were attached at least 10 min before the first measurement was recorded in order to allow the gel reaction on the skin stabilize and hence avoid impedance drift Tronstad et al., 2010. The impedance analyzer was

calibrated before the beginning of the experiment. During the whole experiment, one frequency sweep measurement was recorded every 5 min at the same time as the blood samples were obtained for blood glucose measurement.

2.2. NIR measurement

NIR absorbance in the 880–2200 nm range was measured using a Spektron® (Prediktor AS) together with a tungsten halogen light source (LS-1 model, Ocean Optics) and a custom-made probe for stable contact to the skin. The probe consisted of a black block of plastic designed for being strapped to the arm, having 6 illumination optical fibers surrounding a central pickup fiber. The distance between pickup and illumination fibers was 0.2 mm. During the whole experiment, a spectrum of 256 channels over the whole frequency range was recorded with a sampling rate of 1 spectrum per second.

2.3. Skin temperature measurement

Skin temperature was measured using a Siemens Dräger 5204669 temperature probe taped to the upper arm, not covered by clothing. The temperature value was acquired by a Siemens SC7000 monitor connected to the probe, and was recorded every 5 min at the same time as the blood was drawn for the blood glucose measurement.

2.4. Preprocessing

The preprocessing of the NIR spectra was based on conventional methods frequently used in chemometrics (normalization, centering, and the spectral derivative), with particular details selected according to the experimental design and model validation procedure. More on preprocessing techniques for spectroscopic data can be found for example in Ref. [30]. The NIR spectra were first averaged in 30-s blocks (for noise reduction) before picking each measurement that coincided with the times of the blood samples for BG measurement. In order to subtract systematic noise, inside centering was employed separately on the data within each experiment as in Ref. [14]. For these data from each experiment separately, row normalization (dividing all values in each row by the row sum) was first applied followed by column centering. This was followed by lowpass-filtering (3rd order Butterworth filter with a 0.15 Nyquist rate) and a first order row differentiation (over

Fig. 1. Illustration of the experimental setup and the clamping procedure. Blood samples were taken from a cannula in the right arm, and the blood glucose was regulated by a constant insulin (I) and variable glucose (G) infusion rate to a cannula on the left arm. Skin temperature, NIR spectroscopy and bioimpedance (Z) were measured at different sites on the left arm. Each subject underwent two clamps, one hypoglycemic and one euglycemic clamp in a random order.

the wavelength channels).

The impedance magnitude and phase angle which was provided by the device was calculated into resistance and reactance (as these are separately related to the conductive and capacitive tissue properties respectively), but no further preprocessing was done to these recordings except for in a comparison where column centering was applied. The data from one experiment with erroneous impedance measurements (resistance values of approximately tenfold magnitude compared to the rest probably due to an electrode contact problem), and from one experiment with saturated NIR measurements were excluded, giving a total of 38 datasets from different experiments used in the blood glucose prediction.

The feature set was made of the preprocessed NIR (254 channels), bioimpedance (250 resistance + 250 reactance values) and skin temperature data from each experiment, adding up to a total of 1329 observations from all the experiments.

2.5. Model development

Prediction models were initially explored using partial least squares (PLS) regression and artificial neural network (ANN) regression models, testing the ability to fit such models to a set of training data and the ability to predict the blood glucose on data from other experiments. There are many variants of these methods, especially for ANN. Basic versions of both methods have been employed in this study, and a brief comparison between these approaches is provided in Table 1. For more information on ANN in general, see for example [3]; and on the use of ANN for nonlinear multivariate calibration on spectroscopic data see for example [26] or [17].

Fig. 2 shows an example of predictions on all examples by ten-fold cross-validation using a PLS model with 5 components and an ANN model with one hidden layer of 20 nodes.

