Medical University of Graz

Dissertation

ASSESSMENT OF THE GLUCOSE CONCENTRATION AT THE SUBCUTANEOUS ADIPOSE TISSUE SITE OF INSULIN DELIVERY

INVESTIGATIONS IN TYPE-1 DIABETIC PATIENTS

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Prof. Dr. Thomas Rudolf Pieber

Graz, am ……..…… Unterschrift ……

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3. LIST OF ABBREVIATIONS

CGM = continuous glucose monitoring

CSII = continuous subcutaneous insulin infusion

CV = coefficient of variation

IQR = interquartile range

ISF = interstitial fluid

MDI = multiple daily injections

MP_I = open flow microperfusion probe, perfusate: insulin, inflow = variable, outflow = constant

MP_{M1} = open flow microperfusion probe, perfusate: mannitol, inflow = variable, outflow = constant

MP_{M2} = open flow microperfusion probe, perfusate: mannitol, inflow = outflow = constant

OFM = open flow microperfusion

OGTT = oral glucose tolerance test

PIGR = plasma-to-ISF glucose ratio

rCV = robust coefficient of variation

SAT = subcutaneous adipose tissue

SD = standard deviation

SE = standard error

4. ABSTRACT

Current treatment of type-1 diabetes comprises glucose measurement in capillary blood obtained by finger-pricking and administration of exogenous insulin in the form of a subcutaneous bolus injection or a continuous subcutaneous infusion. A promising approach to reduce finger-pricking burden and simplify the treatment in type-1 diabetic patients would be the integration of insulin delivery and glucose measurement at a single subcutaneous tissue site (i.e. single-port treatment approach). The overall goal of the present thesis was to test the applicability of this approach.

To accomplish this goal we developed a novel method to perform simultaneous insulin delivery and glucose sampling at a single subcutaneous adipose tissue (SAT) site. This method uses a single open-flow microperfusion probe, placed in the periumbilical SAT, together with a standard insulin preparation (i.e. Insulinaspart, 100 U/ml) as a probe perfusate and applies two peristaltic pumps to operate the probe. We used this method in 10 type-1 diabetic patients (2 females, 8 males; age 39.8 ± 2.9 years; BMI 25.3 ± 1.0 kg/m2; $HbA1_c$ 7.6 ± 0.3 %, all C-peptide negative; means ± SE) during both an overnight fasting period and after ingestion of 75g glucose. As controls, two additional microperfusion probes were applied and perfused with an insulin-free perfusate (i.e. mannitol solution, 5%) for glucose sampling only. Furthermore, estimates of plasma glucose levels were calculated from the glucose concentrations obtained with insulin and mannitol-perfused probes using a prospective one-point calibration method. These estimates were then compared with the directly measured plasma glucose concentrations by applying Bland and Altman as well as Error Grid Analysis.

The subcutaneous insulin delivery with the insulin-perfused probe was successful in both achieving and maintaining stable, near normal plasma glucose concentrations during the overnight fasting as well as in reestablishing near normal plasma glucose levels by 4 to 5 hours after glucose ingestion. Bland and Altman Analysis showed that the median residual means and 2SD values for the insulin-perfused probe were 5.6 % (IQR: -6.0–12.4 %) and 31.6 % (26.4–34.6 %) and that they were similar to those obtained with mannitol-perfused control probes ($p > 0.49$ with Friedman test). Furthermore, Error Grid Analysis indicated that the percentage number of estimated plasma glucose values that fall in a clinically acceptable range was 99.6 % for the insulin-perfused probe, and that there were no differences between insulin and mannitol-perfused probes ($p > 0.87$ with Fisher's exact test). Overall, these results indicate that estimation of plasma glucose levels from the glucose concentrations directly

measured at the SAT site of insulin delivery is feasible and its quality is comparable to that of estimating plasma glucose levels from the glucose concentrations obtained in insulin-unexposed adipose tissue.

In conclusion, our studies performed in type-1 diabetic patients showed that the integration of insulin delivery and glucose measurement at a single SAT site is feasible. This novel approach could be used to simplify and improve glucose management in type-1 diabetic patients and may provide the basis for the future development of a simplified closed-loop system, able to self-regulate insulin delivery (artificial pancreas).

5. KURZFASSUNG

BESTIMMUNG DER GLUKOSEKONZENTRATION IM SUBKUTANEN FETTGEWEBE AM ORT DER INSULINZUFUHR

Die derzeitige Behandlung von Typ-1 Diabetikern beinhaltet die Blutglukosemessung im Kapillarblut und die Zufuhr von exogenem Insulin, entweder durch die mehrmals am Tag durchgeführten subkutanen Insulininjektionen oder durch eine kontinuierliche Insulininfusion mittels eines Verweilkatheters. Die Zusammenlegung der Insulinverabreichung und Blutglukosemessung durch einen einzigen subkutanen Verweilkatheter wäre daher ein vielversprechender Ansatz, um die Diabetestherapie zu vereinfachen und die Anzahl der Blutglukosemessungen, die mit schmerzhaftem Fingerstechen verbunden sind, zu reduzieren. Ziel dieser Studie war es daher, die Anwendbarkeit dieses Ansatzes zu testen.

Um dieses Ziel zu erreichen, entwickelten wir eine neue Methode, mit der die Insulinzufuhr und Glukosemessung gleichzeitig am selben Ort im subkutanen Fettgewebe ermöglicht wird. Diese neue Methode besteht aus der Anwendung eines subkutan platzierten Open-flow Mikroperfusionskatheters, der unter Verwendung zweier Peristaltikpumpen mit einer Standardinsulinlösung (Insulinaspart, 100 U/ml) perfundiert wird. Die Methode wurde an 10 Typ-1 Diabetikern (2 weiblich, 8 männlich; Alter 39.8 ± 2.9 Jahre; BMI 25.3 ± 1.0 kg/m2; $HbA1_c$ 7.6 ± 0.3 %, alle C-Peptid negativ; Mittelwerte ± Standardfehler) während einer Fastenperiode und nach Einnahme von 75g Glukose angewandt. Zu Kontrollzwecken wurden zwei zusätzliche Mikroperfusionskatheter ins subkutane Fettgewebe plaziert, mit insulinfreier Flüssigkeit (Mannitlösung, 5%) perfundiert und nur für die Glukosemessung verwendet. Weiters wurden die Blutglukosekonzentrationen durch eine Ein-Punkt Kalibrations-Methode von den mit insulin- und mannitperfundierten Kathetern gemessenen subkutanen Glukosekonzentrationen abgeleitet. Die auf diese Weise abgeleiteten Blutglukosekonzentrationen wurden dann mit den direkt im Blut gemessenen Glukosekonzentrationen mittels Bland and Altman und Error Grid Analyse verglichen.

Wie die Zeitverläufe der Blutglukose zeigten, war die subkutane Insulinzufuhr mit dem insulinperfundierten Katheter sehr gut geeignet, um normoglykämische Blutglukose während des Fastens zu erreichen und aufrechtzuerhalten, sowie annähernd normale Blutglukosewerte 4-5 Stunden nach der Glukoseeinnahme wiederherzustellen. Die Bland and Altman Analyse zeigte, dass der Median der Mittelwerte der Residuen und der Median der 2 Standardabweichungen für den insulinperfundierten Katheter 5.6 % (IQR: -6.0–12.4 %)

bzw. 31.6 % (26.4–34.6 %) waren und sich nicht von den Medianwerten, die mit den mannitperfundierten Kathetern berechnet wurden, unterschieden ($p > 0.49$, getestet mit Friedman Test). Weiters zeigte die Error Grid Analyse, dass der prozentuelle Anteil der klinisch akzeptablen Blutglukoseschätzungen vom insulinperfundierten Katheter 99.6 % war und dass es im Vergleich keinen Unterschied zu den prozentuellen Anteilen der mannitperfundierten Kathetern gab ($p > 0.87$ getestet mit Fisher's Exact Test). Diese Resultate zeigten übereinstimmend, dass die Ableitung der Blutglukosekonzentrationen, basierend auf den Glukosekonzentrationen im subkutanen Fettgewebe am Ort der Insulinzufuhr möglich ist, und dass die Qualität dieser Ableitung sich nicht von der im subkutanen Fettgewebe ohne Insulineinfluss unterscheidet.

Mit dieser Studie konnten wir zeigen, dass Insulinzufuhr und Glukosemessung gleichzeitig am selben Ort im subkutanen Fettgewebe von Typ-1 Diabetikern möglich ist. Dieses Ergebnis bildet daher eine mögliche Grundlage für die weitere Entwicklung eines vereinfachten Closed-Loop Systems, welches die benötigte Insulinzufuhr anhand der subkutanen Glukosekonzentration berechnet und daraufhin automatisch verabreicht (künstliche Bauchspeicheldrüse).

6. INTRODUCTION

Type-1 diabetes is a widespread endocrinological disorder, and even though it accounts for only about 5 to 10 % of all diabetes mellitus cases, its incidence is rapidly increasing[1]. Type-1 diabetes usually appears at a younger age and is caused by an auto-immunological destruction of the insulin-producing β cells in the islets of Langerhans in the pancreas, leading to an absolute insulin deficiency[1,2]. Due to that fact, patients with type-1 diabetes require insulin replacement therapy for survival. The goal in this therapy is to mimic the physiological pattern of insulin secretion in order to avoid metabolic short-term complications (i.e. therapy-induced hypoglycemia[1] or hyperglycemia with ketoacidosis and osmotic diuresis[3]) as well as vascular long-term complications (i.e. microvascular alterations like retinopathy, nephropathy and neuropathy and macrovascular alterations like cardiovascular, cerebrovascular and peripheral vascular diseases[1,4]) and to reduce the mortality rates in type-1 diabetic patients[5].

For replacement of endogenous insulin, the majority of the type-1 diabetic patients self-administer exogenous insulin in their subcutaneous adipose tissue (SAT), either in the form of multiple daily injections (MDI) using a hypodermic needle (e.g. syringe, insulin pen) or in the form of a continuous subcutaneous insulin infusion (CSII) using an special indwelling catheter connected to an insulin pump[1,2,6-9]. Usually the insulin delivery pattern for both MDI and CSII comprises a basal insulin supply (injection of long-acting insulin 1-2 times a day or continuous infusion of regular or rapid-acting insulin[10,11]) as well as pre-prandial insulin boluses of regular or rapid-acting insulin[1,2].

To adjust insulin dosage accordingly, type-1 diabetic patients have to self-monitor their glucose levels several times a day[1,2]. The common way of glucose self-monitoring is the measurement of blood glucose concentration in capillary blood obtained by finger-pricking with a portable blood glucose monitoring device[12].

In recent years, ISF of the adipose tissue has received considerable attention as an alternative site for the measurement of glucose levels. Continuous glucose monitoring (CGM) in SAT was introduced in clinical care as supportive device[13-15], additionally to capillary blood glucose monitoring. With this relatively new method, glucose levels in the interstitial fluid of the SAT can be measured continuously by means of a flexible needle sensor, which is placed under the skin. This needle sensor is linked to a non-implanted transmitter transferring the glucose data to a portable radio receiver that displays the glucose levels. Thus, CGM provides

benefits like reducing finger-pricking burden, display of real-time glucose values, trend-assessment, detection of hypo- and hyperglycemic events by acoustic signals and transfer of glucose data to a personal computer for analysis and management[1,6,15-17].

Several studies have been conducted in type-1 diabetic patients to assess the clinical effectiveness and reliability of CGM in patients with CSII treatment[13,14,18] as well as to test an automated closed-loop insulin delivery systems[15,19,20], thereby indicating that glucose measurement and insulin delivery at the same time in the SAT were feasible, but at two separate locations (i.e. dual-port treatment). Furthermore, Hermanides et al.[21] recently performed CGM in the vicinity of CSII in type-1 diabetic patients, showing that there was no significant local effect of insulin on the glucose measurement in the SAT, whereas the mean insertion distance between insulin infusion catheter and the closest insertion point of the glucose sensor (Glucoday S, Menarini Diagnostics, Firenze, Italy) was 0.9 ± 0.2cm.

In order to avoid such a complex dual-port treatment as well as to reduce the finger-pricking burden in diabetes therapy, the integration of insulin delivery and glucose measurement at a single subcutaneous tissue site (i.e. single-port treatment) would be a promising approach to simplify the therapy in type-1 diabetic patients.

As already shown in recent studies by our group, an open-flow microperfusion (MP) probe can be used both to deliver exogenous agents (e.g. insulin) to the SAT[22] as well as to sample interstitial solutes (e.g. glucose, sodium, potassium)[23-26]. The MP probe is a perforated double lumen catheter, which allows the exchange between the perfusate (fluid, which is pumped through the probe) and the ISF of certain tissues. However, by operating the MP probe conventionally by means of a single pump (i.e. *single-pump operation mode,* **Figure 6-1**) and by using insulin as a perfusate, subcutaneous insulin-delivery would be feasible, but not at variable rates, as required for an appropriate treatment in type-1 diabetic patients.

Therefore, **objective #1** of our study was to develop a method, which allows variable and controlled insulin delivery and simultaneous glucose sampling at a single subcutaneous adipose tissue site in type-1 diabetic patients. To achieve this objective, we used a conventional rapid-acting insulin solution as perfusate and two autonomous pumps to operate the MP probe: One pump responsible for the inflow, the other one for the outflow (i.e. *dual-pump operation mode*).

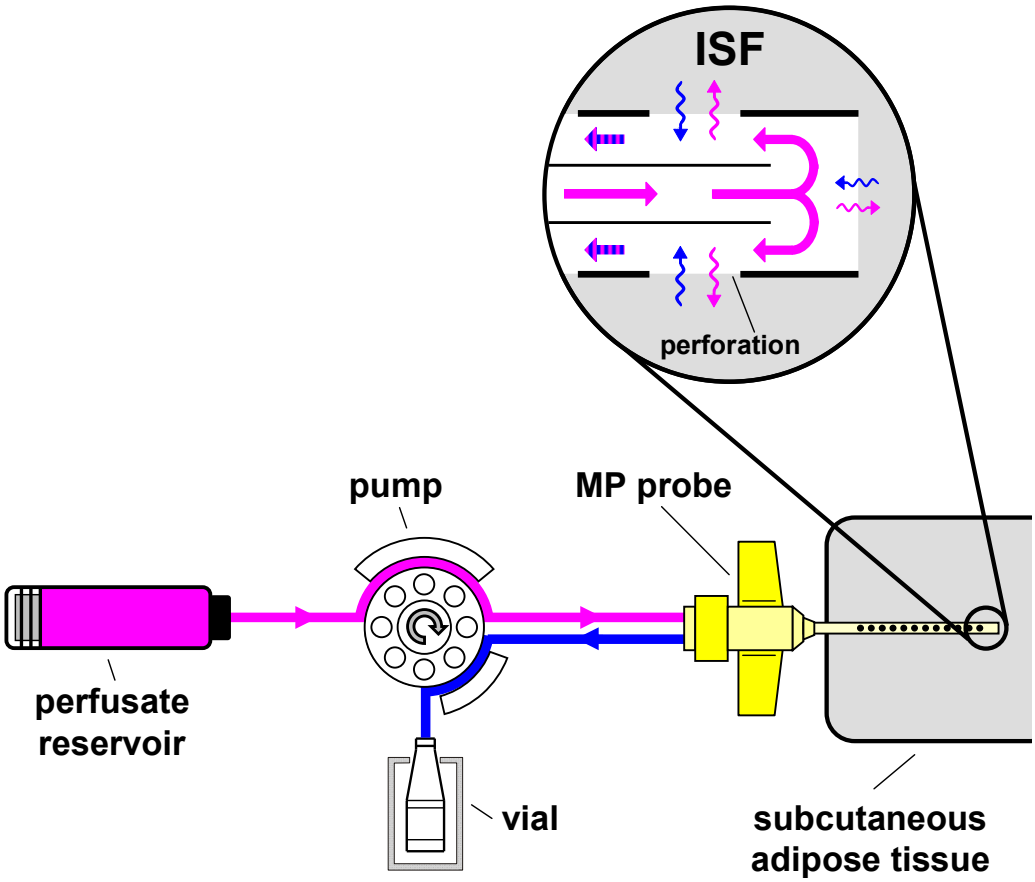


Figure 6-1 Scheme of the conventional single-pump mode of MP probe operation: Thereby the perfusate (fluid, which is pumped through the probe) is pumped from the reservoir via the inlet tubing to the MP probe, moves through the inner cannula to the tip of the probe, and streams back through the annular space between the inner cannula and the perforated outer cannula. Partial equilibration occurs via diffusion (thin wavy arrows) between the perfusate and the surrounding ISF as the perfusate passes by the perforations of the outer cannula. The partially equilibrated perfusate is then pumped through the outlet tubing into sampling vials for further analysis. In- and outflow rates applied in the single-pump operation mode are always equal and are controlled by means of a single pump. Furthermore, in order to assure steady sampling conditions, the pump rate of this pump is fixed to a certain value.

When insulin is secreted from the pancreas or absorbed from the subcutaneous injection site into the circulation, the resulting rise in the plasma insulin level acts to lower the plasma glucose concentration by both suppressing glucose production in the liver[27] and enhancing glucose uptake in insulin-sensitive tissues (i.e., muscle and adipose tissue)[28]. Moreover, in vitro studies showed that insulin exposure to isolated fat cells increases glucose uptake[29], and hence, insulin may have a local effect on glucose concentration in the ISF of the SAT.
Thus, to investigate if there is a local effect of insulin on the glucose concentration in the SAT in diabetic patients, **objective #2** of this study was to compare the ISF glucose levels observed in SAT at the insulin delivery site with those observed in insulin-unexposed SAT. Therefore, in addition to the insulin-perfused MP probe used with the dual-pump operation mode, we employed two additional MP probes, perfused with an insulin-free perfusate (i.e. 5% mannitol solution): One probe was used with the dual-pump operation mode (i.e. similar to the insulin-perfused probe), the other probe was used with the conventional single-pump operation mode.

As already mentioned above, type-1 diabetic patients have to measure their glucose levels several times a day, to appropriately adjust the insulin dosage[1,2]. Due to the fact that the patients usually measure glucose levels in blood obtained by finger-pricking, **objective #3** of this study was to asses the feasibility of estimating blood glucose levels from the ISF glucose levels observed at the insulin delivery site.

7. OBJECTIVES

Objective #1 of our study was to develop a new method (dual-pump operation mode), which allows variable and controlled insulin delivery and simultaneous glucose sampling under steady conditions at a single subcutaneous adipose tissue site in type-1 diabetic patients.

Objective #2 was to compare the ISF glucose levels observed in adipose tissue at the insulin delivery site with those observed in insulin-unexposed adipose tissue.

Objective #3 was to asses the feasibility of estimating blood glucose levels from the ISF glucose levels observed at the insulin delivery site.

8. METHODS

8.1. MICROPERFUSION PROCEDURES

8.1.1. FABRICATION

Open-flow microperfusion probe:

The open-flow microperfusion (MP) probe consists of an inner and outer cannula:

The outer cannula was made of a conventional catheter (Neoflon, Becton Dickinson, Helsingborg, Sweden. Outer diameter: 0.65 mm or 24 gauge, shaft length: 19 mm), that was perforated with 27 macroscopic holes (3 rows of 9 holes, over a catheter length of 11mm), using Excimer Laser Technique (Laserzentrum Hannover e.V., Hannover, Germany). The diameter of each hole was 0.3 mm. The inner cannula with the in- and outlet was produced from Crusius Company, Peggau, Austria.

Probe perfusate:

For the perfusion of the MP probes, we used two types of perfusates:

One perfusate was a rapid acting-insulin (Novorapid®, 100 U/ml; Insulinaspart), which was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The other perfusate was a sterile 5% mannitol solution (275 mmol/l) filled in 15-ml vials. The vials were obtained from the Pharmacy of the Medical University Graz. There the mannitol solution was prepared under aseptic conditions from a conventional 550-mmol/l mannitol solution purchased from Fresenius Kabi, Graz; Austria.

Probe tubing:

In- and outflow tubings were fabricated to connect the in- and outlet of the probe with the perfusate reservoir and the sampling vial, respectively. Therefore, Tygon tubing (Outer diameter: 2.0 mm, inner diameter: 0.25 mm, sterilizable with ethylene oxide) winded on to rolls was purchased from Cole-Parmer, Vernon Hills, Illinois, USA. Using a sterile surgical blade, tube pieces with a length of 750 mm were cut off from the Tygon tubing on the roll. For making the connections to the perfusate reservoir and to the sampling vials, hubs of 21-gauge syringe needles (0.5 x 25 mm BD Microlance, Becton Dickinson, Drogheda, Ireland) were cut off, and the excised needle shafts were then pushed into the ends of the Tygon tubes.

8.1.2. DUAL-PUMP MODE OF PROBE OPERATION

As was mentioned in chapter 6, the single-pump mode of MP probe operation is not feasible to accomplish *variable* insulin delivery under steady sampling conditions. Thus, to perform variable and controlled insulin delivery in the presence of steady sampling conditions by means of a single subcutaneous MP probe, a dual-pump mode of probe operation (**Figure 8-1**) was developed. This method comprises the use of two autonomous pumps: One pump responsible for the probe-inflow, the other one for the probe-outflow.

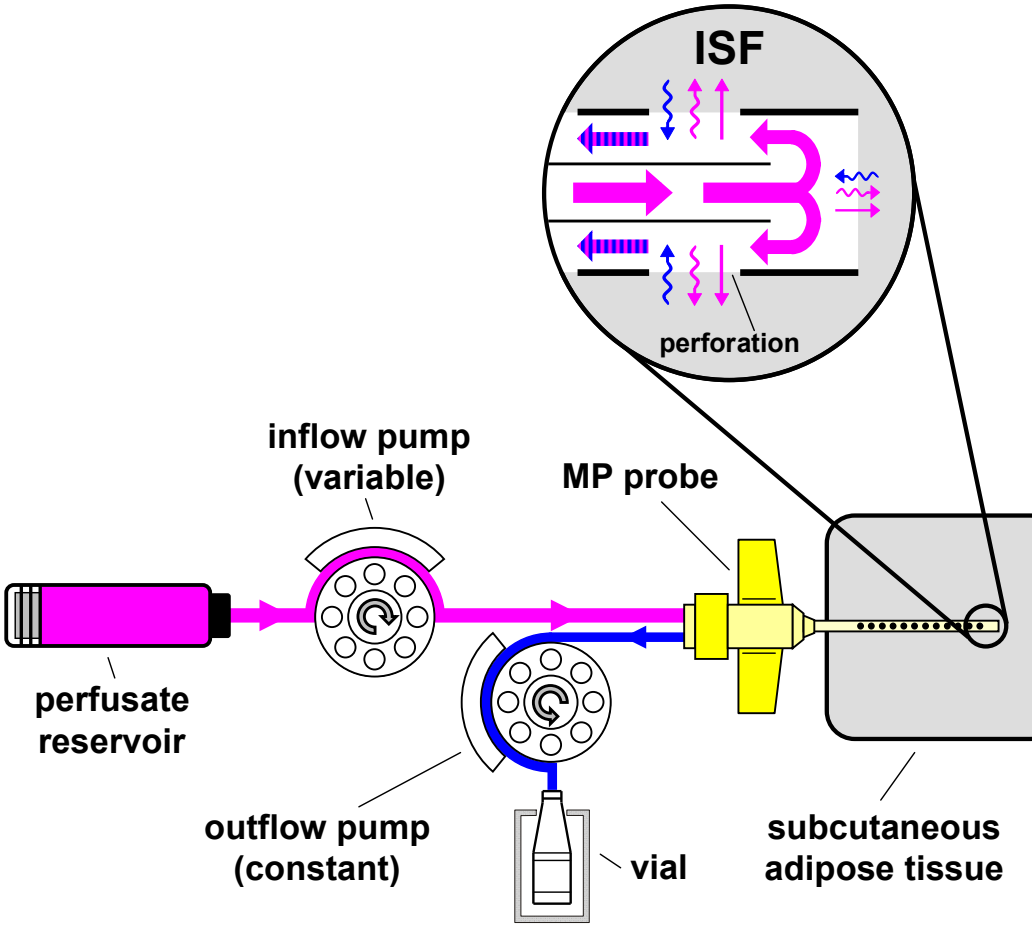


Figure 8-1 Scheme of the dual-pump mode of MP probe operation: This method comprises the use of two autonomous pumps: The pump rates of the inflow pump were variable and always higher than or at least equal to the pump rate of the outflow pump, which was kept constant at a pump rate of ~0.5 µl/min. This dual-pump operation mode allowed the perfusate entering the probe (bold arrow) to be split into two main flow fractions: One flow fraction was directed to the surrounding tissue (thin straight arrow) for insulin delivery and the other flow fraction was guided along the annular probe exchange region to the outlet of the probe (striped bold arrow) for partially equilibration with the surrounding ISF via diffusion (thin wavy arrow). Then the partially equilibrated perfusate (now called effluent fluid) was pumped through the outlet tubing in a sampling vial for further analysis.

By using that dual-pump operation mode, it is possible to supply a variable amount of insulin to the tissue via diffusion *and* convection as well as to obtain partially equilibrated effluent samples, which are reflecting a percentage number (i.e. exchange efficiency) of the glucose concentration in the ISF surrounding the probe. To determine the glucose exchange efficiency, ionic reference method was applied and is now described in the following paragraph.

8.1.3. PROBE CALIBRATION WITH IONIC REFERENCE METHOD

As already mentioned, the equilibration between perfusate and surrounding ISF is not complete (i.e. the effluent glucose concentration is lower than the ISF glucose concentration) at an outflow rate of ~ 0.5µl/min, which was used in this study. Therefore, we used a method that allows the determination of the efficiency of the glucose exchange between the perfusate and ISF. This method, termed ionic reference method[23], is based on the use of ions (e.g. Na^+ and K^+) as endogenous markers for the glucose exchange efficiency. Ionic concentrations in the blood are tightly regulated and are very close to the concentrations in the ISF. Therefore, the exchange efficiency of an ion can be estimated as the ratio of the ion concentration in the probe effluent to the ion concentration in plasma.

In vivo experiments have shown that the exchange efficiency of ions, like Na^+ and K^+, equals the exchange efficiency of glucose under various conditions[23]. Because of this property, the *glucose concentration in the ISF can be estimated as the glucose concentration in the probe effluent divided by the ionic exchange efficiency*.

The ionic exchange efficiency itself can be easily monitored by applying the electrical conductivity measurement. This is possible, because the electrical conductivity equals the measured sum of all ionic concentrations in a fluid[23,24].

8.2. STUDY PROTOCOL

To accomplish the objectives in our study, three MP probes were inserted into the periumbilical subcutaneous adipose tissue of 10 diabetic subjects. One probe was used for insulin delivery and simultaneous glucose sampling (in the following *MP_1 probe*) and, as controls, two additional probes were used for glucose sampling only (in the following *MP_{M1}* and *MP_{M2} probes)*.

The study design included basal insulin delivery during a fasting period lasting overnight (~10 hours) followed by an 8-hour period (i.e. OGTT period) of insulin delivery after ingestion of 75g glucose (**Figure 8-2**).

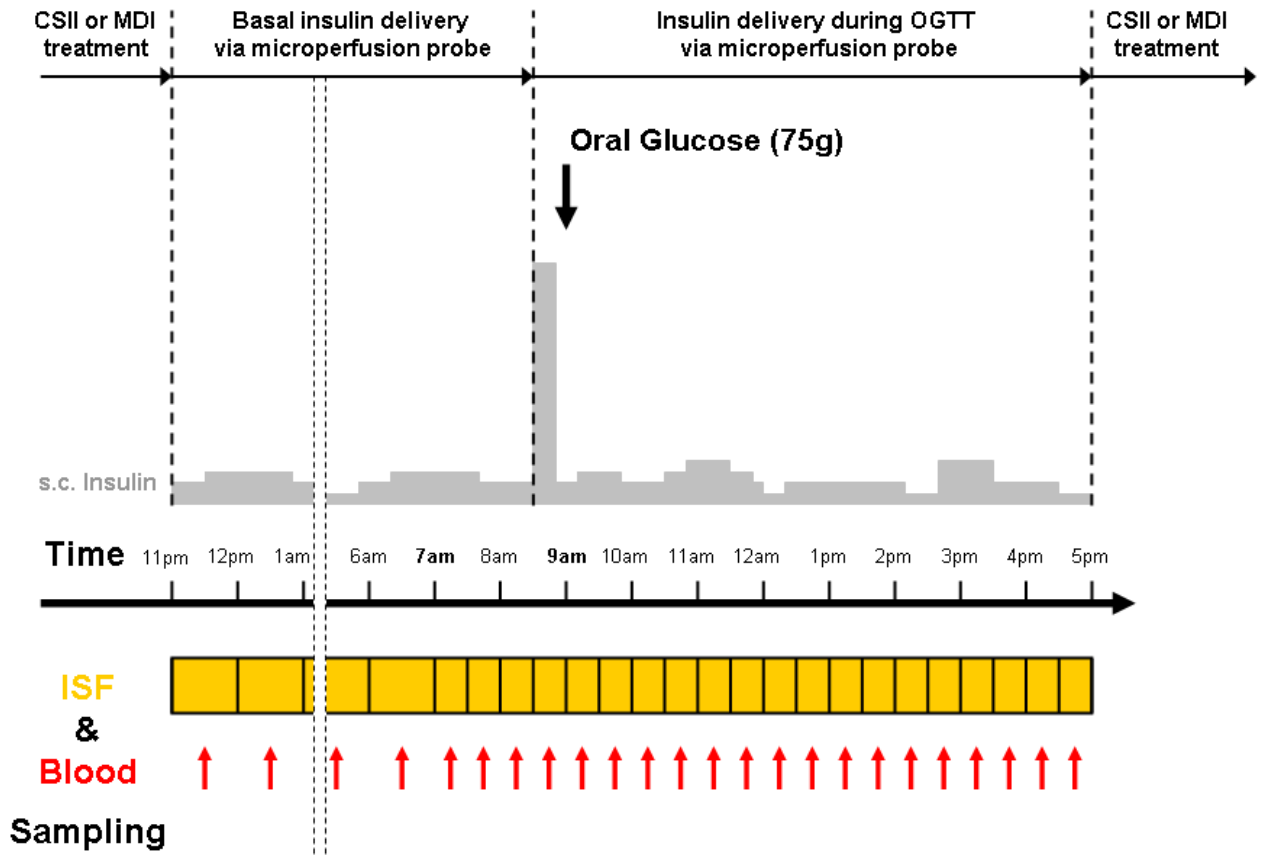


Figure 8-2 Scheme of the study design for providing subcutaneous insulin delivery and simultaneous ISF sampling via a microperfusion probe in 10 type-1 diabetic patients during a basal period lasting overnight and after an oral glucose load (75g glucose).

8.2.1. SUBJECTS

Study was conducted in ten type-1 diabetic subjects, 2 females and 8 males, with a mean age of 39.8 ± 2.9 years (range 27 - 57 years), and a mean BMI of 25.3 ± 1.0 kg/m^2 (range 21.1 – 29.5 kg/m^2). Their mean duration of diabetes was 22.9 ± 2.6 years (range 7 - 35 years) and their percent HbA_{1C} averaged 7.6 ± 0.3 % (range 5.7 - 8.6 %, normal range 4.3 - 5.9 %). Data represented as means ± SE

Seven subjects were treated with multiple daily injections (MDI) and three subjects with continuous subcutaneous insulin infusion (CSII). Patients were all without residual endogenous insulin secretion, as indicated by undetectable C-peptide levels in blood (i.e. < 22 pmol/l). At the time of the study, patients had no evidence of clinically overt diabetic complications and, apart from insulin, were not taking any medication known to influence carbohydrate metabolism.

Written informed consent was obtained after the purpose, nature, and potential risks of the study were explained to the subjects. The studies were approved by the ethics committee of the Medical University of Graz.

8.2.2. STUDY DAY

Admission:

Each subject came to the Clinical Research Center between 9:30 and 10:30 pm. Subjects treated with multiple daily injections (MDI) did not inject their long acting insulin dose for this night and subjects with continuous subcutaneous insulin infusion (CSII) treatment stopped their insulin pump on arrival.

Intravenous catheter placement and arterialized venous blood sampling:

At ~ 10:45 pm the subject received an intravenous catheter (TriCath; 18-gauge, Codan GmbH, Lensahn, Germany) in a vein of the forearm. The forearm was then placed in a thermoregulated box and maintained at 55°C to ensure the arterialization of the venous blood. For measurement of plasma glucose concentrations, the arterialized venous blood samples were drawn every 30 minutes until 2 hours before ingestion of the standardized glucose load. Thereafter blood samples were drawn every 15 minutes.

If the gradient of the blood glucose was too high, the blood sampling intervals were shortened in order to have an appropriate temporal resolution. During the experiment, the patency of the intravenous catheter was maintained by a slow infusion of 0.9% NaCl solution.

Subcutaneous probe placement, tubings, perfusates, sampling vials and pumps setup:

Shortly after intravenous catheter insertion, three MP probes were placed in the periumbilical subcutaneous adipose tissue (distance between adjacent probes were > 35 mm). An in- and outflow tubing was then used to connect the in- and outlet of each probe to a reservoir containing the perfusate (either rapid acting insulin [Novorapid®, Insulinaspart 100U/ml] or insulin-free isotonic solution [275 mmol/l mannitol in aqueous solution]) and a sampling vial (Microvial; CMA/Microdialysis, Solna, Sweden), respectively. Afterwards the tubes were inserted into peristaltic pumps (Minipuls 3; Gilson, Villiers-le-Bel, France), which were continuously pumping perfusate from the perfusate reservoir in the MP probes, and sucking out the partially equilibrated perfusate from the probe through the outflow tubing into the sampling vials. Perfusion of the probes started immediately, but collecting the samples began ~60 min (equilibration period and activation of metabolic effects of insulin) after the insertion of the probes. Pictures of a study day are shown in **Figure 8-3**.

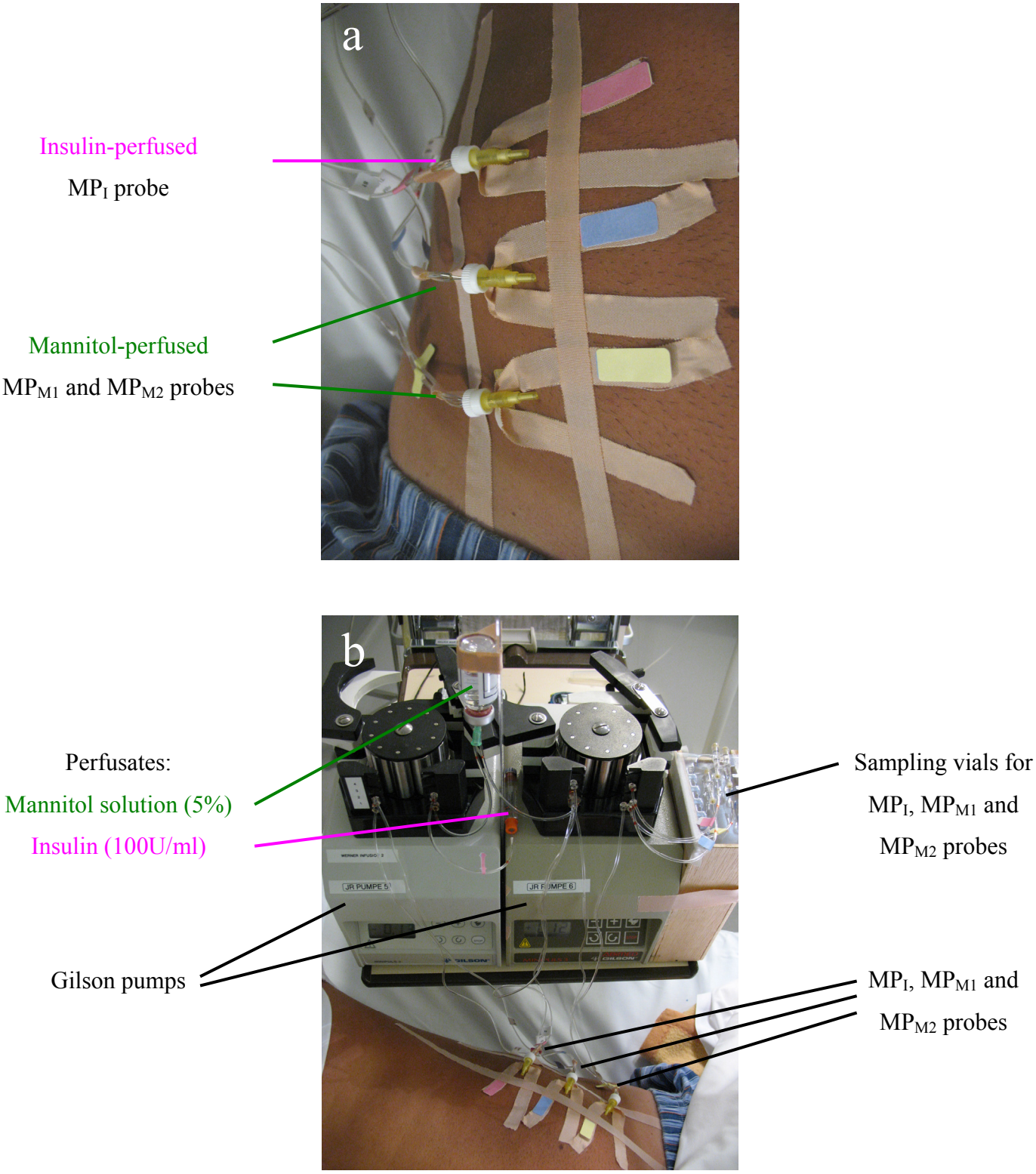


Figure 8-3 Pictures of a study day: (a) MP_I, MP_{M1} and MP_{M2} probes in the periumbilical subcutaneous adipose tissue. (b) Experimental setup: Perfusates, Gilson pumps, microperfusion probes, sampling vials and flexible tubes.