The initial testing revealed that the glucose predictions tended to change mostly in one direction during the course of an experiment, following the initial trend in glucose but often failing to reflect the trends later in the experiments, in particular the upswing following the hypoglycemic excursions. As shown in Fig. 3a, models with low complexity would always show this behavior, indicative of underfitting or modeling of spurious features that drift over time along with the glucose change, but not having predictive ability. More complex PLS models would also struggle to reflect the changes in glucose trends, but ANN models would show cases of bidirectional trend agreement with the reference glucose, as shown in Figs. 2 and 3b, which we believe is a more likely sign of true glucose prediction. When the predictions change in only one direction while glucose changes bi-directionally, it is very unlikely a true prediction of glucose, even though the point-wise errors such as the root mean square error (RMSE) are small. Therefore, we chose to look for trend agreement as a property in model assessment instead of conventional performance parameters such as the RMSE. Due to the nature of the experiments, the glucose trends are mostly in the negative direction, with a flatter part during the euglycemic clamps and a shorter rising part during the hypoglycemic clamps. Because of the relatively short

rising part (skewed distribution of trends), we used a trend agreement calculation which was weighted evenly between falls (<-1 mmol/L per 20min), rises (>1 mmol/L per 20min) and flatter fluctuations in blood glucose. Trend agreement was determined by the mean of a moving two-point slope over 20 min (as used in Ref. [24]) within each experiment, after applying a noise-removal filter to better represent the relevant trends. In the example figure below, conventional performance parameters would not favor the model with best trend agreement, as they are independent on the order of the reference-prediction pairs. As shown in the example, the RMSE may increase due to larger level-variability in the more complex models that represent trends better. The same applies for the predicted residual error sum of squares (PRESS), and the R^2 shows a good improvement for increased PLS complexity, but only a slight improvement for ANN. The weighted trend agreement however, gives a substantial error reduction in favor of the model exhibiting cases of bidirectional trend agreement. Thus, we believe that selecting complexity based on the weighted trend agreement would produce the most correct model.

These observations suggested that ANN could possibly be used to develop a model for predicting trends in blood glucose, and that such performance is very dependent on the complexity of the model. In order to optimize the model complexity and try to estimate a realistic performance on new observations (within the same population), the repeated double cross validation (RDCV) strategy [15] was employed. In addition, inside mean centering was changed to centering by the first row level (first measurement in a new experiment), not using data from observations that are not yet known in a real-time setting.

2.6. Internal validation of blood glucose prediction

The repeated double cross-validation strategy was implemented as shown in Fig. 4.

Within $N = 100$ runs, the selected set of predictors and the blood glucose target was first randomly permuted according to experiment number, ensuring that there were no observations from the same experiment used in training the model and testing its performance. In an outer loop of $k = 10$ iterations, the observations were partitioned into a $(k-1)/k$ part for learning and a $1/k$ part outer test set. Using the learning set, an inner $l = 5$ -fold loop was used to cross-validate models produced with different complexity (number of hidden layer nodes or PLS components) ranging from 1 to 50, training the regression models for each complexity level. The PLS models were trained using the *training + validation* data shown in Fig. 4, but the ANN model was trained with early stopping for regularization using a $(l-2)/l$ training partition and an $1/l$ validation partition divided randomly using the scaled-conjugate gradient algorithm. The last $1/l$ partition of the learning set, the inner test set, was extracted from the learning set based on the experiment number, so that this set contained no data from the same experiments as the training and validation sets. Each of the models trained were used to make predictions based on the inner test set, and the mean inner test performance was used to select the complexity level for the current iteration of the outer loop based on the standard error method, as described in Ref. [15], using the

Table 1
Brief overview of the PLS and ANN regression methods and their main properties.

Method:	PLS regression	ANN regression
Dimensionality reduction:	Orthogonal latent variables	Input weights
Model type:	Linear combination	Multilayer perceptron
Model complexity:	Number of components	Network size (layers and nodes)

Fig. 2. Predictions on all experiments using PLS (purple) and ANN (orange) models with 5 components and 20 hidden layers, respectively, presenting the mean predictions of 10-fold cross-validation over 10 repetitions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Examples of predictions on seven validation experiments (Exp1–7) using PLS and ANN models trained on 27 experiments using low complexity (PLS components and ANN layer size = 1) shown in a) and high complexity models (PLS components and ANN layer size = 10) shown in b). Performance parameters RMSE, PRESS, R^2 and WTE (weighted trend agreement) are given as the mean over the experiments.

weighted trend agreement as an error criterion.

In the outer k -fold CV loop, the learning set was used to train models using the whole range of hidden layer sizes (1 to $S = 50$), and these models were used to generate outer test predictions for each of the complexity levels. After k iterations, these outer test predictions were concatenated into $S = 50$ predictive time-series encompassing all the experiments. After $N = 100$ runs with different permutations of the observations, a 3-dimensional matrix of $N \times S$ time-series vectors was generated. Using the $N \times k$ complexity level selections from the inner CV-loop, the most frequent value of this matrix was used as a final selection of for the model complexity, and the N outer test prediction vectors with this selection of complexity was extracted from the 3-dimensional matrix and used for the performance evaluation. The procedure was implemented in Matlab, 2016b; using functions from the Neural Network and Statistics and Machine Learning Toolboxes.

2.7. Performance evaluation

Using the N test prediction vectors together with the BG target data, selected performance measures were calculated for each run, and the mean and standard deviation over the 100 runs was used to assess the performance. Due to the skewed nature of the data, the weighted trend agreement was used as the main performance indicator in comparison between model variants. For further evaluation of trend agreement, the Rate Error Grid [25]; adapted to mmol/L units) was used to calculate percentages of rates falling within different clinically relevant zones of agreement based on the rate and direction of change. 15-minute increments were used for the rate analysis (as suggested in Ref. [25]). For visual representation, the pooled reference and mean prediction test results were plotted against each other in the grids. In addition to the trend performance measures, the Mean absolute relative deviation (MARD) and root mean square error (RMSE) was calculated, and the Clarke-error grid analysis was provided for comparison to other studies on non-invasive glucose prediction. The MARD provides a measure of the overall accuracy of glucose prediction when compared to a reference, given as a percentage of deviation from the reference (presumably true) glucose value. MARD was calculated by taking the mean of the vector of absolute values of the difference between the reference and predicted blood glucose divided by the reference blood glucose. The Clarke-error grid [12]

provides a picture of the clinically relevant accuracy of glucose predictions with respect to misclassifications within defined regions (e.g. predicting hyperglycemia when the patient has hypoglycemia, being the most adverse error). The clinically relevant agreement of the predicted blood glucose level was assessed by the percentages of predictions falling within the A to E zones of the Clarke-error grid.

The performance was compared between PLS and ANN, and for four ANN model variants. For comparison between feature selections, models using only the NIR and temperature data, only the Z and temperature data, only the NIR and Z data, were compared to the full model. For the full model, the performance was also compared between centering of the Z data and without Z data centering.

3. Results

A summary of the trend agreement and the other selected performance parameters are presented in Table 2, where different selections of model type and training method are compared. The RDCV resulted in quite different levels in complexity of the final model among the cases compared, making a direct comparison difficult. As mentioned earlier, we regard the weighted trend error as the most reliable parameter for assessing glucose prediction in this study. In addition, the amount of predictions falling into the D zone of the Clarke- or rate error grid is an indicator of predictions failing to follow hypoglycemic excursions. Comparing the final models, ANN outperformed PLS in these measures. Comparing against different partial models, removing bioimpedance or NIR from the predictors resulted in models with minimum complexity and lower errors in level prediction (e.g. MARD and RMSE), but worse trend performance. Centering of the bioimpedance predictors reduced the performance of the full model. Removing skin temperature from the full model gave a less complex model with an increase in trend error, but with minimal change in the Clarke and rate error grid D zones. The model with only bioimpedance and temperature had the worst performance among the models compared, seen in both level and trend performance parameters. In total, the full ANN model achieved the best performance of the parameters most relevant to predict glucose trends during hypoglycemia. The distribution in performance parameters over repetitions of the RDCV is indicated by the standard deviations given in

Fig. 4. The implementation of repeated double cross validation implemented in the study for training, validation of the model and assessment of the prediction performance. The diagram shows the implementation for the ANN regression case. The process is equal for PLS regression, except for the training/validation split of the learning data, which was necessary for the early stopping method in ANN training.

the table. With $N = 100$, the 95% confidence intervals of the means can easily be found by adding and subtracting one fifth ($2/\sqrt{100}$) of the standard deviation.

The training and inner test errors for the final complexity level of each model are also provided in the table for the weighted trend error and the RMSE. The training error would in most cases be

Table 2
Summary of the mean $\pm$ standard deviation of performance over the 100 RDCV runs for different model selections, showing the performance in predicting trends (slope errors and the Rate Error Grid zone percentages) and levels (MARD and Clarke-error Grid zone percentages) of blood glucose during the experiments. Abbreviations: WTE=Weighted trend error, REG = Rate error grid, MARD = Mean absolute relative deviation, RMSE = Root mean square error, CEG=Clarke-error grid.