Inflow and outflow pump rates, insulin delivery and glucose sampling:

To slowly achieve and maintain normoglycemia during the basal insulin delivery period lasting overnight, inflow rates of the insulin-perfused MP_I probe were variable, but always equal to or higher than the constant outflow rate of ~0.5 µl/min (**Figure 8-4**). Based on the periodically measured plasma glucose concentrations, the required amount of insulin was estimated and delivered to the subject. The amount of insulin convectively transported to the tissue, was controlled by controlling the difference between the inflow and outflow rates of the probe. Due to the low outflow rate of ~0.5 µl/min, the insulin delivery via diffusion was neglected in this controlling procedure.

The inflow rates of the mannitol-perfused MP_{M1} and MP_{M2} probes were either equal to the inflow rates applied in the MP_I probe or kept constant at a value of ~0.5 µl/min (i.e. equal to the outflow rate), respectively.

The outflow rates of all three probes were fixed to a value of ~0.5 µl/min. The effluent sampling delay time introduced by the dead space volume (i.e. 25µl) of the probe outflow tubing was taken into account when the sample collection was begun. The effluent samples were collected in 60-min fractions until 2 hours before the ingestion of the glucose. Afterwards the sampling interval was 30-min. Effluent fluid was collected in sampling vials on ice and frozen for assay. To determine the exact sample volume and to monitor the outflow of the perfusate solution, the vials were weighed before and after sampling.

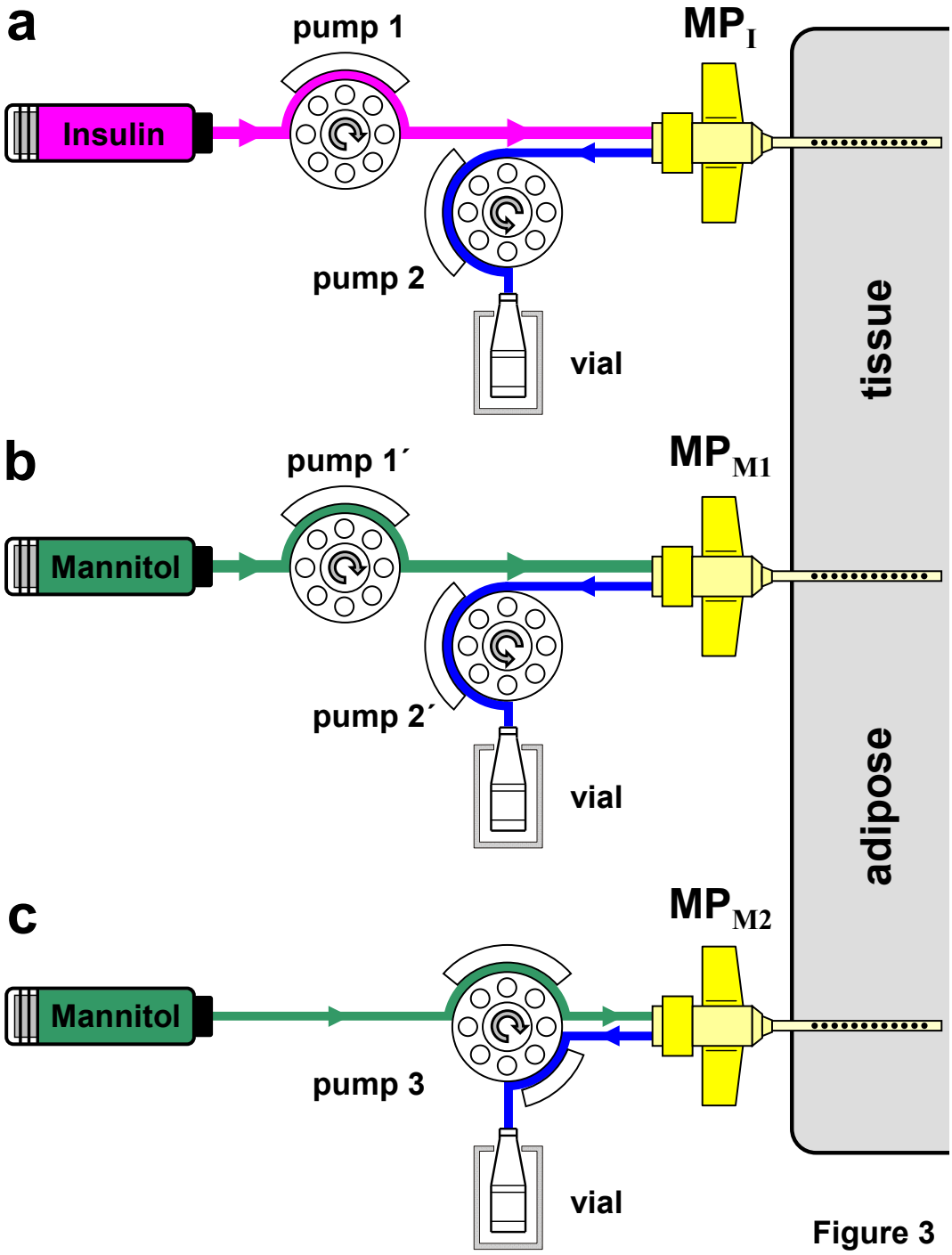


Figure 8-4 Schematic of the experimental set-up. Three probes were inserted into the periumbilical subcutaneous adipose tissue of 10 diabetic patients. (**a**) One probe (MP_I) was used for variable insulin delivery and simultaneous glucose sampling. This probe was perfused with a rapid-acting insulin solution (100 U/ml, Aspart) using two peristaltic pumps, with one attached to the inflow tubing and one to the outflow tubing (dual-pump operation mode). Insulin delivery rate was adjusted by adjusting the difference between the inflow and outflow rate of the probe. (**b-c**) Additionally, two control probes (MP_{M1} and MP_{M2}) were used for glucose sampling only and were perfused with an insulin-free solution (5 % mannitol) using either (**b**) dual-pump operation mode where the inflow and outflow rates equaled those applied in the insulin-perfused MP_I probe or (**c**) single-pump operation mode where the inflow equaled the outflow rate. (**a-c**) The effluent fluid conveying the extracted glucose was collected in sampling vials.

Bolus application and ingestion of standardized glucose load:

In the morning at ~9 am, the subject was asked to ingest 75g glucose (6.25 units carbohydrate) dissolved in 300ml of water (Glucoral; Unipack, Wr. Neustadt, Austria). Twenty minutes before glucose ingestion, an insulin bolus was administered via the insulin-perfused MP_I probe. Due to the fact that at a sufficiently high inflow rate insulin flows back up the catheter shaft[30], leaking to the surface and reducing the delivery to the tissue itself, the insulin bolus was administered over a period of 15 minutes at a relatively low inflow rate.

The amount of insulin administered as a bolus was determined by using medical records on the subject's insulin sensitivity factor (i.e. subject's insulin-to-carbohydrate ratio). After administration of the insulin bolus, the basal insulin delivery via the insulin-perfused MP_I probe was continued and periodically adjusted so as to reestablish normal plasma glucose by ~5 h after glucose ingestion.

If the plasma glucose levels measured during the experiment decreased to values lower than 3.22 mmol/l, the subject was asked to ingest additional glucose.

End of the experiment:

At the end of the experiment (i.e. eight hours after the glucose ingestion) the subcutaneous and intravenous probes were removed. After switching back to the usual treatment (i.e. MDI or CSII), the subject was discharged.

A phone call was made to the subjects 24-72 hours after probe removal to document any subsequent adverse events.

8.3. SAMPLE ANALYSIS

Plasma glucose concentration was measured at the bedside using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA) with a coefficient of variation (CV) of 2 %.

The probe effluent glucose levels were measured using an automated CMA 600 analyzer (CMA Microdialysis AB, Solna, Sweden) with a within-run CV of 2 % (**Figure 8-5**).

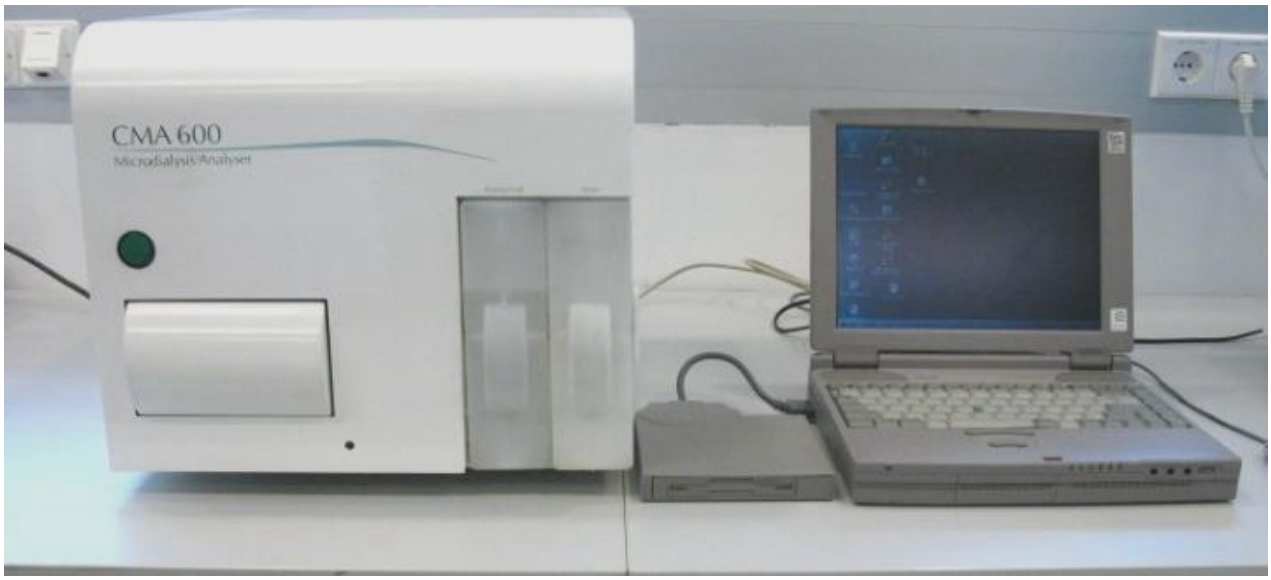


Figure 8-5: CMA 600 analyzer with Laptop

The conductivity levels in plasma and effluent probe samples as well as in the perfusates were measured using a contactless conductivity detector (TraceDec, I.S.T., Strasshof, Austria, **Figure 8-6**) positioned on a fused silica capillary (inner diameter 50 µm, outer diameter 360 µm; Agilent Technologies, Santa Clara, CA, USA). Aliquots of the samples (~1 µl) were drawn from the sample vial into the capillary by using a peristaltic pump coupled to the capillary. The electrical conductivity was determined with a within-run CV of <1 %

a **b**

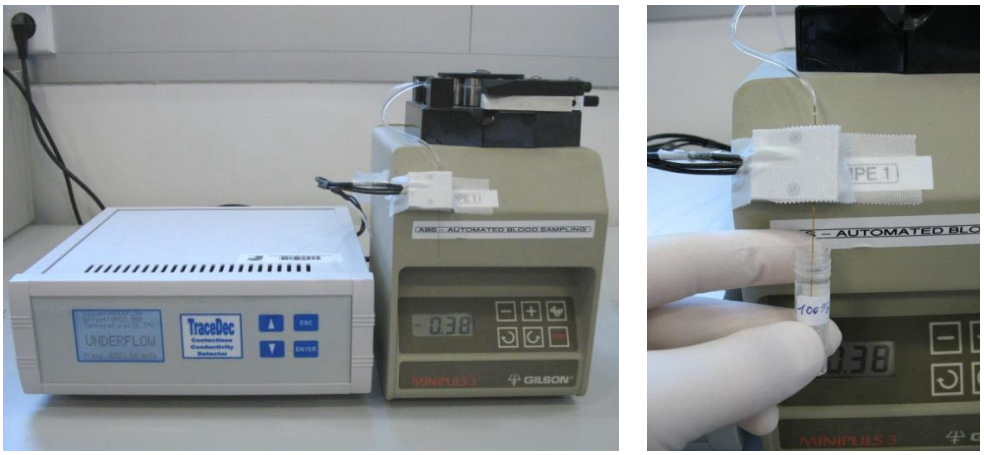


Figure 8-6 (**a**) Conductivity detector with peristaltic pump. (**b**) Contactless conductivity measurement of samples.

The C-peptide concentrations were determined using ELISA kits (Mercodia, Uppsala, Sweden). The C-peptide assay has a lower limit of quantification of 22 pmol/l.

HbA_{1C} levels were measured by high-performance liquid chromatography (Menarini HA-8160, Menarini Diagnostics, Florence, Italy).

8.4. DATA ANALYSIS

8.4.1. CALCULATION OF ISF GLUCOSE LEVELS

By using ionic reference method (chapter 8.1.3. on page 19), the ISF glucose concentration was calculated as the glucose concentration in the probe effluent sample divided by the glucose exchange efficiency (EE_G) of the probe, which is equal to the ionic exchange efficiency (EEI). Glucose exchange efficiency was determined for each sample as

$$EE_G = EEI = (C_{out} - C_{in}) / (C_{pl} - C_{in})$$

C_{in}, C_{out}, and C_{pl} are the measured electrical conductivities in the perfusate, the effluent sample, and the corresponding plasma sample, respectively. Application of the ionic reference method was possible, because the electrical conductivities in the used probe perfusates were either negligible (Mannitol solution: 0 % of average C_{pl}) or low compared to that in plasma (Aspart: ~22 % of average C_{pl}).

8.4.2. STATISTICAL METHODS

Normality of the data was assessed using normal probability plots. Homogenity of the variances in the linear regression data was assessed using plots of residuals against fitted values. P-values below 0.05 were considered to indicate statistical significance.
Data analysis was performed using MATLAB (The MathWorks Inc., Natick, MA, USA) and SPSS (SPSS Inc., Chicago, IL, USA) software packages.

Assessment of the relationship between ISF and plasma glucose levels

To assess the relationship between plasma (x-axis) and ISF (y-axis) glucose concentrations, correlation analysis was performed using Pearson's product-moment correlation coefficient and linear regression analysis was performed by least squares method[31].
For further examination, the plasma-to-ISF glucose ratio was calculated from each pair of observed plasma glucose and corresponding ISF glucose levels. Then, the median plasma-to-ISF glucose ratio (PIGR) and the robust coefficient of variation (rCV) of the median PIGR was determined for each subject and probe. The rCV of the median PIGR is the nonparametric aequivalent to the parametric coefficient of variation (CV) and is calculated as follows: rCV = 100 * IQR * 0.7413 / median.
The results of correlation, linear regression, and PIGR analysis obtained for insulin and mannitol-perfused probes were then analysed with Friedman's test.
If significance was achieved in Friedman's test, post-hoc comparison of the medians was performed with Wilcoxon signed rank test.

Assessment of agreement between ISF-derived plasma glucose estimates and directly measured plasma glucose levels

Agreement between tissue glucose (i.e. ISF-derived estimates of plasma glucose) and corresponding plasma glucose concentrations was assessed by applying the method of residuals[32], error grid analysis[33] and calculating the median absolute difference.
Results, obtained from the application of the method of residuals and the error grid analysis, were examined with Friedman's test and Fisher's exact test, respectively.

9. RESULTS:

9.1. INSULIN DELIVERY WITH THE MICROPERFUSION PROBE

Insulin delivery to the subcutaneous adipose tissue with the MP_I probe was successful in both achieving and maintaining a stable, near normal plasma glucose during the overnight fasting as well as in reestablishing near normal plasma glucose levels by 4 to 5 hours after ingestion of 75g glucose (**Figure 9-1**). The average basal insulin delivery rates during the fasting period and after the oral glucose load (i.e. OGTT period) were 1.04 ± 0.11 U/h and 0.85 ± 0.16 U/h, respectively. Furthermore, the bolus amount of insulin, subcutaneously delivered before the ingestion of the glucose load, was 7.22 ± 0.48 U. Thus, as 75g of glucose were ingested, an average insulin-to-carbohydrate ratio of 1.16 ± 0.12 can be calculated for the patients.

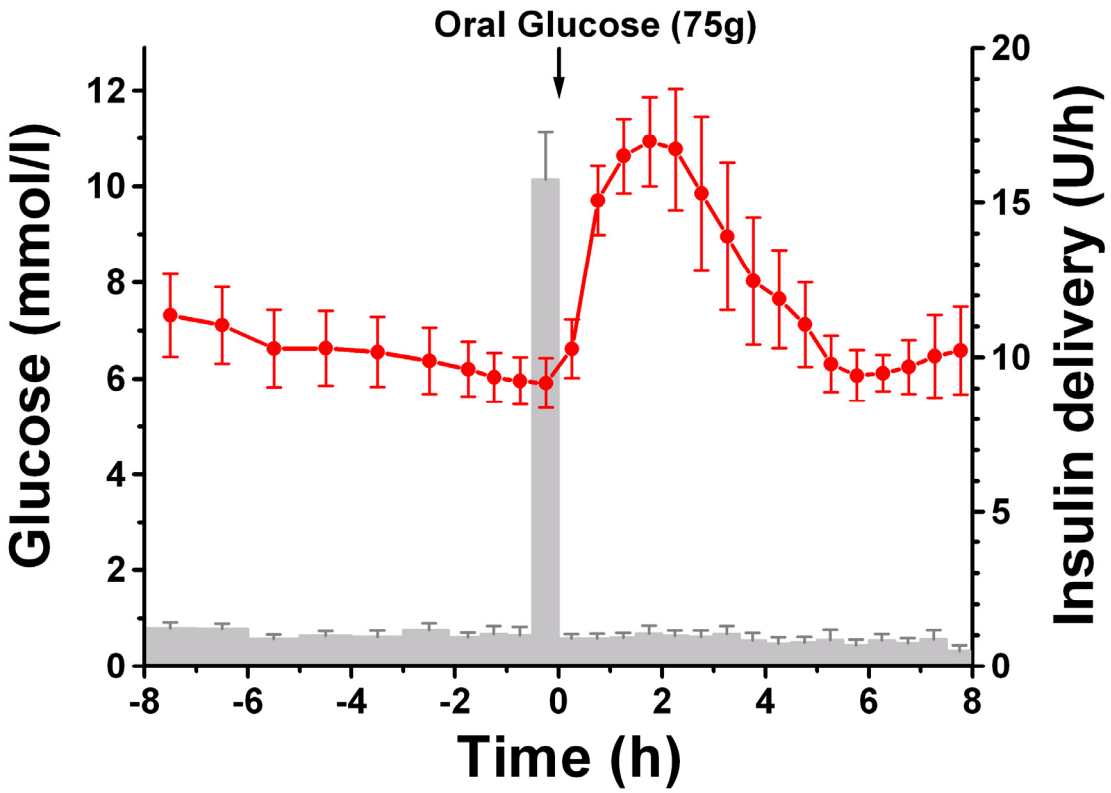


Figure 9-1: Average time course of plasma glucose (red circle) and subcutaneous insulin delivery rate (grey bars) during an overnight fast and a subsequent oral glucose tolerance test in diabetic subjects. Data are shown as means ± SE, n = 10.

As already mentioned in chapter 8.2.2, the insulin delivery rates were controlled by controlling the difference between the inflow and outflow rates of the insulin-perfused MP_I probe. The average time course of the inflow and outflow rates is shown in **Figure 9-2**.
During the fasting period and after the oral glucose load, the average inflow rates were 0.68 ± 0.03 µl/min and 0.64 ± 0.03 µl/min, respectively, whereas the average inflow rate during the bolus administration period (from t = -20 to -5 min) was 5.33 ± 0.49 µl/min. The average outflow rate applied during the experiments was 0.51 ± 0.02 µl/min (**Figure 9-2)**.

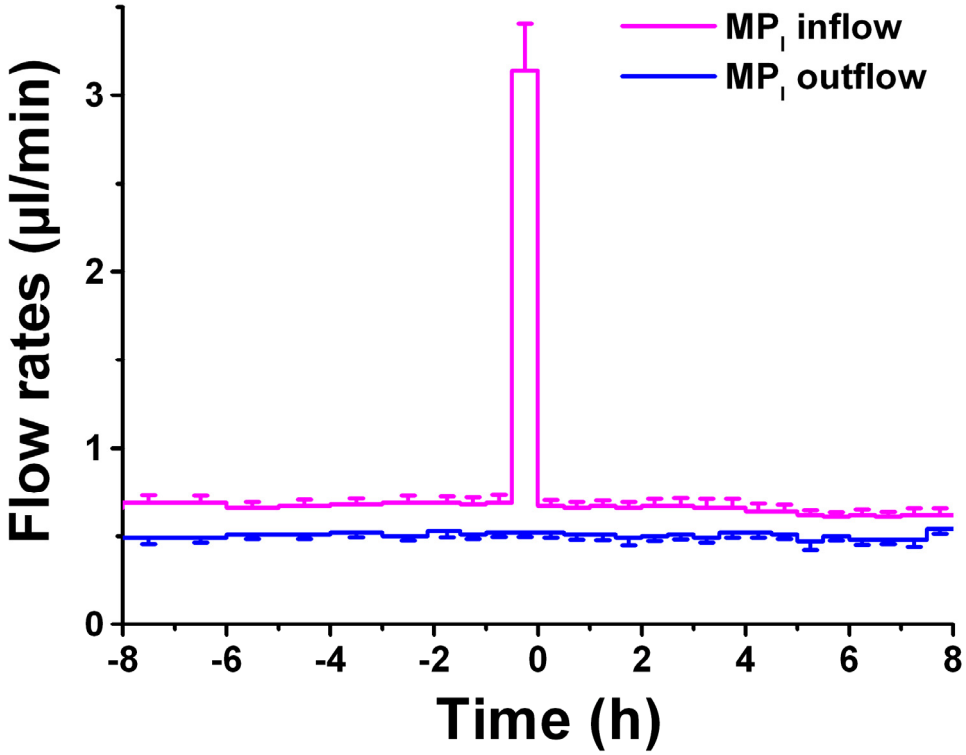


Figure 9-2: The average time course of in- and outflow rates of the insulin-perfused MP_I probe. Rates were averaged over 60-min intervals (overnight) and 30-min intervals (OGTT phase). Data are shown as means ± SE, n = 10.

9.2. PROBE-DERIVED ISF GLUCOSE LEVELS

9.2.1. TIME COURSES OF ISF AND PLASMA GLUCOSE LEVELS

The average time course of plasma and ISF glucose obtained by MP_I, MP_{M1}, and MP_{M2} probes are depicted in **Figure 9-3**. As can be seen, during the fasting as well as during the OGTT period, the ISF glucose concentration obtained with the MP_I probe paralleled the glucose levels observed in plasma. Also shown are the average time courses of effluent glucose and exchange efficiency, from which the ISF glucose levels were derived.
Additionally, time course of plasma and ISF glucose for each subject and probe are depicted in the **Appendix.**

To investigate the relationship between ISF and plasma glucose levels, several statistical analyses have been performed and will be described in the following chapter.

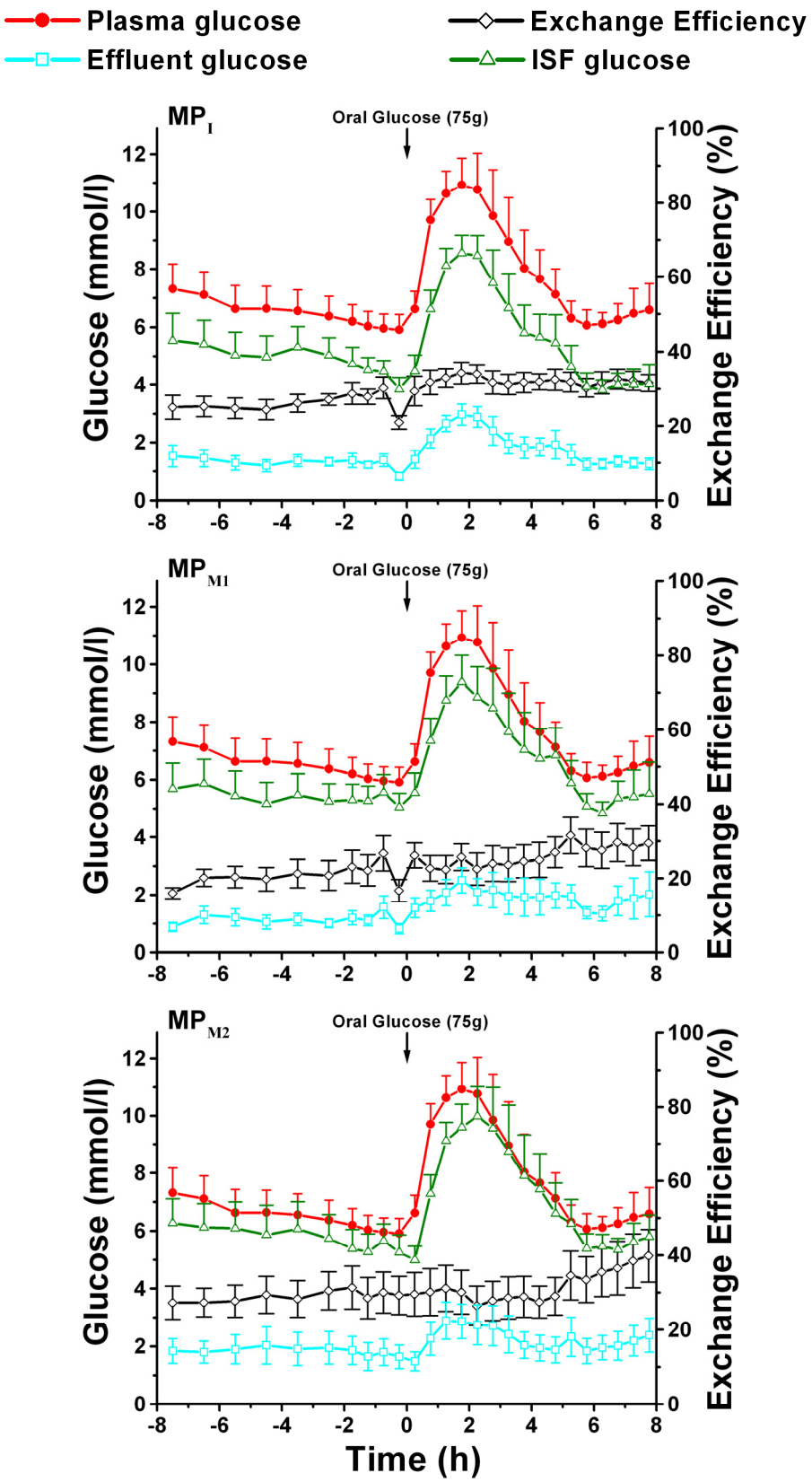


Figure 9-3: Average time course of plasma glucose as well as ISF glucose, effluent glucose and exchange efficiency obtained with insulin perfused probe (MP_I) and mannitol perfused control probes (MP_{M1} and MP_{M2}) during an overnight fast and after ingestion of a standardized glucose load (75g). Data are shown as means ± SE, n = 10.

9.2.2. RELATIONSHIP BETWEEN ISF AND PLASMA GLUCOSE LEVELS

9.2.2.1. PEARSON'S CORRELATION COEFFICIENT

To quantify the effect of insulin on the relationship between ISF and plasma glucose levels, the Pearson coefficient of correlation (r) was derived for each probe using plasma (x-axis) and ISF (y-axis) glucose concentrations obtained from the experiments. The median correlation coefficient value for the insulin-perfused MP_I probe was found to be high [0.93 (interquartile range: 0.91–0.97)] and did not differ from that obtained for the mannitol-perfused probes [vs. MP_{M1}: 0.94 (0.88–0.97) and MP_{M2}: 0.95 (0.91–0.98); p = 0.67 with Friedman's test; **Table 9-1**].

Table 9-1: Correlation Coefficients derived for insulin perfused (r_I) and mannitol perfused probes (r_{M1} and r_{M2}).

Subject No.	r_I*	r_{M1}*	r_{M2}*
01	0.99	0.98	0.98
02	0.98	0.97	0.94
03	0.94	0.88	0.87
04	0.91	0.92	0.96
05	0.91	0.97	0.98
06	0.96	0.96	0.98
07	0.85	0.95	0.91
08	0.55	0.77	0.88
09	0.92	0.86	0.92
10	0.97	0.94	0.97
Median [a]	0.93	0.94	0.95
25th percentile	0.91	0.88	0.91
75th percentile	0.97	0.97	0.98
Interquartile range (IQR)	0.06	0.09	0.07

* all Pearson coefficient values were significantly different from zero; $P < 0.001$

[a] no difference between the median r_I, r_{M1} and r_{M2} values; $p = 0.67$ with Friedman test.

9.2.2.2. LINEAR REGRESSION ANALYSIS

In addition to the analysis of the Pearson's correlation coefficients, a linear regression analysis was conducted for MP_I, MP_{M1} and MP_{M2} probes. The intercepts and slopes derived for each subject and probe are shown in **Table 9-2** and **Table 9-3**. The median intercepts for MP_I, MP_{M1}, and MP_{M2} probes were -0.13 (-0.71–0.10), 0.31 (-0.27–1.20), and -0.03 (-0.37–0.18), respectively, thereby indicating that for both insulin- and mannitol-perfused probes the derived straight-lines may pass through the origin of the regression graph, i.e., the median intercept values for the insulin-perfused probes and the mannitol-perfused probes are not different from zero (MP_I vs zero, $p = 0.193$, MP_{M1} vs zero $p = 0.492$, MP_{M2} vs zero $p = 0.922$, tested with Wilcoxon signed rank test). Thus, the linear regression equation $Y=B*X + A$ may be simplified to $Y=B*X$.

Table 9-2: Regression analysis according to the equation $Y=B*X + A$. Intercept (A) and standard error (SE) of the intercept derived for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2})

	MP_I		MP_{M1}		MP_{M2}	
Subject	A_I mmol/l	SE_I mmol/l	A_{M1} mmol/l	SE_{M1} mmol/l	A_{M2} mmol/l	SE_{M2} mmol/l
01	-0.38	0.17	-0.68	0.29	-0.06	0.35
02	0.10	0.25	0.27	0.40	-0.37	0.63
03	-0.08	0.46	-0.18	0.91	2.15	1.12
04	-0.09	0.70	1.20	0.55	-0.09	0.57
05	0.13	0.48	0.40	0.41	-0.42	0.30
06	-0.71	0.32	-0.27	0.37	0.01	0.27
07	0.70	0.55	0.36	0.34	0.42	0.49
08	-1.42	1.79	1.51	0.75	0.00	0.69
09	-1.75	0.58	-2.89	0.73	-1.37	0.56
10	-0.18	0.42	1.72	0.49	0.18	0.42
Median [a]	-0.13	0.47	0.31	0.45	-0.03	0.52
25th	-0.71	0.32	-0.27	0.37	-0.37	0.35
75th	0.10	0.58	1.20	0.73	0.18	0.63
IQR	0.81	0.26	1.47	0.36	0.55	0.28

[a] median intercepts of insulin-perfused probes (A_I) and mannitol-perfused probes (A_{M1} and A_{M2}) were not different from zero ($p \geq 0.193$ with Wilcoxon signed rank test).

Table 9-3: Regression analysis according to the equation Y=B*X + A. Slopes (B) and standard error (SE) of the slopes derived for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2})

	MP_I		MP_{M1}		MP_{M2}	
Subject	B_I	SE_I	B_{M1}	SE_{M1}	B_{M2}	SE_{M2}
01	0.792	0.023	0.948	0.041	1.085	0.049
02	0.706	0.027	0.890	0.044	0.896	0.068
03	0.783	0.057	0.920	0.108	0.807	0.122
04	0.941	0.084	0.783	0.066	0.957	0.071
05	0.626	0.056	0.928	0.048	0.791	0.035
06	0.813	0.046	0.898	0.053	0.976	0.039
07	0.643	0.075	0.755	0.047	0.781	0.067
08	0.758	0.233	0.581	0.098	0.815	0.090
09	1.096	0.095	0.992	0.118	1.048	0.091
10	0.727	0.041	0.642	0.050	0.842	0.041
Median	0.771	0.056	0.894	0.052	0.869	0.067
25th	0.706	0.041	0.755	0.047	0.807	0.041
75th	0.813	0.084	0.928	0.098	0.976	0.090
IQR	0.107	0.043	0.173	0.051	0.169	0.049

Due to this possible simplification, we performed a second linear regression analysis according to the equation Y=B*X with the *straight line forced through the origin of the regression graphs.* The median slopes for MP_I, MP_{M1} and MP_{M2} probes were 0.726 (0.712–0.774), 0.862 (0.801–0.916), and 0.859 (0.833–0.977), respectively, and are shown in **Figure 9-4** and **Table 9-4**. Furthermore, there was a significant difference between at least two of the derived median slopes (p = 0.002, tested with Friedman test). Post-hoc analysis suggests that the median slope for the MP_I probe exhibited a tendency to be lower (~16 %) than the median slope of the MP_{M1} probe (p = 0.084, tested with Wilcoxon signed rank test). However, the median slope for the MP_I probe was 15.5 % lower than the median slope for the MP_{M2} probe (p = 0.002, tested with Wilcoxon signed rank test). Finally there was no difference between the median slopes of the mannitol perfused control probes MP_{M1} and MP_{M2} (p = 0.160, tested with Wilcoxon signed rank test).

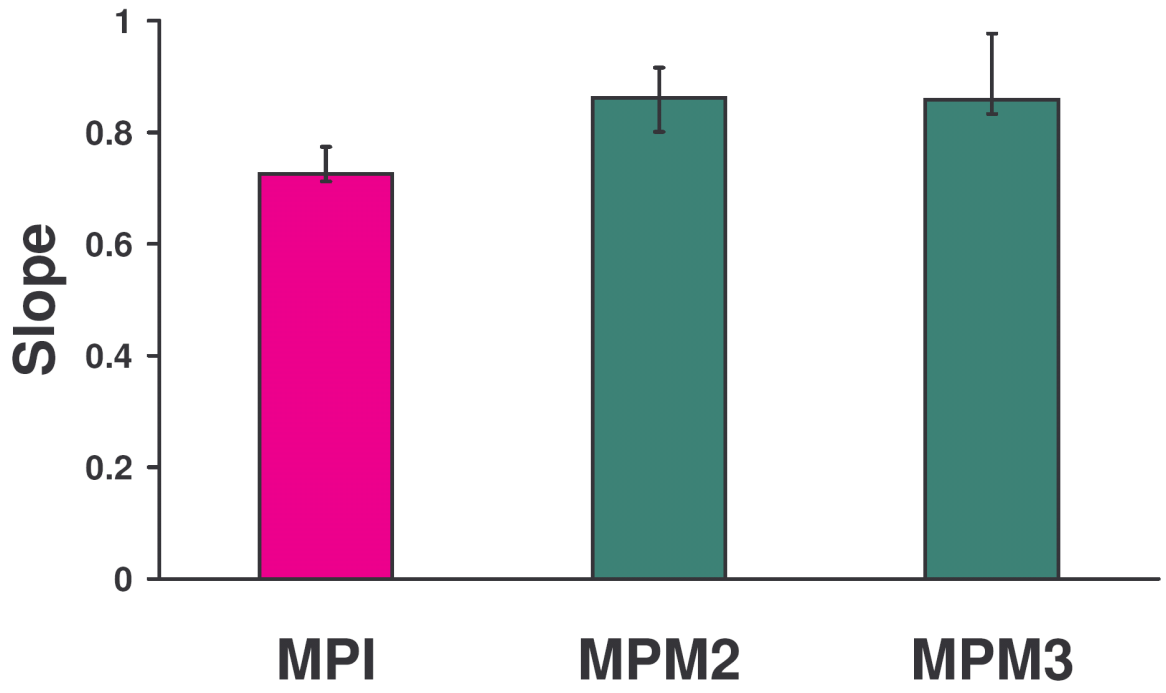


Figure 9-4 Slopes obtained from the regression analysis derived for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2}). Data are shown as medians with IQR, n = 10.