Model variant	Features	NIR + Z + T	NIR + Z + T	NIR + Z + T	NIR + Z	NIR + T	Z + T
	Centering	NIR	NIR	NIR, Z	NIR	NIR	None
	Model type	ANN	PLS	ANN	ANN	ANN	ANN
	Complexity	13	2	1	6	1	1
Trend performance	WTE	1,04 $\pm$ 0,04	1,11 $\pm$ 0,02	1,10 $\pm$ 0,03	1,07 $\pm$ 0,04	1,07 $\pm$ 0,02	1,20 $\pm$ 0,01
	REG A + B [%]	88,3 $\pm$ 1,1	93,1 $\pm$ 0,2	88,2 $\pm$ 1,1	88,4 $\pm$ 1,2	88,4 $\pm$ 1,0	88,0 $\pm$ 0,9
	REG C [%]	4,8 $\pm$ 0,7	0,1 $\pm$ 0,0	4,1 $\pm$ 0,8	4,7 $\pm$ 0,8	4,5 $\pm$ 0,6	0,8 $\pm$ 0,6
	REG D [%]	4,8 $\pm$ 0,6	6,7 $\pm$ 0,2	5,8 $\pm$ 0,7	4,9 $\pm$ 0,5	5,3 $\pm$ 0,5	10,1 $\pm$ 0,6
	REG E [%]	2,1 $\pm$ 0,5	0,1 $\pm$ 0,0	1,9 $\pm$ 0,4	2,0 $\pm$ 0,5	1,8 $\pm$ 0,4	1,1 $\pm$ 0,6
	REG uD + uE [%]	4,6 $\pm$ 0,7	3,8 $\pm$ 0,1	5,3 $\pm$ 0,7	4,5 $\pm$ 0,6	4,9 $\pm$ 0,6	8,9 $\pm$ 0,5
	WTE train	0,72 $\pm$ 0,10	1,11 $\pm$ 0,01	1,01 $\pm$ 0,05	0,81 $\pm$ 0,08	1,00 $\pm$ 0,02	1,17 $\pm$ 0,03
	WTE inner test	1,06 $\pm$ 0,04	1,11 $\pm$ 0,01	1,10 $\pm$ 0,03	1,07 $\pm$ 0,04	1,08 $\pm$ 0,03	1,21 $\pm$ 0,02
	MARD [%]	42,5 $\pm$ 2,9	35,0 $\pm$ 0,3	34,7 $\pm$ 1,7	39,0 $\pm$ 2,7	32,7 $\pm$ 1,6	41,0 $\pm$ 2,8
	RMSE	2,64 $\pm$ 0,15	2,09 $\pm$ 0,02	2,24 $\pm$ 0,09	2,51 $\pm$ 0,15	2,09 $\pm$ 0,08	2,72 $\pm$ 0,18
Level performance	CEG A + B [%]	85,9 $\pm$ 1,3	83,5 $\pm$ 0,3	82,0 $\pm$ 0,5	85,0 $\pm$ 1,4	82,2 $\pm$ 0,4	81,6 $\pm$ 1,1
	CEG C [%]	1,6 $\pm$ 0,7	0,0 $\pm$ 0,0	0,4 $\pm$ 0,4	1,5 $\pm$ 0,6	0,4 $\pm$ 0,3	0,8 $\pm$ 0,9
	CEG D [%]	12,1 $\pm$ 1,2	16,5 $\pm$ 0,3	17,5 $\pm$ 0,4	13,1 $\pm$ 1,3	17,3 $\pm$ 0,3	17,2 $\pm$ 1,3
	CEG E [%]	0,4 $\pm$ 0,3	0,0 $\pm$ 0,0	0,1 $\pm$ 0,2	0,4 $\pm$ 0,3	0,1 $\pm$ 0,3	0,4 $\pm$ 0,4
	RMSE train	0,92 $\pm$ 0,15	1,98 $\pm$ 0,02	1,55 $\pm$ 0,21	1,04 $\pm$ 0,15	1,52 $\pm$ 0,08	2,17 $\pm$ 0,15
	RMSE inner test	2,70 $\pm$ 0,19	2,10 $\pm$ 0,03	2,27 $\pm$ 0,12	2,55 $\pm$ 0,19	2,16 $\pm$ 0,10	2,81 $\pm$ 0,25

substantially lower than the inner and outer test errors, despite the use of early stopping in ANN training. This can be explained by the training process using validation data from within the same set of experiments for early stopping, while the inner and outer test data are from different sets of experiments.