Table 9-4: Regression through the origin according to the equation Y=B*X. Slopes (B) and standard errors (SE) of the slopes derived for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2})

	MP_I		MP_{M1}		MP_{M2}	
Subject	B_I	SE_I	B_{M1}	SE_{M1}	B_{M2}	SE_{M2}
01	0.746	0.010	0.862	0.018	1.077	0.020
02	0.716	0.012	0.916	0.019	0.860	0.029
03	0.774	0.017	0.900	0.030	1.033	0.036
04	0.931	0.028	0.917	0.024	0.947	0.025
05	0.639	0.021	0.971	0.019	0.746	0.014
06	0.718	0.018	0.862	0.020	0.977	0.014
07	0.734	0.025	0.802	0.015	0.836	0.022
08	0.577	0.042	0.776	0.019	0.816	0.016
09	0.821	0.028	0.539	0.038	0.833	0.026
10	0.712	0.018	0.801	0.026	0.858	0.018
Median [a,b]	0.726	0.020	0.862	0.019	0.859	0.021
25th percentile	0.712	0.017	0.801	0.019	0.833	0.016
75th percentile	0.774	0.028	0.916	0.026	0.977	0.026
Interquartile range	0.062	0.011	0.115	0.007	0.144	0.010

[a] significant difference between at least two of the three median slopes derived with MP_I, MP_{M1} and MP_{M2} probes $p = 0.002$ with Friedman test.

[b] no difference between median SE_I, SE_{M1} and SE_{M2} values; $p = 0.74$ with Friedman test.

Both the median standard errors (**Table 9-4**) and the median root mean square errors (**Table 9-5**) of the slopes obtained from this regression analysis were similar for the insulin-perfused and mannitol-perfused probes (p = 0.74 for standard errors and p = 0.50 for mean square errors, tested with Friedman test), suggesting that the strength of the proportional relationship between plasma and ISF glucose values derived with the MP_I, MP_{M1} and MP_{M2} probes were similar.

Table 9-5: Regression through the origin according to the equation Y=B*X – Root Mean Square Error derived for insulin-perfused ($RMSE_I$) and mannitol-perfused probes ($RMSE_{M1}$ and $RMSE_{M2}$)

Subject No.	$RMSE_I$ mmol/l	$RMSE_{M1}$ mmol/l	$RMSE_{M2}$ mmol/l
01	0.38	0.69	0.75
02	0.56	0.91	1.39
03	0.72	1.29	1.37
04	1.30	1.11	0.89
05	0.99	0.87	0.64
06	0.66	0.71	0.51
07	0.99	0.61	0.86
08	1.67	0.75	0.63
09	0.91	1.25	0.84
10	0.95	1.31	0.95
Median [a]	0.93	0.89	0.85
25th percentile	0.66	0.71	0.64
75th percentile	0.99	1.25	0.95
Interquartile range	0.33	0.54	0.31

[a] no difference between median $RMSE_I$, $RMSE_{M1}$ and $RMSE_{M2}$ values; p = 0.50 with Friedman test

9.2.2.3. PLASMA-TO-ISF RATIO

The group median plasma-to-ISF ratio (PIGR) were 1.38 (1.30–1.44), 1.17 (1.09–1.23), and 1.15 (1.02–1.22) for MP_I, MP_{M1}, and MP_{M2} probe, respectively. Results are shown in **Figure 9-5** and **Table 9-6.** Furthermore, there was a significant difference (p = 0.020, tested with Friedman test) between at least two of the derived median slopes. Post-hoc analysis indicated that the group median PIGR of the MP_{M1} probe had a tendency to be lower (~15 %) than that of the MP_I probe (p = 0.084, tested with Wilcoxon signed rank test). The group median PIGR of MP_{M2} probe was 16.6 % lower than the group median PIGR of the MP_I probe (p = 0.010, tested with Wilcoxon signed rank test). Furthermore there was no difference between the group median PIGR of the mannitol perfused probes MP_{M1} and MP_{M2} (p = 0.375, tested with Wilcoxon signed rank test).

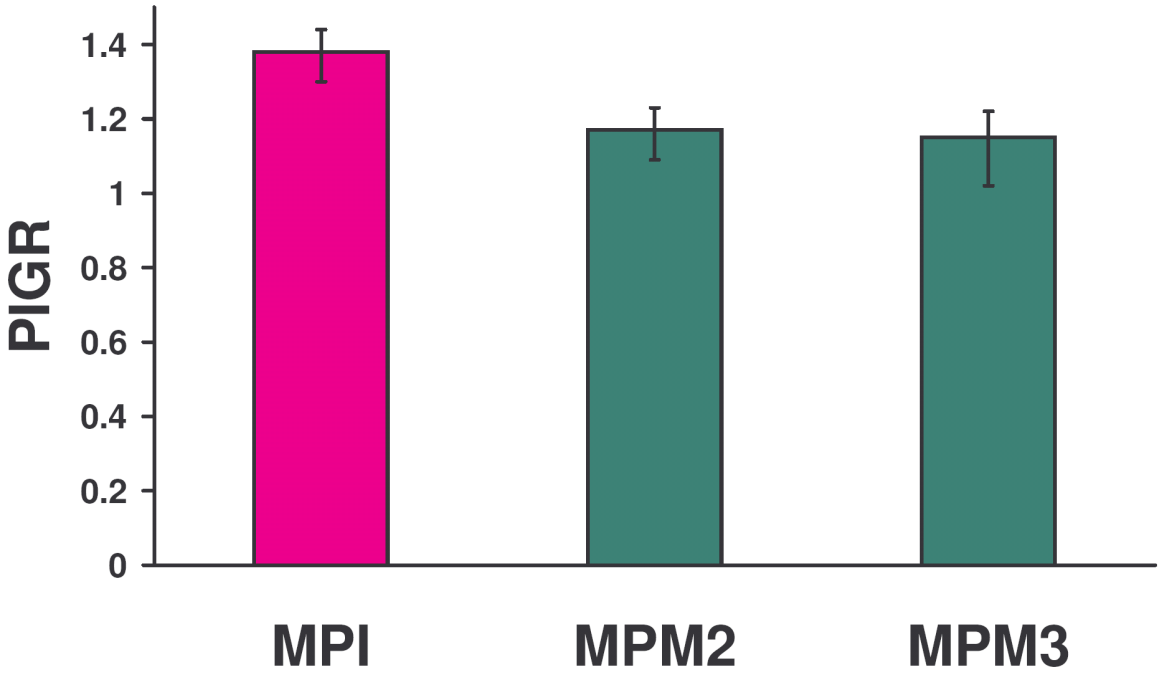


Figure 9-5 Plasma-to-ISF glucose ratio obtained for insulin-perfused (MP_I) and mannitol-perfused (MP_{M1} and MP_{M2}) probes. Data are shown as medians with IQR, n = 10

Table 9-6: Median Plasma-to-ISF Glucose Ratio Values (PIGR) and Interquartile Range (IQR) for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2})

	MP_I		MP_{M1}		MP_{M2}	
Subject	$PIGR_I$ %	IQR_I %	$PIGR_{M1}$ %	IQR_{M1} %	$PIGR_{M2}$ %	IQR_{M2} %
01	1.36	0.16	1.17	0.21	0.94	0.11
02	1.41	0.15	1.09	0.25	1.22	0.32
03	1.30	0.14	1.11	0.32	0.91	0.20
04	1.13	0.24	1.05	0.21	1.14	0.17
05	1.55	0.33	1.00	0.16	1.42	0.22
06	1.44	0.21	1.21	0.15	1.02	0.09
07	1.30	0.36	1.23	0.12	1.15	0.14
08	1.67	1.32	1.30	0.11	1.22	0.13
09	1.25	0.33	2.09	0.95	1.25	0.26
10	1.39	0.30	1.17	0.32	1.14	0.12
Group Median[a]	1.38	0.27	1.17	0.21	1.15	0.15
25th	1.30	0.16	1.09	0.15	1.02	0.12
75th	1.44	0.33	1.23	0.32	1.22	0.22
IQR	0.14	0.17	0.14	0.17	0.20	0.10

[a] significant difference between at least two of the three group median PIGR derived with MP_I, MP_{M1} and MP_{M2} probes; $p = 0.020$ with Friedman test.

The median rCV values derived for MP_I, MP_{M1} and MP_{M2} probes were 15.60 (8.98–19.43), 14.16 (9.13–20.19) and 9.97 (8.16–15.33), as shown in **Table 9-7**. The statistical analysis indicated that the median rCV of the insulin-perfused probe MP_I is similar to the median rCV of the mannitol-perfused probes MP_{M1} and MP_{M2} ($p = 0.150$, tested with Friedman test).

Table 9-7: Robust Coefficient of Variation of median ratios obtained for insulin-perfused (rCV_I) and mannitol-perfused probes (rCV_{M1} and rCV_{M2})

Subject No.	rCV_I	rCV_{M1}	rCV_{M2}
	%	%	%
01	8.98	13.38	8.53
02	7.82	16.74	19.19
03	8.24	21.41	16.14
04	15.53	14.93	11.01
05	15.68	11.85	11.53
06	10.98	9.13	6.52
07	20.37	7.36	8.93
08	58.74	6.24	8.16
09	19.43	33.80	15.33
10	16.02	20.19	8.01
Median [a]	15.60	14.16	9.97
25th percentile	8.98	9.13	8.16
75th percentile	19.43	20.19	15.33
Interquartile range	10.45	11.06	7.17

[a] no difference between rCV_I, rCV_{M1} and rCV_{M2} values; $p = 0.150$ with Friedman test

9.3. ESTIMATION OF PLASMA GLUCOSE CONCENTRATIONS

As mentioned in chapter 9.2.2.2, the linear regression equation simplified to Y = B * X. Thus, due to the fact that proportionality prevails, *tissue glucose levels* (i.e. ISF-derived estimates of plasma glucose levels) have been calculated from the ISF glucose levels by using a prospective one-point calibration procedure, that consisted of multiplying the ISF glucose values with the plasma-to-ISF glucose ratio, calculated from the ISF and corresponding plasma glucose concentrations observed at the beginning of each experiment.

9.3.1. TIME COURSES OF ISF-DERIVED ESTIMATES OF PLASMA GLUCOSE LEVELS

The averaged time course of plasma and tissue glucose obtained with MP_I, MP_{M1} and MP_{M2} probes as well as the insulin delivery rates of the MP_I probe are depicted in **Figure 9-6**. Additionally, time courses of plasma and tissue glucose levels as well as insulin delivery rates for each subject and each probe are depicted in the **Appendix.**

As shown in **Figure 9-6**, the tissue glucose concentrations obtained by MP_I, MP_{M1} and MP_{M2} probes agreed well with the glucose concentration observed in plasma during fasting period as well as during the 8-hour period after the ingestion of 75g glucose.

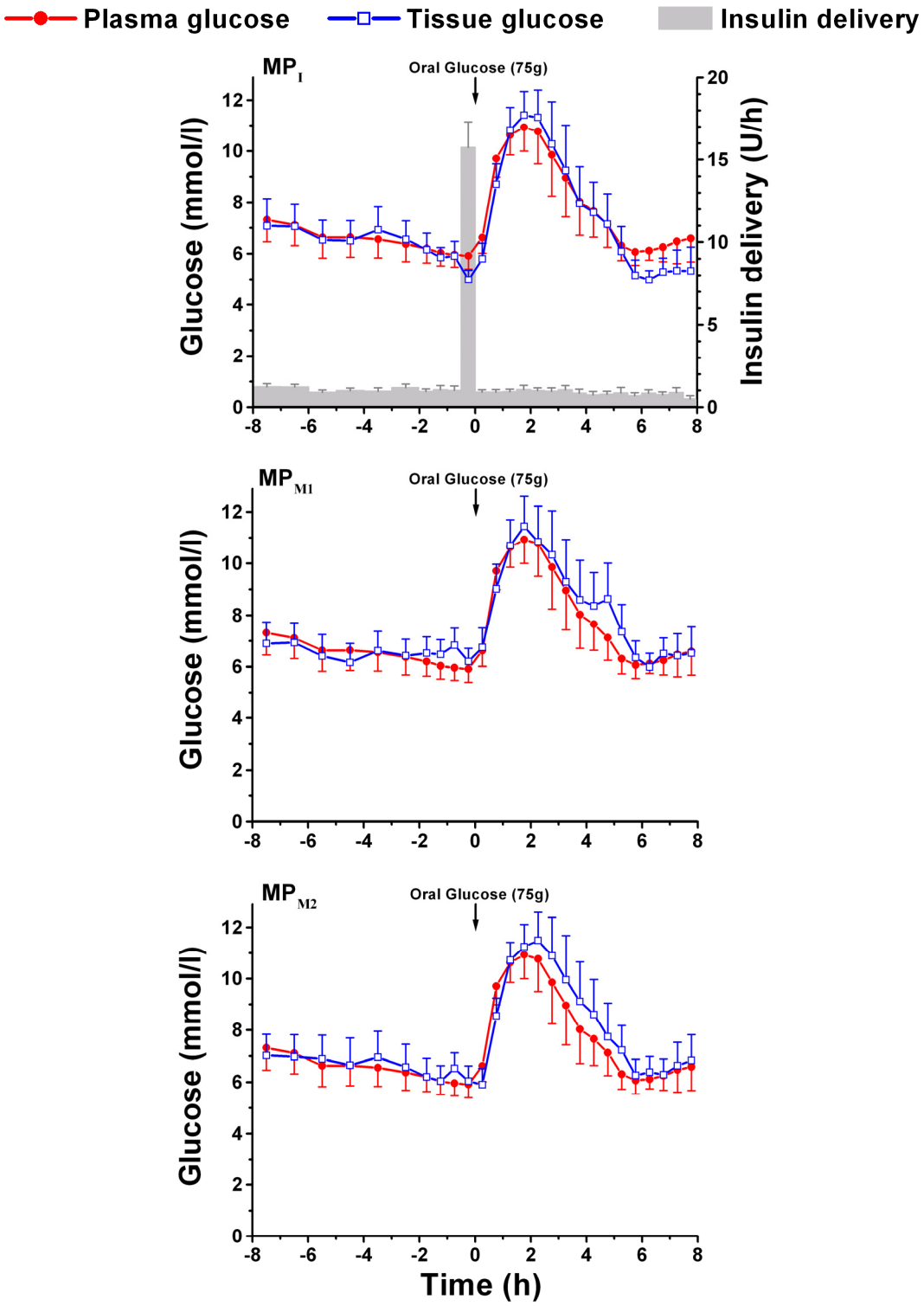


Figure 9-6: Time courses of plasma glucose and tissue glucose obtained with insulin-perfused probe (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2}) during an overnight fast and a subsequent oral glucose tolerance test. Additionally, the insulin delivery rates of the MP_I probe are shown in the upper graph. Data are shown as means ± SE, n = 10.

9.3.2. AGREEMENT BETWEEN ISF-DERIVED ESTIMATES AND DIRECTLY MEASURED PLASMA GLUCOSE LEVELS

The analysis of the agreement between plasma glucose and tissue glucose concentration was performed using Bland and Altman analysis of residuals, median absolute difference and Error grid analysis.

9.3.2.1. BLAND & ALTMAN ANALYSIS OF RESIDUALS:

Bland and Altman Analysis of residuals is a common method to compare different measurement techniques[32]. The means and two standard deviations (2SD) of the residuals were calculated for each subject and probe (**Table 9-8**) The median residual means and 2SD values calculated for the insulin-perfused probes were 5.6 % (-6.0–12.4 %) and 31.6 % (26.4–34.6 %), respectively. The statistical analysis showed, that there was no difference between the median means of the residuals of the insulin perfused-probe and the mannitol-perfused probes ($p = 0.67$, tested with Friedman test). The 2SD of the residuals also indicated no difference between the insulin perfused-probe and the mannitol-perfused probes ($p = 0.50$, tested with Friedman test).

Table 9-8: Bland and Altman analysis of residuals – Means and 2 standard deviations (2SD) of residuals obtained for insulin-perfused (MP_I) and mannitol-perfused probes (MP_{M1} and MP_{M2})

	MP_I		MP_{M1}		MP_{M2}	
Subject	$Means_I$ %	$\pm 2SD_I$ %	$Means_{M1}$ %	$\pm 2SD_{M1}$ %	$Means_{M2}$ %	$\pm 2SD_{M2}$ %
01	5.5	18.2	6.2	30.2	-6.4	17.6
02	-14.1	22.9	-10.9	31.2	7.9	36.3
03	-6.5	26.4	-2.3	35.6	-19.4	29.7
04	12.4	35.6	3.2	29.2	9.1	38.1
05	-0.4	34.6	-0.2	22.5	6.6	19.5
06	-6.0	30.2	-15.5	27.8	-2.0	15.1
07	12.9	33.4	-3.4	23.9	-15.2	29.2
08	5.7	74.2	-5.3	34.5	-6.9	25.8
09	18.1	32.9	21.6	61.6	-6.8	39.2
10	11.5	27.0	-21.3	42.2	1.0	21.8
Median [a,b]	5.6	31.6	-2.9	30.7	-4.2	27.5
25^{th}	-6.0	26.4	-10.9	27.8	-6.9	19.5
75^{th}	12.4	34.6	3.2	35.6	6.6	36.3
IQR	18.4	8.2	14.1	7.8	13.5	16.8

[a] no difference between median $Means_I$, $Means_{M1}$ and $Means_{M2}$ values; $p = 0.67$ with Friedman test

[b] no difference between median $2SD_I$, $2SD_{M1}$ and $2SD_{M2}$ values; $p = 0.50$ with Friedman test

9.3.2.2. MEDIAN ABSOLUTE DIFFERENCE

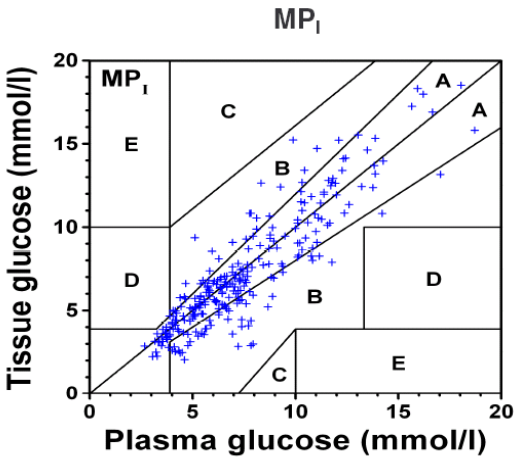
The median absolute difference calculated for the insulin-perfused probe (10.9 %) was comparable to that obtained for the mannitol-perfused probes (vs. 10.5 % and 10.0 % for MP_{M1} and MP_{M2}, respectively).