The prediction test result for the full ANN model according to each experiment is presented in Fig. 5 as the mean prediction over all runs. Based on visual inspection, the plots are marked according to good (green), poor (red) or ambiguous (blue) predictions according to trend agreement. Visually, there are obvious errors and predictions tend to be noisy, but for many cases (green) there is a decent trend agreement between the predicted and reference curves. Of special importance are the simultaneous changes in curvatures in most of the plots, indicating that the prediction is sensitive to changes in blood glucose and not merely spurious correlations with other effects.

A Clarke-error grid based on the mean test prediction vector for the full ANN model is shown in Fig. 6a. Most of the predictions fall in the A and B zones, and few predictions fall in the C (leading to unnecessary treatment) or E (confusing hyper- and hypoglycemia) zones. More important for the purpose of this study, 12% of the predictions fall in the D zone, and most of these are in the left part where the predictions fail to detect hypoglycemia. These data are also presented in the rate error grid for evaluation of glucose rates and direction as shown in Fig. 6b. Of particular importance for hypoglycemia detection are the uD_R and uE_R zones, and the sum of these percentages are given in Table 2 for comparison between different models.

4. Discussion

The results show that a global model using non-invasive measurements of NIR, bioimpedance and skin temperature may have the potential to predict blood glucose trends during hypoglycemia. Expectedly, there are large inaccuracies in predicting the BG levels, an error which becomes pronounced in threshold-based hypoglycemia detection. While most part of the predictions fall within the clinically acceptable A and B zones of the Clarke-error grid, the hypoglycemic part suffers from a large portion within the D zone, hampering the sensitivity of detection. This performance is poor compared to invasive continuous glucose monitors, but comparable to other results reported from non-invasive measurements, particularly in the non-hypoglycemic range. The full model using all

the measurements (NIR, bioimpedance and skin temperature) gave the best prediction of trends during hypoglycemia. Although the less complex models gave lower RMSE and MARD errors, their reduced ability to follow trends indicate that the prediction might not reflect true glucose changes, as shown in the example in Fig. 3a.

The results indicate that complementing NIR spectroscopy with bioimpedance measurement may improve the blood glucose trend prediction. However, adding column centered bioimpedance measurements as predictors did not produce the same gain in performance, being quite equal to the NIR + temperature model, but with an increase in the trend error. Also considering the poor prediction performance for the bioimpedance + temperature model, it is likely that the bioimpedance serves mainly as a correction for individual tissue or skin properties which influence the NIR measurement rather than being a direct predictor of blood glucose, thereby facilitating a global model without individual calibration. It is well known that the NIR measurement is very sensitive to tissue properties such as hydration and the thickness and composition of the skin layers [38], which may vary to a large extent between subjects and over time, all of which are properties known to correlate with bioimpedance [5,27]. Thus the role of bioimpedance could be mainly to adjust for confounders more than directly catching the blood glucose variation. Omitting skin temperature from the model did not produce a large change in performance, indicating that this feature was not very important. The site of measurement (upper arm) in this study may have been too far apart from the NIR and bioimpedance sensors in order to provide optimal temperature correction.

While the full ANN model was able to obtain decent trend agreement for several cases, the final PLS model would predict unidirectional changes as demonstrated in Fig. 3a, also leading to larger amounts of Clarke and rate error grid points falling in the D zones. The reason for this is probably that ANN regression allows for combinations of nonlinear relationships between the input and output of the algorithm, while PLS uses a linear regression model. Being confined to linear relationships in the PLS model, it is more likely that the calibration gives more weight to time-dependent confounders which by chance are linearly correlated with the blood glucose evolution such as temperature drift, buildup of hydration at the probe surface or skin-electrode reactions for the bioimpedance measurement. The improved performance of ANN over PLS has also been found in other studies having compared these methods on multivariate spectroscopic data [1,2,20].

Fig. 5. Plots of reference plasma glucose (dotted lines) and mean predicted plasma glucose (solid blue lines) plotted over time for each experiment. Predictions are based on the ANN model using bioimpedance, NIR and skin temperature as predictors. Experiments E1–E19 were hypoglycemic clamps while E20–E38 were euglycemic clamps. The vertical axes represent blood glucose in mmol/L. With respect to overall trend agreement, problematic cases are marked with red lines, cases with good trend agreement are marked with green lines, and ambiguous cases are marked with blue lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Clarke-error grid (a) and Rate Error Grid (b) representation of the mean test prediction vector of the NIR + Z ANN model. With respect to both grids, the A and B zones are clinically acceptable in the way that they would not lead to inappropriate treatment, zone C leads to unnecessary treatment, zone D indicates failure to detect hypo- or hyperglycemia and zone E is the most dangerous zone where hypoglycemia and hyperglycemia are confused. The percentage of predictions falling within the different zones is given in Table 2.

4.1. Comparison with literature

These results are comparable to previous studies of similar approaches to noninvasive prediction of blood glucose, but far poorer than certain laboratory studies using NIR spectroscopy such as [16] (however not using a global model). A similar sensor system has been used in Refs. [7,9,42]; and the results in this study is comparable to the latest of these studies [42], with approximately 87% (Zanon et al.) vs 86% (this study) of predictions within the A or B zones of the Clarke-error Grid.

4.2. Performance and usefulness

It is clear from the results, in particular the percentage of predictions in the D zone of the Clarke-error grid, that threshold-based hypoglycemia detection based only on this method is unreliable. The method seems to perform better for trend prediction, which could be helpful when combined with other sensors. For the purpose of detecting onset and recovery from hypoglycemia, it is however important that this trend prediction is reliable in the hypoglycemic range (below 4.0 mmol/L), but it is clear from inspecting Fig. 5 that there are many cases of poorer trend agreement during the valleys of the hypoglycemic clamp (e.g. E10–E14). The trend prediction during hypoglycemia seems to be the biggest challenge with the method. With respect to NIR measurement, and possibly also the bioimpedance measurement, the concentration of the glucose molecule is so low that it gives a very small signal of interest compared to confounding sources. This “needle in the haystack” becomes an even bigger problem during hypoglycemia making accurate predictions even more difficult. Some studies have shown that correlations between NIR and blood glucose may also be attributed to changes in scattering of the light [13,22], but it is not clear how this pertains to the hypoglycemic range. Nevertheless, the detection of drops in blood glucose while still in the normoglycemic range may be a valuable addition to a multisensory system for detection of hypoglycemia.

4.3. Sources of errors and confounding factors

It is well known that the signals of interest, both the changes in the NIR absorption spectrum and the bioimpedance spectrum which are directly or indirectly related to changes in blood sugar, are very small compared to changes in these spectra due to error sources in the measurement. For the NIR measurement, such errors are mainly changes in humidity in the skin below the probe, changes in the mechanical contact between the probe and the skin, and changes in temperature. For bioimpedance, such errors may be changes in the impedance of the electrode-skin contact area, sweating and changes in skin hydration, or changes in perfusion (which may also be indirectly related to blood glucose changes). All of these errors produce larger variation in the measurement than what originates from changes in glucose concentration, and they may also be correlated to the glucose concentration trend, especially when the clamp is going mainly in one direction. Consequently, it is crucial to include different glucose trends in the time-series used for learning data. In this work, most of the experiments had two trends: falling and then rising for the hypoglycemic clamps, and falling and then flattening for the euglycemic clamps. The learning data always included many (at least 27) experiments with an equal amount of hypoglycemic and euglycemic clamps, providing trends in both directions, but it is still possible that confounding effects on the individual level could lead to inclusion of spurious components in the model. We also cannot exclude that physiological responses during hypoglycemia (e.g. skin blood perfusion) could have been picked up by the NIR or bioimpedance measurement and possibly been spuriously included in the prediction models. We therefore underline the importance of further testing in more realistic settings.

4.4. Limitations and further work

Although the model is global (requiring no individual calibration) and was tested on data from different experiments than those used in calibration using repeated double cross-validation, it is

important to note that all data came from the same study in a controlled laboratory setup using the same equipment, protocol and patient population, and the reproducibility in other settings is not tested. Thus, these results should not be regarded as a final validation of the prediction performance, especially not concerning more realistic settings such as wearable and ambulatory use of a sensor system. Ideally, more subjects should be included for improved model development and validation, but the experiments are resource-demanding.