9.3.2.3. ERROR GRID ANALYSIS

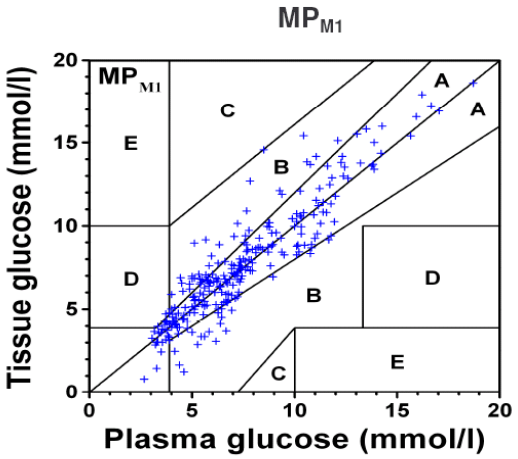
To further evaluate whether the tissue glucose concentrations observed with MP_I, MP_{M1} and MP_{M2} probes can be clinically used to estimate plasma glucose concentrations, tissue glucose was compared with the corresponding plasma glucose using Error Grid Analysis[33] (**Table 9-9** and **Figure 9-7)**. This analysis indicates that the percentage number of the tissue glucose values that fall in the clinically acceptable range (i.e. zones A and B) is high for the insulin-perfused probes (99.6 %) and comparable to that obtained for the mannitol-perfused probes (vs. 99.2 % for MP_{M1} and 98.8 % for MP_{M2}, $p > 0.87$ with Fisher's exact test).

Table 9-9: Error grid analysis results – Numbers and percentages of samples in region A, B, C, D & E for MP_I, MP_{M1} and MP_{M2} probes.

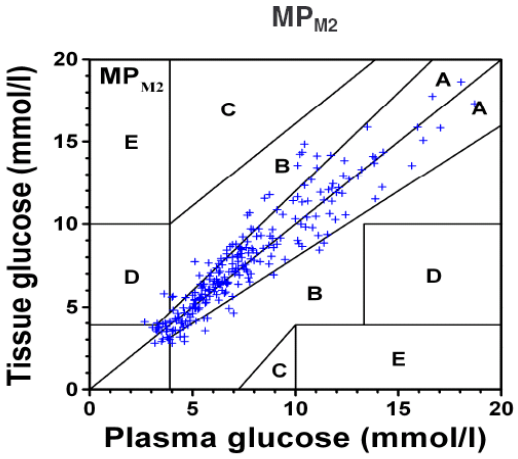
	MP_I	MP_{M1}	MP_{M2}
Region A	191 (71.2 %)	206 (77.4 %)	203 (81.9 %)
Region B	76 (28.4 %)	58 (21.8 %)	42 (16.9 %)
Region C	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Region D	1 (0.4 %)	2 (0.8 %)	3 (1.2 %)
Region E	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Overall	268 (100 %)	266 (100 %)	248 (100 %)



A: 71.2% B: 28.4% C: 0.0% D: 0.4% E: 0.0%



A: 77.4% B: 21.8% C: 0.0% D: 0.8% E: 0.0%



A: 81.9% B: 16.9% C: 0.0% D: 1.2% E: 0.0%

Figure 9-7: Error Grid Analysis results for insulin-perfused MP_I probe and mannitol-perfused MP_{M1} and MP_{M2} probes. As the results for MP_I probe show, one of 268 data points (0.4%) fell outside the clinically acceptable region A and B. Furthermore, the results for MP_{M1} and MP_{M2} probe show that there were two of 266 (0.8%) and three of 248 (1.2%) data points outside region A and B, respectively.

10. DISCUSSION

One objective of the present study was to develop a method, which allows variable and controlled insulin delivery and simultaneous glucose sampling at a single subcutaneous adipose tissue site (i.e. single-port treatment) in type-1 diabetic patients (**Objective #1**). For this reason, we devised the dual-pump mode to operate a microperfusion (MP) probe: This method comprises the use of two autonomous pumps: One pump is responsible for the probe-inflow, the other one for the probe-outflow. The inflow pump rate of the insulin-perfused MP probe is adjusted periodically, whereas the outflow pump rate is kept constant at a slow pump rate of ~0.5µl/min, to achieve relatively high glucose exchange efficiency. The insulin delivery rates to the subcutaneous adipose tissue are then calculated from the difference between the adjusted inflow and outflow rates of the MP_I probe. Due to the low outflow rate of ~0.5µl/min, the insulin delivery to the SAT via diffusion is neglected in this controlling procedure.

As shown in the plasma glucose time course of **Figure 9-1**, insulin delivery with the MP probe operated in the dual-pump mode was successful both in achieving and maintaining a stable, near normal plasma glucose during the overnight fasting as well as in reestablishing near normal plasma glucose by 4 to 5 hours after ingestion of a standardized glucose load (75 g). Furthermore, as can be seen in **Figure 9-3**, it was also possible to measure glucose and conductivity levels at the subcutaneous insulin delivery site. By applying the ionic reference method, the measured glucose and conductivity levels were then used to derive the ISF glucose concentration. As also shown in **Figure 9-3**, the ISF glucose paralleled the glucose levels observed in plasma during the fasting period as well as after the oral glucose load (i.e. OGTT period).

To compare the ISF glucose levels at the insulin delivery site with those observed in insulin-unexposed SAT (**Objective #2),** we assessed the relationship between the observed plasma and ISF glucose values by calculating Pearson's coefficients of correlation and applying linear regression analysis.

The correlation coefficients indicated that there was a strong linear relationship between plasma glucose and ISF glucose derived with insulin and mannitol-perfused MP probes, and that there was no difference between the coefficients of correlation obtained for insulin and mannitol-perfused probes (**Table 9-1**). Furthermore, the linear regression analysis showed

that the intercepts on the y-axis of the derived lines were not different from zero, indicating that there was a proportional relationship (i.e., Y = B * X) between plasma (X) and ISF (Y) glucose levels for insulin and mannitol-perfused probes (**Table 9-2**). Thus, we performed an additional regression analysis with the straight line forced through the origin of the regression graphs and calculated the plasma-to-ISF glucose ratio (PIGR) for each subject and probe. The results of these analyses showed, that there were no differences between the insulin and mannitol-perfused probes regarding the standard errors (i.e. SE, **Table 9-4**) and root mean square errors (i.e. RMSE, **Table 9-5**) of the slopes as well as the robust coefficients of variation (i.e. rCV, **Table 9-7**) of the PIGR. These results, together with the results from the correlation analysis, indicate that there was *a strong linear relationship between plasma glucose levels and probe-derived ISF glucose values and, most importantly, tissue exposure to insulin did not reduce the strength of this relationship*. In other words: The ISF glucose measurement at the site of variable insulin delivery is as stable as it is in insulin-unexposed tissue.

In contrast to the similar SE and RMSE of the slopes and similar rCV of the PIGR obtained for insulin and mannitol-perfused probes, the analyses further showed that there was a difference of ~16 % in the median slopes (**Table 9-4**) and PIGR (**Table 9-6**) values observed with insulin-perfused probes compared to that observed with mannitol-perfused probes. This difference indicates that the average glucose concentration in the ISF surrounding the insulin-perfused probe was ~16 % lower than that in the ISF surrounding the mannitol-perfused probes. The decrease of the glucose concentration in the ISF surrounding the insulin-perfused probe may occur because of the action of insulin. Previous in vitro studies have shown that insulin increases the glucose uptake in isolated muscle and fat cells[29]. Furthermore, insulin increases arteriovenous glucose difference[34], blood flow[34] and glucose uptake[34,35] in the human forearm and leg as well as it increases the whole body glucose uptake in man[28,34,35]. Therefore, in view of the higher arteriovenous glucose difference, blood flow and glucose uptake in insulin-exposed peripheral tissues, it seems very likely that the glucose concentration in the adipose ISF surrounding the insulin-perfused probe is lower than the glucose concentration in the adipose ISF surrounding the mannitol-perfused probes.

However, contrary to these results, Hermanides et al.[21] showed in recently published data, obtained by using a dual-port treatment approach consisting of a microdialysis probe and a conventional insulin infusion catheter inserted in adipose tissue about 9 mm apart from each other, that insulin did not have a significant influence on the microdialysis-based glucose sensing. By means of ^{125}I-labeled insulin and a scintillation detector, Linde et al.[36] showed

that, after a typical bolus administration, the average cross-sectional area of the insulin depot was ~150 mm² (i.e. radius ~7 mm, assuming a spherical depot[37]). Therefore, the insulin-exposed tissue layer around the shaft of a conventional insulin infusion catheter may have a width of ~7 mm. Thus, a possible reason for not observing a significant effect of insulin on the local tissue glucose concentration by Hermanides et al.[21] may be that, due to the considerable length (25 mm) of the applied microdialysis membrane[21,38], the whole or most parts of the glucose-exchanging membrane - especially the membrane part near the probe outlet - may not have been positioned in the insulin-exposed tissue layer surrounding the insulin infusion catheter.

In our study we could ensure that the tissue surrounding the insulin-perfused probe was exposed to insulin due to the presence of macroscopic perforations in the open-flow microperfusion probe (i.e. no agent-exchange-constricting microdialysis membrane present) and applying the dual-pump operation mode (inflow rate ≥ outflow rate).

Finally, **Objective #3** of our study was to assess the feasibility of estimating plasma glucose concentrations from the ISF glucose levels observed at the insulin delivery site. As linear regression analysis indicated a proportional relationship between plasma and ISF glucose levels for both insulin and mannitol-perfused probes, we calculated the tissue glucose levels (i.e. ISF-derived estimates of plasma glucose levels) from the ISF glucose levels by using a prospective one-point calibration procedure. The analysis of the agreement between plasma glucose and tissue glucose concentration was performed using Bland and Altman analysis of residuals, median absolute difference and Error grid analysis.

The results from Bland and Altman analysis showed that the residual means and 2 standard deviations of the residual means obtained with insulin-perfused probes did not differ from that obtained with mannitol-perfused probes (**Table 9-8**), thereby indicating that the ability of estimating plasma glucose concentrations from the ISF glucose levels observed with the insulin-perfused probes is comparable to that of the mannitol-perfused probes. The median absolute difference calculated for the insulin-perfused probe was also comparable to that of mannitol-perfused probes, confirming the results of the Bland and Altman analysis.

To further evaluate whether the ISF glucose concentrations observed with insulin-perfused probes can be clinically used to estimate plasma glucose concentrations, the tissue glucose levels were compared with the corresponding plasma glucose values using the Error Grid Analysis. The results of this analysis indicate that the percentage number of the tissue glucose values that fall in the clinically acceptable range is high (99.6 %) for the insulin-perfused

probe and comparable to that obtained for the mannitol-perfused probes (**Table 9-9** and **Figure 9-7**).

Thus, taken together, results from the Bland and Altman analysis of residuals, median absolute difference and Error Grid Analysis suggest that estimation of plasma glucose concentrations from the ISF glucose levels directly observed at the adipose tissue site of insulin delivery is feasible and its quality is comparable to that of estimating plasma glucose concentrations from the ISF glucose levels measured in insulin-unexposed tissue.

The surprising result that the glucose concentration, measured at the site of *variable* insulin delivery, can be used to estimate plasma glucose levels, may be explained by the known pharmacodynamics of insulin in adipose tissue. It has been shown that the response to increasing insulin concentration in isolated fat cells[29] is a continuous increase in glucose uptake until a maximal response is reached. This maximal response to insulin is also observed in healthy human subjects at a plasma concentration of 300-1,000 µU/ml[28,34,35]. Further increase in the prevailing insulin concentrations beyond this threshold level (i.e. maximal effective insulin level) does not further increase glucose uptake rates.

In our study we used a standard insulin solution with a concentration of 100 U/ml as probe perfusates. Thus, this extreme high insulin concentration in the perfusates may have evoked insulin concentrations in the tissue surrounding the probe, which were much higher (~ 100,000 times) than the maximally effective insulin concentration. Therefore, despite variable insulin infusion rates, maximally stimulated glucose uptake rates of the adipose tissue cells at the insulin infusion site may have ensured stable plasma-to-ISF glucose gradients and hence stable glucose measurements in the ISF at the site of insulin infusion.

11. CONCLUSION

In our study we developed a new method (i.e. dual-pump operation mode) to operate an open-flow microperfusion probe to accomplish appropriate insulin delivery and glucose sensing at a single subcutaneous adipose tissue site in 10 type-1 diabetic subjects during a fasting period and after ingestion of a standardized glucose load. The insulin delivery with the insulin-perfused probe was successful in achieving glycemic control during the whole duration of the experiment. Furthermore, the ISF glucose concentration obtained with the insulin- and mannitol-perfused probe paralleled the glucose levels observed in plasma, even though the ISF glucose levels obtained with the insulin-perfused probe were ~16 % lower than the glucose levels obtained with the probes perfused with insulin-free solution. As the results of the statistical analysis showed, there was a strong linear and proportional relationship between plasma glucose levels and probe-derived ISF glucose values and, most importantly, tissue exposure to insulin did not reduce the strength of this relationship. Hence, estimates of plasma glucose levels have been calculated from the ISF glucose levels obtained with insulin- and mannitol-perfused probes by using a prospective one-point calibration procedure. The results from the Bland and Altman analysis of residuals, median absolute difference and error grid analysis suggest that estimation of plasma glucose concentrations from the glucose levels directly observed at the adipose tissue site of insulin delivery is feasible and its quality is comparable to that of estimating plasma glucose concentrations from the glucose levels measured in insulin-unexposed adipose tissue.

Thus, the integration of insulin delivery and glucose measurement at a single subcutaneous adipose tissue site is feasible. This single-port treatment approach could be used to simplify and improve glucose management in type-1 diabetic patients and may provide the basis for the future development of a simplified closed-loop system, able to self-regulate insulin delivery (artificial pancreas).

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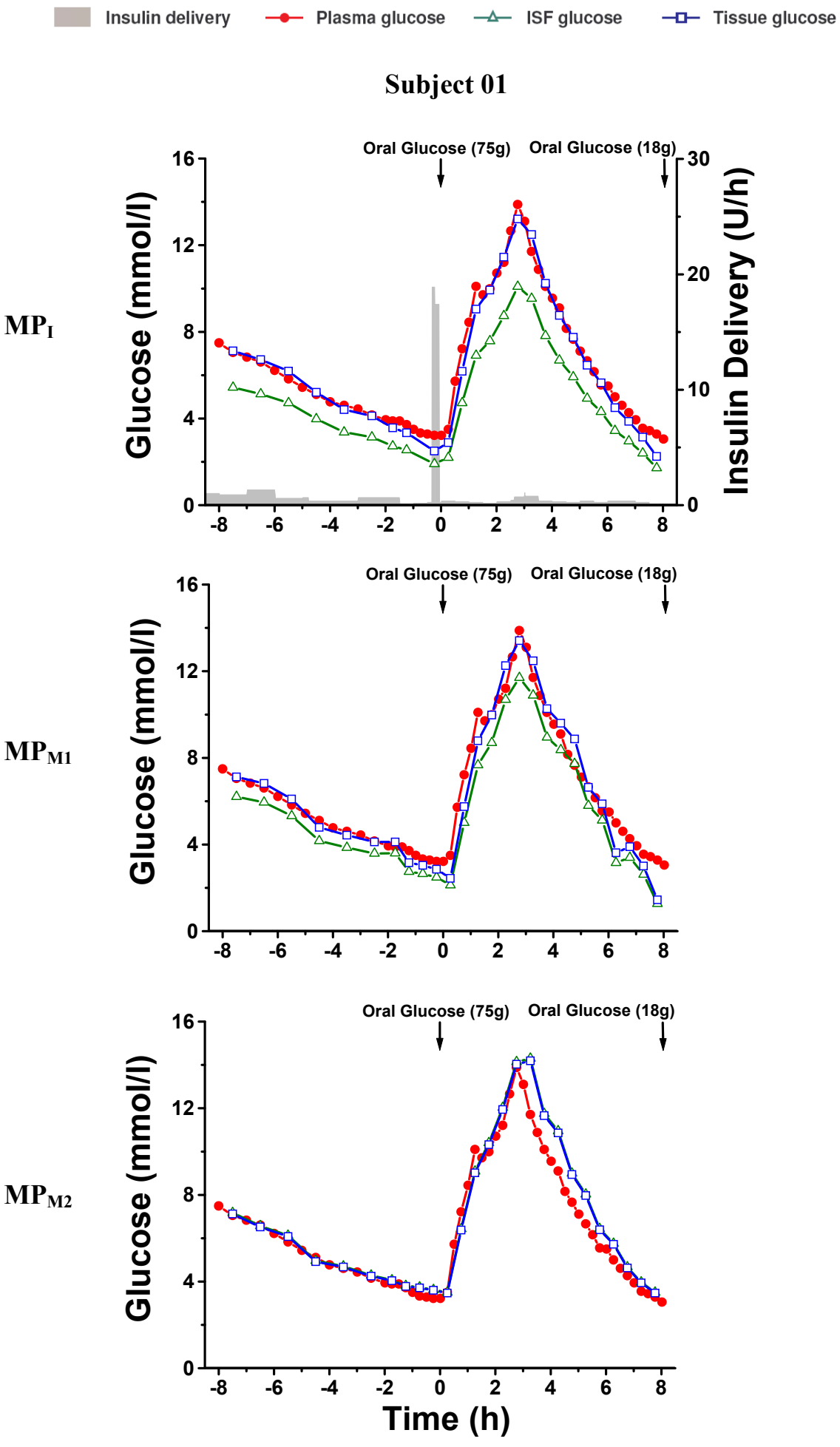
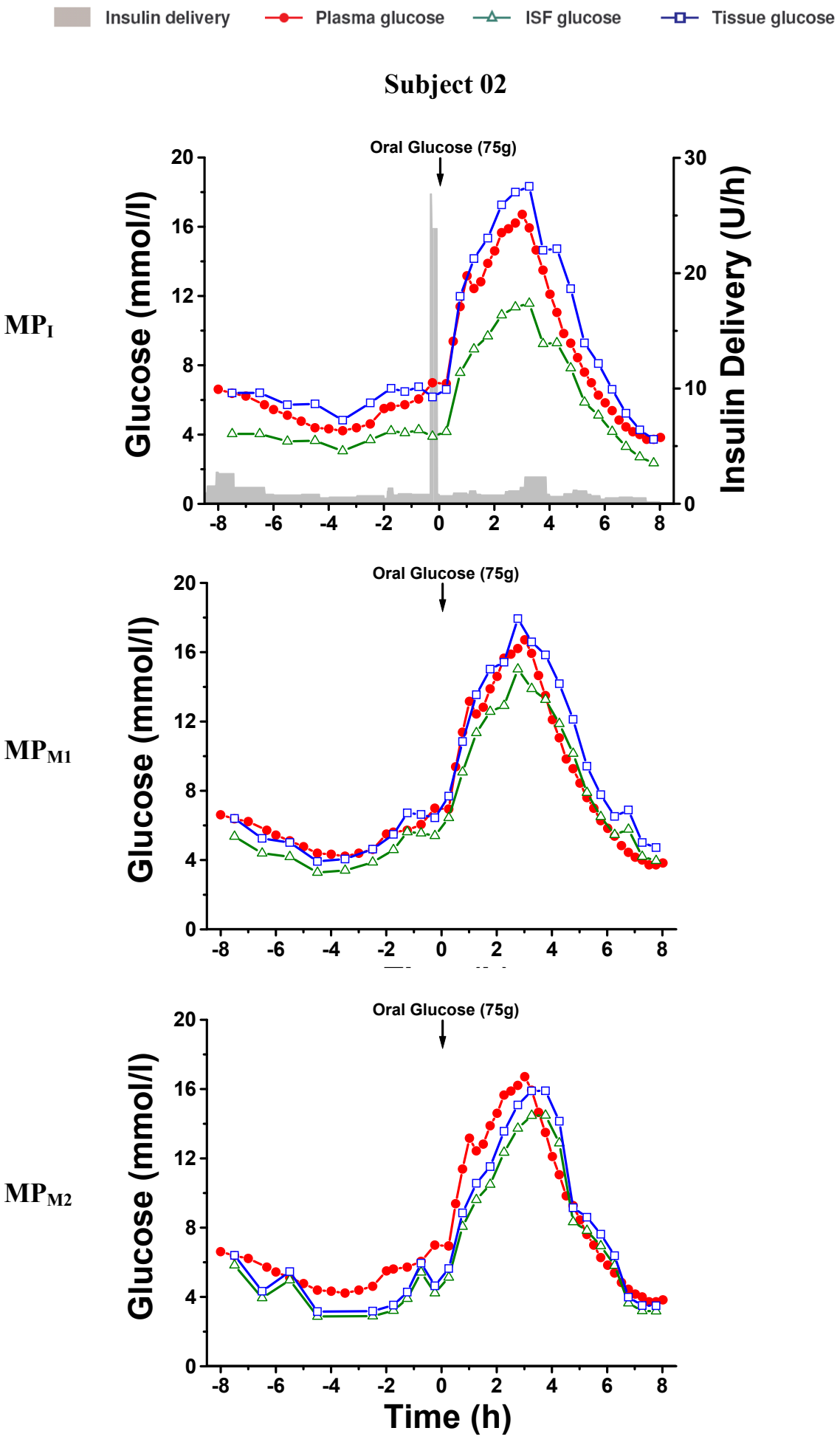
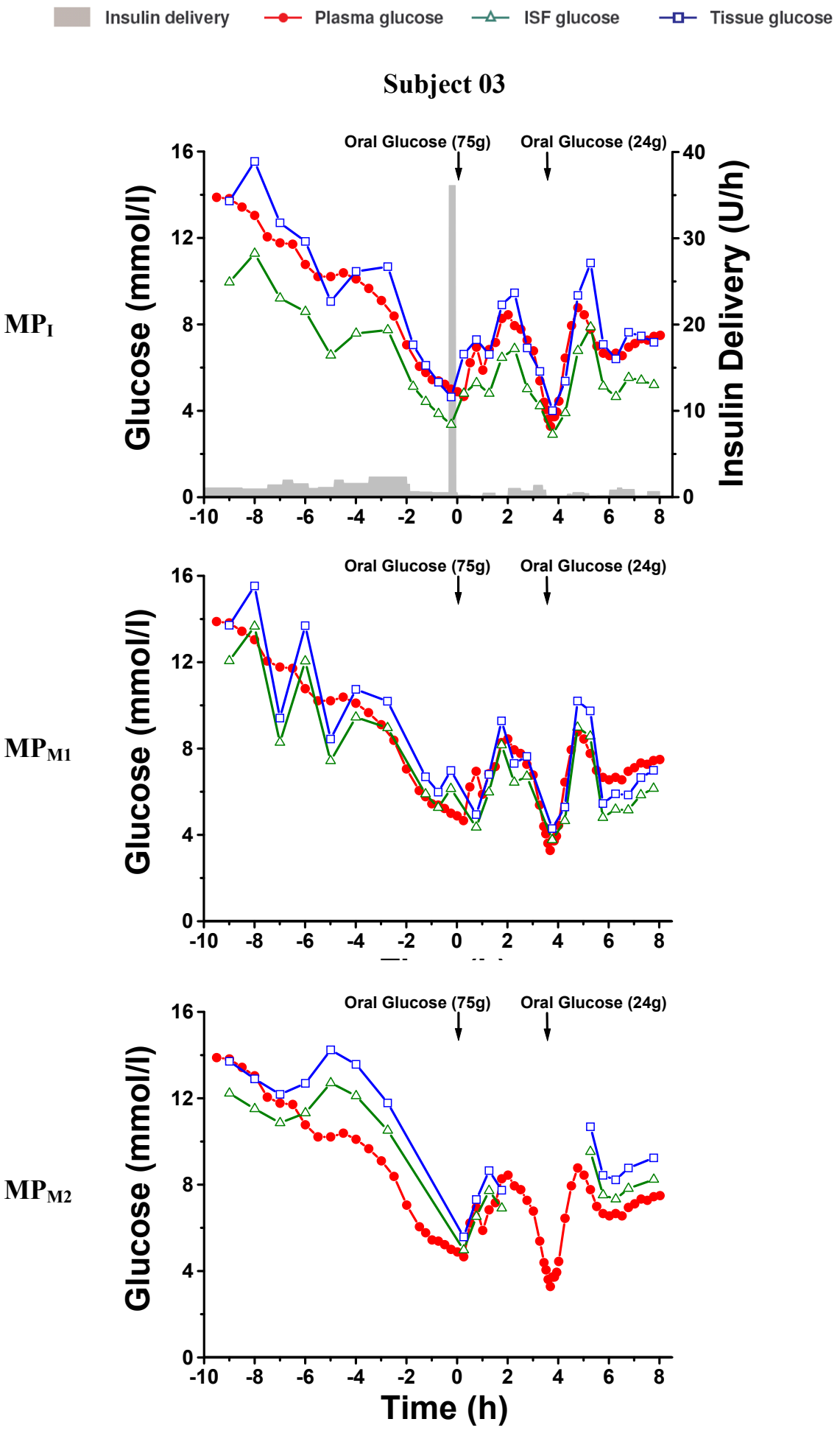
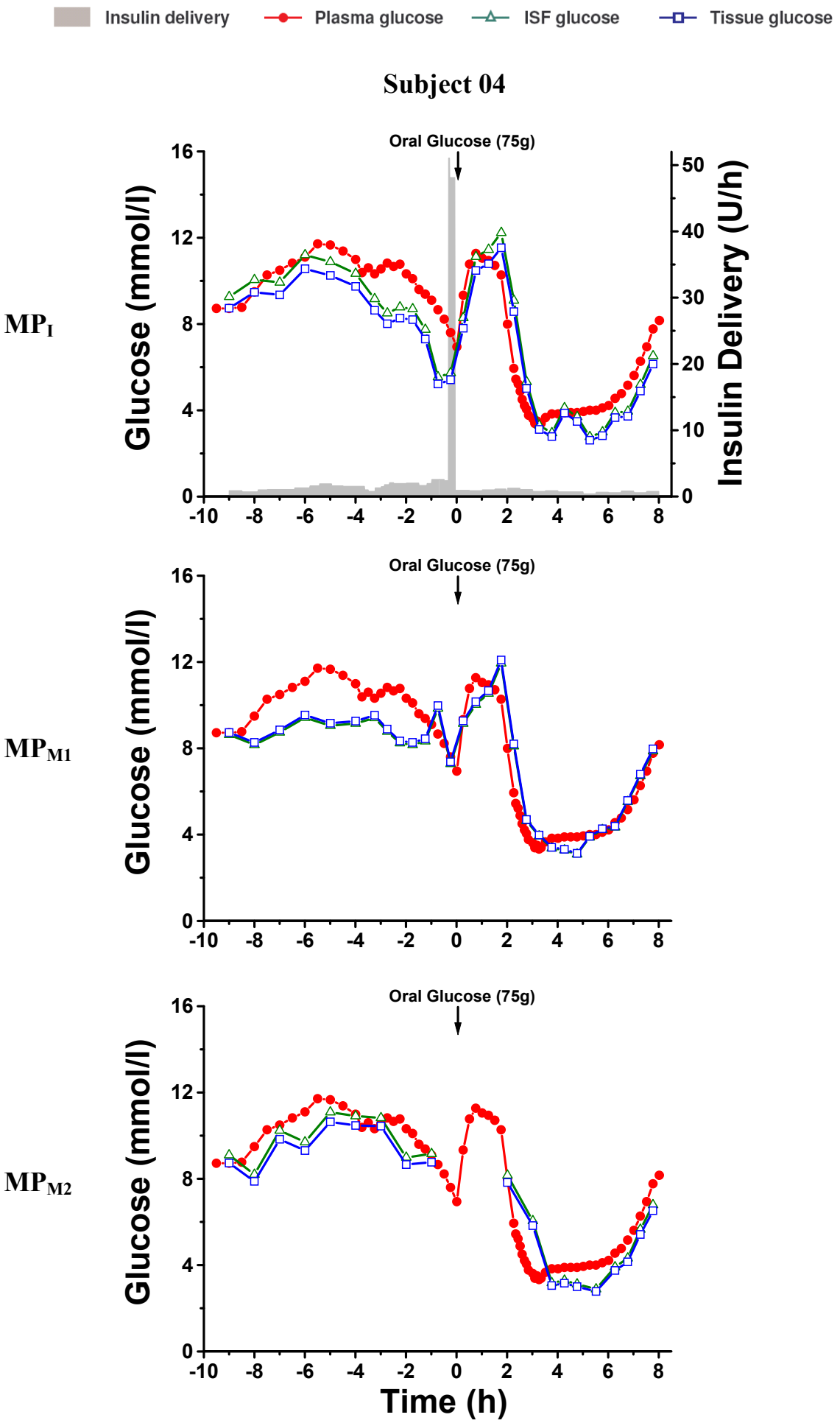
13. APPENDIX

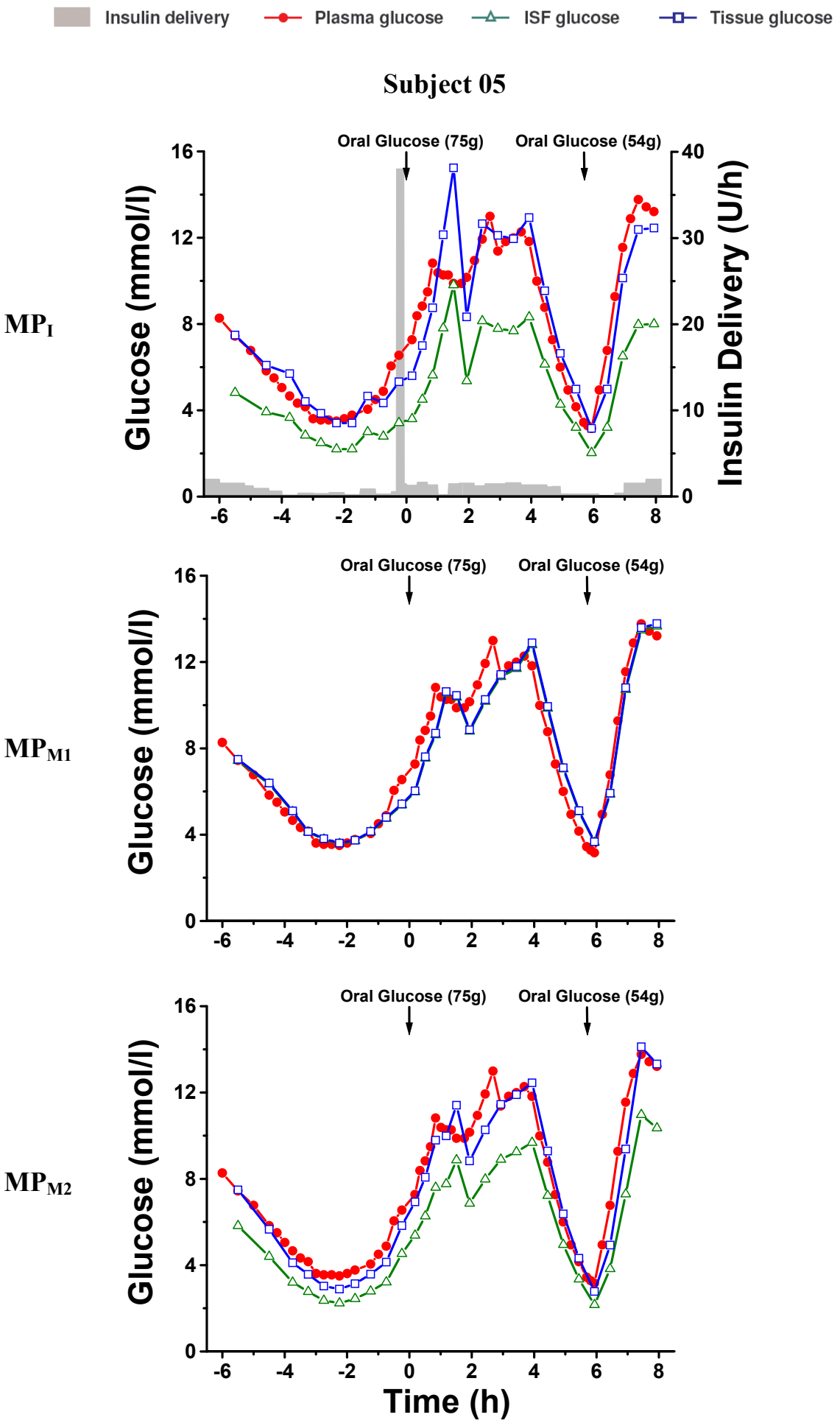
Comparison of plasma and ISF-derived glucose concentrations observed during an overnight fast and a subsequent oral glucose tolerance test in 10 diabetic subjects. Upper panels show the time courses of the plasma glucose and the ISF glucose levels obtained with the MP_I probes used for glucose sampling and simultaneous insulin delivery. Also shown are the time courses of the calibrated ISF-glucose concentration (i.e. tissue glucose) and the insulin delivery rates applied to accomplish glycemic control during the experiments. Middle and lower panels show the time courses of the plasma glucose as well as the ISF and tissue glucose levels obtained with the mannitol-perfused control probes MP_{M1} and MP_{M2}.