Having mentioned the likely positive bias from the setup, it is also possible that the method has a higher potential for prediction performance than reflected in this study, and could be improved by further research in for example sensor design, sensor placement and model calibration. The clamping procedure in this study generated an overweight of negative trends compared to positive and flat trends, and the curves were monophasic. It is likely that the calibration of the model would have been improved if the blood glucose was clamped downwards and upwards in more equal time periods and ideally in two phases such as in Ref. [8]. This would however require a very long clamping procedure which was not feasible in this study. Another possible improvement is the inside centering of the NIR predictors. The initial testing where inside mean centering was used (shown in Fig. 2) gave better predictions both in trends and levels than the final result obtained from RDCV and first-row centering (WTE = 0.95 vs 1.04, MARD = 32% vs 43%). The first-row centering used in order to not use any yet unknown data for a simulated real-time prediction, but was probably not giving a good estimate of the average spectrum. It is possible that the performance would be more like what is shown in Fig. 2 if better solutions for average spectrum estimation and real-time centering are used, probably requiring longer measurement durations.

In this study, the feature set included the whole ranges of the bioimpedance and NIR spectra. It is possible that the model could be improved by limiting the feature set to a subspace where the redundant features are excluded, but more data should preferably be gathered for a study on feature relevance.

The probe used for the NIR measurement was an off-the-shelf reflection probe (FCR-7IR400-2-ME from Avantes), and not designed for optimal separation of scattering and absorption properties (i.e. spatially resolved spectroscopy). The fixed separation distance was also lower than optimal, according to Ref. [36] (optimally between 0.4 and 4.5 mm depending on the wavelength). Thus, the results in blood glucose prediction based on NIR have likely been restricted by the probe design. It is also possible that other effects correlating with blood glucose has been included in the prediction model.

With respect to the design of NIR measurement configuration for a miniaturized sensor, the optimal wavelengths for glucose measurement in vivo fluctuate between subjects and even daily for the same person [39]. High spectral resolution is therefore necessary in order to facilitate a global model for glucose trend prediction, and we aim to employ a miniaturized spectrometer rather than use a limited fixed selection of particular wavelengths. Over the last years and ongoing today, new solutions for miniaturized and wearable NIR spectrometers are under development (for example the NeoSpectra Micro by Si-Ware Systems, [10,40]. For the purpose of adding specificity to a multisensor system for the detection of hypoglycemia, the blood glucose trend prediction part is however not limited to NIR and bioimpedance, but any technique that can be miniaturized and can provide continuous measurement is suitable. To the understanding of the authors, NIR and bioimpedance are among the most investigated and tested methods to this date, but other candidates may be considered in the future, such as Raman spectroscopy [32], photoacoustic spectroscopy [29]

or one of the many other technologies which have been presented as possible candidates for non-invasive glucose measurement [37].

Compared with similar studies, the strengths of this study was that the blood sugar level covered the whole range from moderate hypoglycemia (2.5 mmol/L) to hyperglycemia (up to 17 mmol/L) in a standardized clamping protocol, the number of experiments (20 subjects $\times$ 2 clamps – 2 excluded = 38 experiments), and that the performance of a global model was tested using repeated double cross-validation with inside centering of the NIR spectra without using anything from future and yet unknown measurements.

5. Conclusion

In conclusion, plasma glucose prediction based on NIR and bioimpedance using a global model was inaccurate and had an unreliable performance in threshold-based hypoglycemia detection, but seems to provide trend predictions which could be useful in combination with other sensors for the purpose of hypoglycemia detection. Being superior in prediction performance alone, NIR seems to be the main predictor of blood glucose trends while bioimpedance seems to act as correction for individual confounding properties, improving a global prediction model.

Conflicts of interest

The authors are collaborating with Prediktor Medical Inc. aiming to develop a commercial non-invasive glucose sensor. OM Staal is with Prediktor Medical and is partially funded by this company and The Research Council of Norway.

Acknowledgements

The authors would like to thank the nursing staff at the Diabetes Laboratory, Oslo University Hospital including: Åse Halsne, Gørild Vinje, Karin Eide, and Gro Boezelin.

The authors would also like to thank Prediktor Medical for technical cooperation and lending the SPEKTRON[®] NIR analyzer used in the experiments.

The study was funded by South-Eastern Norway Regional Health Authority grant nr 2013051. OM Staal is partially funded by The Research Council of Norway.

We acknowledge the Norwegian Diabetes Association and Simon K Fougner's fund for a research grants for laboratory equipment used in the study.

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