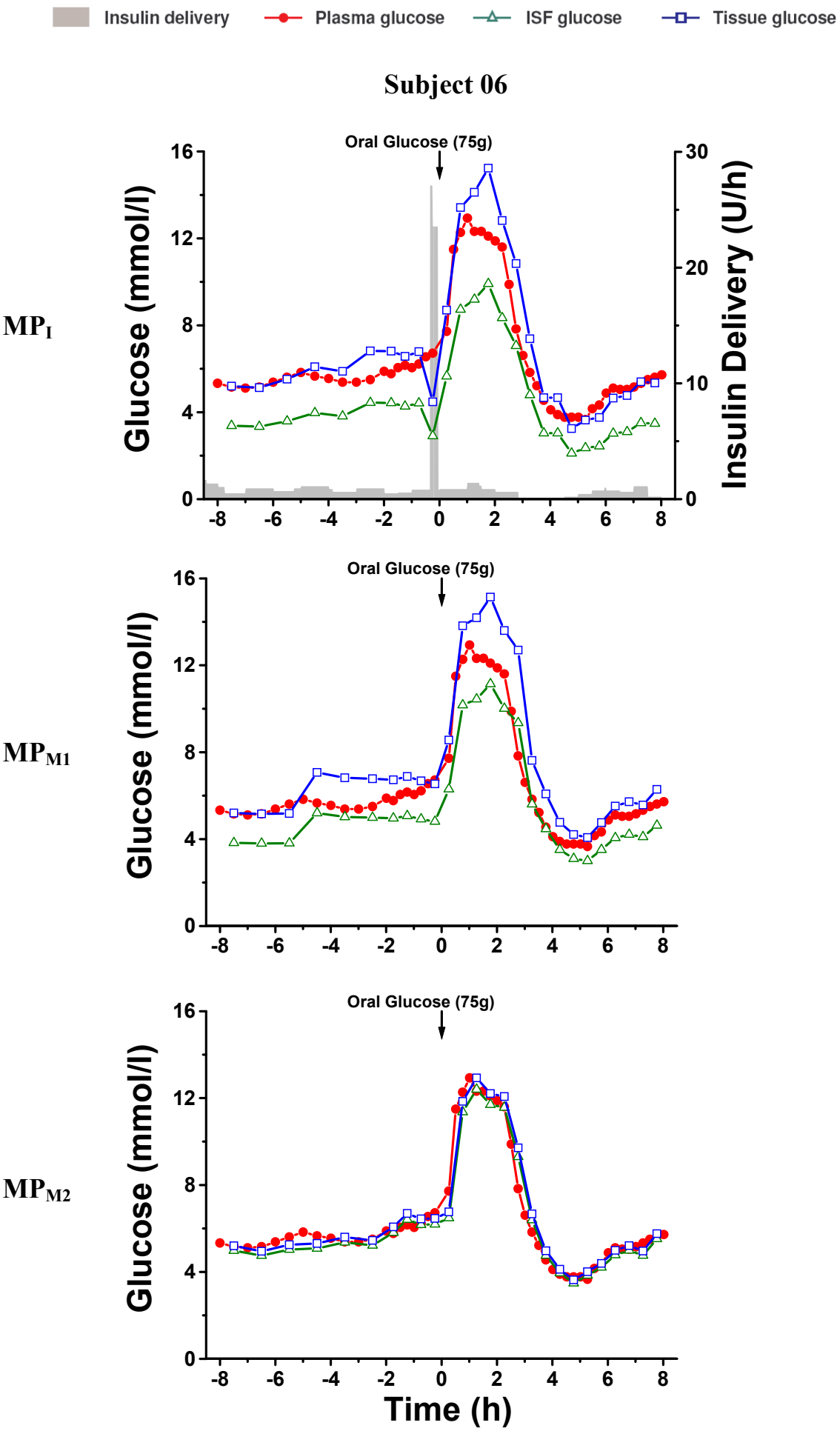

Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 01
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Oral Glucose (18g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)

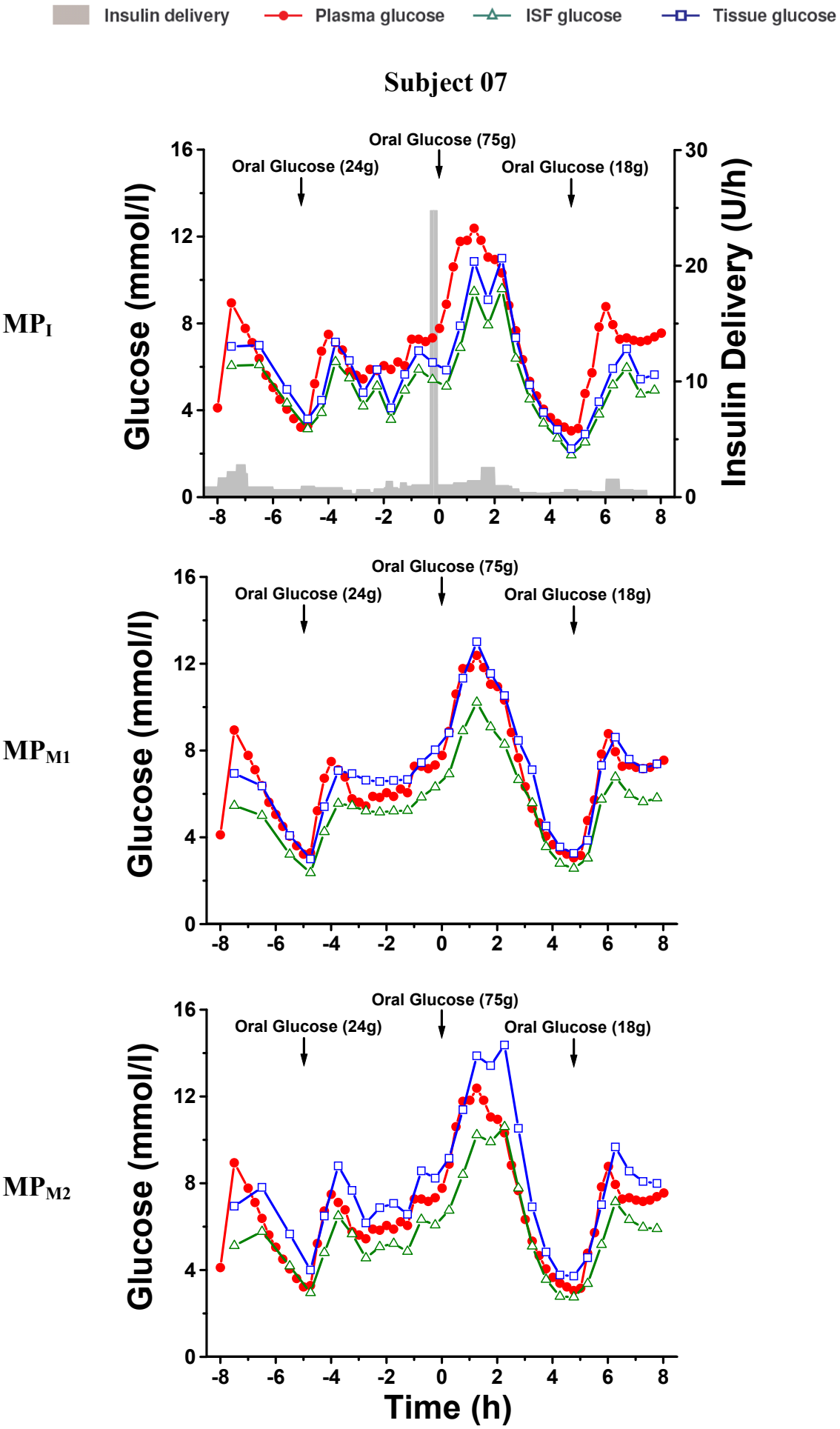

Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 02
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)

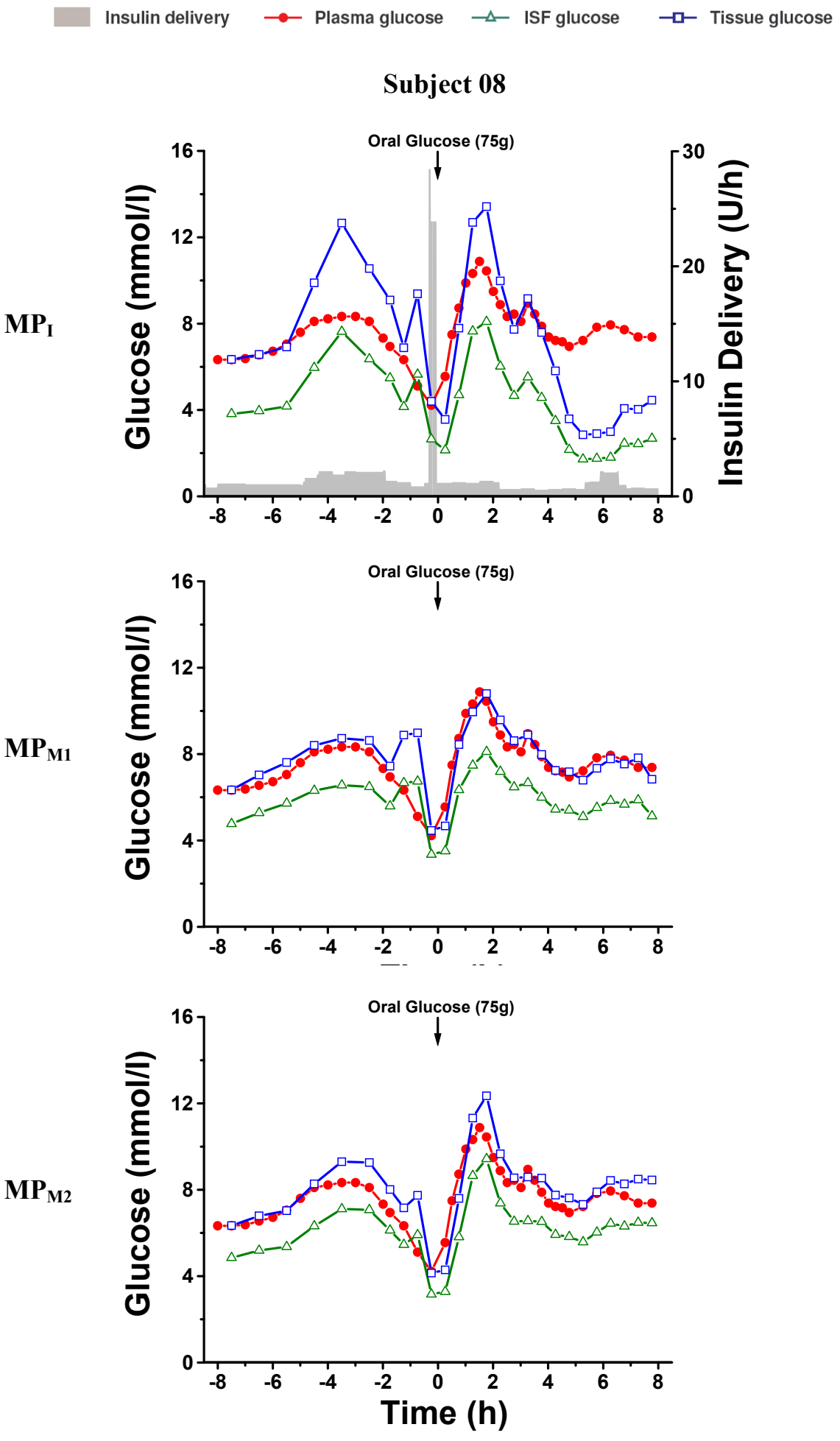

Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 03
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Oral Glucose (24g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)

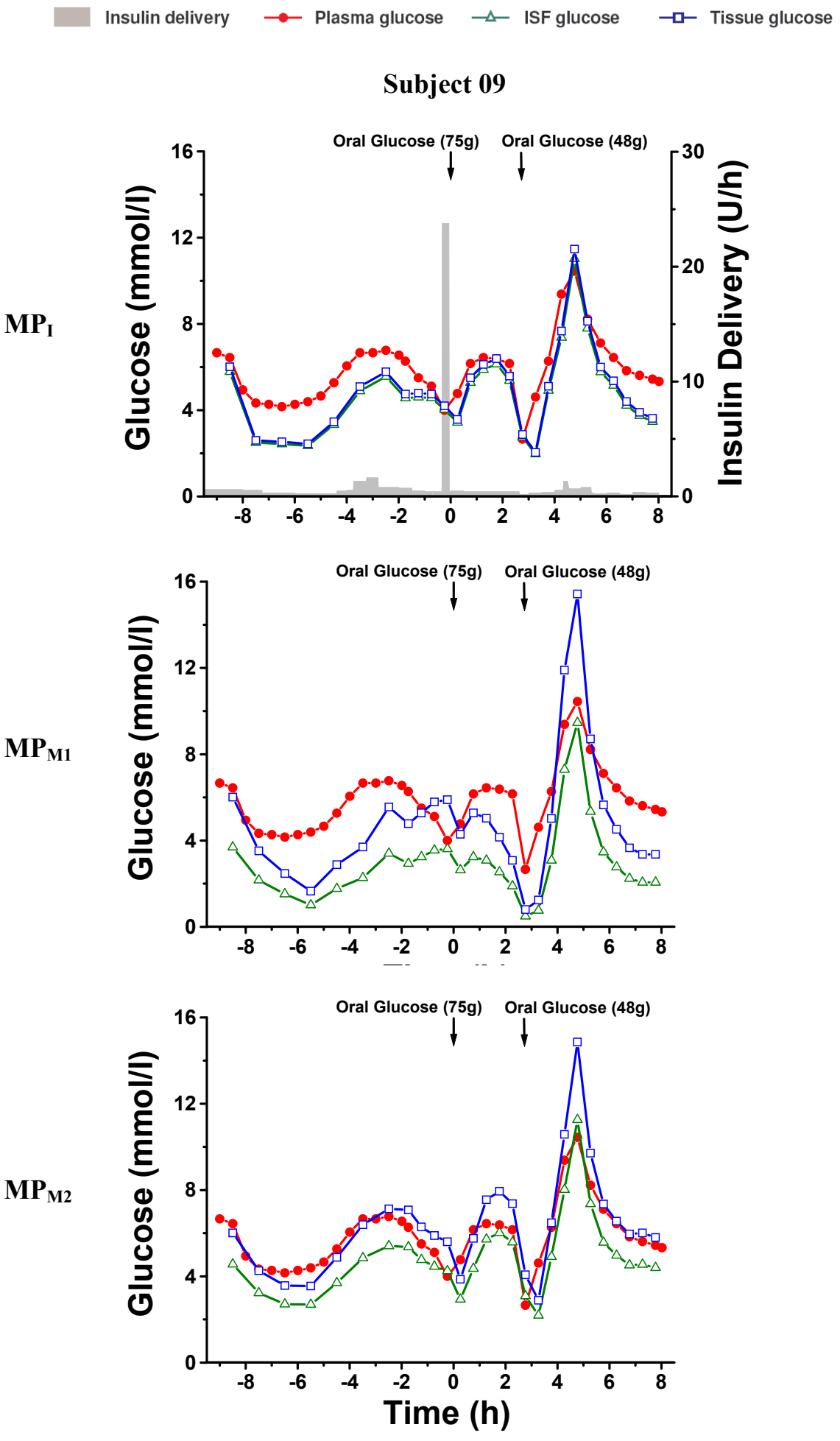

Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 04
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
0
4
8
12
16
0
10
20
30
40
50
-10
-8
-6
-4
-2
0
2
4
6
8

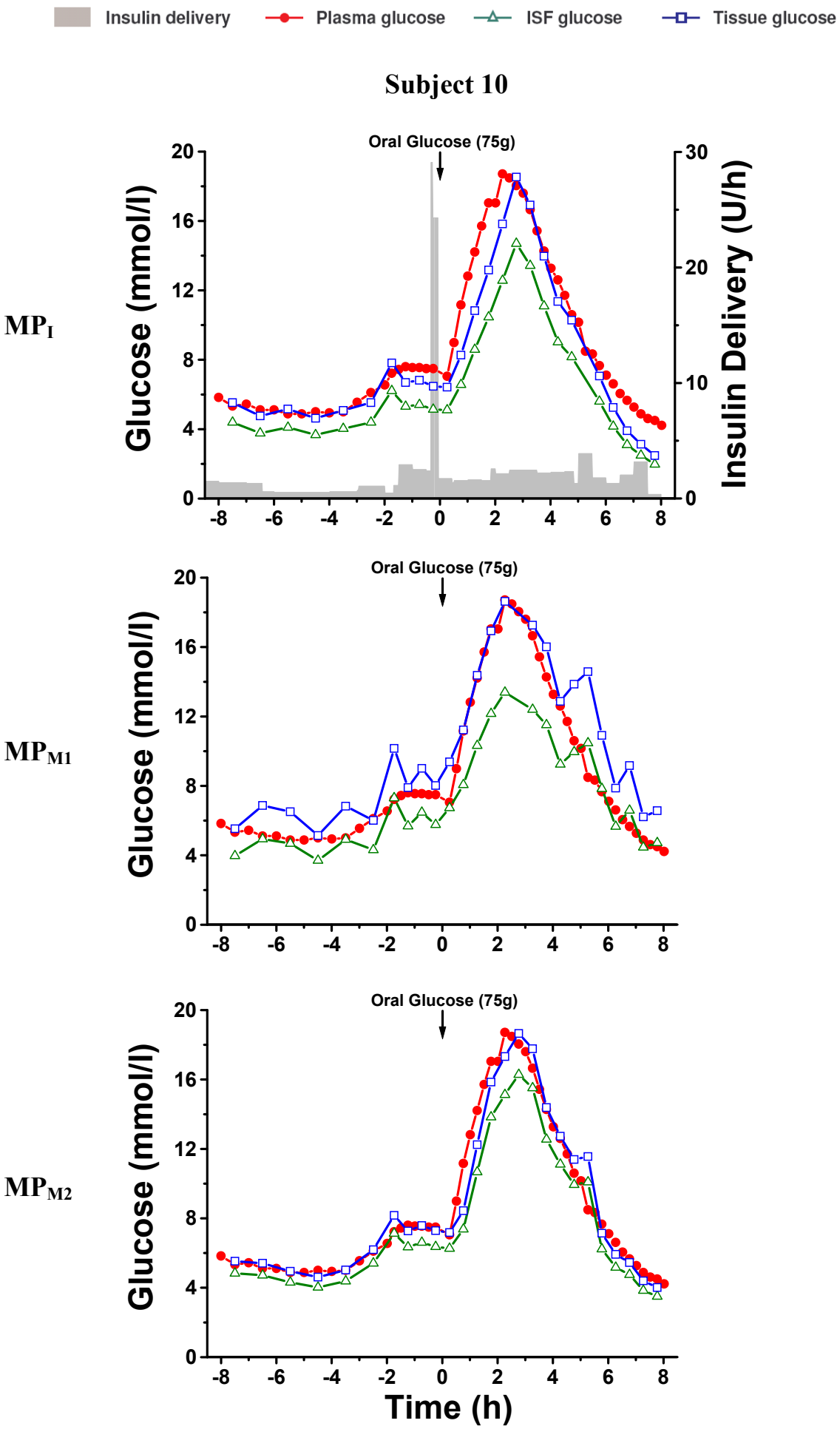

Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 05
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Oral Glucose (54g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
0
4
8
12
16
0
10
20
30
40
-6
-4
-2
0
2
4
6
8


Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 06
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
16
12
8
4
0
30
20
10
-8
-6
-4
-2
0
2
4
6
8


Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 07
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Oral Glucose (24g)
Oral Glucose (18g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)


Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 08
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
0
4
8
12
16
0
10
20
30
-8
-6
-4
-2
0
2
4
6
8


Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 09
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Oral Glucose (48g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
0
4
8
12
16
20
30
10
-8
-6
-4
-2
0
2
4
6
8


Insulin delivery
Plasma glucose
ISF glucose
Tissue glucose
Subject 10
MP$_I$
MP$_{M1}$
MP$_{M2}$
Oral Glucose (75g)
Glucose (mmol/l)
Insulin Delivery (U/h)
Time (h)
0
4
8
12
16
20
-8
-6
-4
-2
0
2
4
6
8
10
20
30

14. CURRICULUM VITAE

Stefan Lindpointner, M.D.

PERSONAL DATA:

Date and Place of Birth:	May 27, 1978, Graz, Austria
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EDUCATION:

1988 - 1996	BRG Seebachergasse (Highschool)
2005	Clinical Traineeship in Bangkok / Thailand and Wenzhou / China
1997 - 2006	Study of Medicine, Medical University of Graz (M.D. 2006)
2006 - 2009	Doctoral Degree Programme in Medical Science, Medical University of Graz

POSITIONS:

1996 - 1997	Civilian Service, Diakonissen Hospital, Schladming
2005 - 2006	Assistance of Surgery and postoperative Treatment, Private Hospital Hansa, Graz
06 / 2006 – 12 / 2008	Scientist at the Clinical Research Center, Medical University of Graz, Diabetes and Metabolism