DERMAL OPEN FLOW MICROPERFUSION (dOFM)
DESIGN, EVALUATION, RESEARCH

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DOCTORAL THESIS

to achieve the university degree of
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Graz, June 2017

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Preface

The work reported herein was carried out from January 2001 – June 2017. The thesis summarizes (i) the design of (dermal) open-flow microperfusion sampling devices, (ii) the clinical evaluation of the devices for pharmaceutical research on human subjects and (iii) describes the clinical and preclinical pharmaceutical research these novel devices have enabled. Most of the technical advances reported herein have been published by the author in international patent applications, biomedical conference proceedings and peer-reviewed papers in medical journals with a methodological focus. The clinical pharmaceutical research subsequently performed with these devices has been published in peer-reviewed research papers in renowned international pharmaceutical journals. As most of the research applications have already been published in issue-specific journals, this thesis aims to complement that information summarizing the methodological achievements from the perspective of medical device design when faced with the biological, pharmaceutical and regulatory challenges linked with these applications. The central chapter describes the design process of the main components thus providing a chronology of the step-by-step device development and in vivo evaluation. This central design chapter also summarizes the most important clinical studies in order to link device design with scientific applications and publications. By describing also some unpublished experiments important for the design process, the author provides insight into the scientific problems and the experimental knowledge gain as the main driver of innovation.

This doctoral thesis is based on a selection of technical and scientific publications reporting the invention of sampling devices (patents), the design of sampling devices (proceedings), their clinical evaluation and their utilization in clinical-pharmaceutical research (peer-reviewed research papers, book chapter).

The author's contribution to these publications is described in the central chapter of this thesis (Chapter 2, OFM Design and Pharmaceutical Research). The publications are provided in copies in the Appendix as far as permitted by the copyrights of the journals.

An overview of all publications as first author and co-author is provided in the section "PUBLICATIONS".

PUBLICATIONS

(i) This thesis is based on the following technical and scientific publications:

PATENTS

M. Bodenlenz and L. Schaupp, “Catheter having an oblong slit” WO002007131780A12007. EP;USA;CA; **2007**. [1]

M. Bodenlenz, C. Hoefferer, T. Birngruber, and L. Schaupp, “Filament-based catheter” WO2010/0315152010. EP;USA;CA; **2010**. [2]

M. Bodenlenz, C. Hoefferer, T. Birngruber, R. Schaller, J. Priedl, F. Feichtner, S. Schock, P. Tkaczyk, and L. Schaupp, “Pump for medical applications” DE102011090210A12011. EP;US;CA; **2011**. Patent pending. [3]

RESEARCH PAPERS (peer-reviewed, in medical/pharmaceutical journals)

F. Kolbinger, C. Loesche, M.-A. Valentin, X. Jiang, Y. Cheng, P. Jarvis, T. Peters, C. Calonder, G. Bruin, F. Polus, B. Aigner, D. M. Lee, M. Bodenlenz, F. Sinner, T. R. Pieber, and D. Patel, “ß-defensin-2 is a responsive biomarker of IL-17A-driven skin pathology in psoriasis,” J Allergy Clin Immunol, vol. 139, no. 3., pp. 923-932.e8, Mar. **2017**.[4]

M. Bodenlenz, K. I. Tiffner, R. Raml, T. Augustin, C. Dragatin, T. Birngruber, D. Schimek, G. Schwagerle, T. R. Pieber, S. G. Raney, I. Kanfer, and F. Sinner, “Open Flow Microperfusion as Dermal Pharmacokinetic Approach to Evaluate Topical Bioequivalence” Clin. Pharmacokinet., vol. 56, no. 1, pp.91-98, Jan. **2017**. [5]

M. Bodenlenz, C. Dragatin, L. Liebenberger, B. Tschapeller, B. Boulgaropoulos, T. Augustin, R. Raml, C. Gatschelhofer, N. Wagner, K. Benkali, F. Rony, T. Pieber, and F. Sinner, “Kinetics of Clobetasol-17-Propionate in Psoriatic Lesional and Non-Lesional Skin Assessed by Dermal Open Flow Microperfusion with Time and Space Resolution” Pharm. Res., vol. 33, no. 9, pp.2229-2238, Sep. **2016**. [6]

C. Dragatin, F. Polus, M. Bodenlenz, C. Calonder, B. Aigner, K. I. Tiffner, J. K. Mader, M. Ratzer, R. Woessner, T. R. Pieber, Y. Cheng, C. Loesche, F. Sinner, and G. Bruin, “Secukinumab distributes into dermal interstitial fluid of psoriasis patients as demonstrated by open flow microperfusion.,” Exp. Dermatol., vol. 25, no. 2, pp. 157–9, Feb. **2016**. [7]

M. Bodenlenz, M. Ellmerer, L. Schaupp, L. V Jacobsen, J. Plank, G. A. Brunner, A. Wutte, B. Aigner, S. I. Mautner, and T. R. Pieber, “Bioavailability of insulin detemir and human insulin at the level of peripheral interstitial fluid in humans, assessed by open-flow microperfusion.,” Diabetes. Obes. Metab., vol. 17, no. 12, pp. 1166–72, Dec. **2015**. [8]

M. Bodenlenz, B. Aigner, C. Dragatin, L. Liebenberger, S. Zahiragic, C. Höfferer, T. Birngruber, J. Priedl, F. Feichtner, L. Schaupp, S. Korsatko, M. Ratzer, C. Magnes, T. R. Pieber, and F. Sinner, “Clinical applicability of dOFM devices for dermal sampling.,” Skin Res. Technol., vol. 19, no. 4, pp. 474–483, Apr. **2013**. [9]

M. Bodenlenz, C. Höfferer, C. Magnes, R. Schaller-Ammann, L. Schaupp, F. Feichtner, M. Ratzer, K. Pickl, F. Sinner, A. Wutte, S. Korsatko, G. Köhler, F. J. Legat, E. M. Benfeldt, A. M. Wright, D. Neddermann, T. Jung, and T. R. Pieber, “Dermal PK/PD of a lipophilic topical

drug in psoriatic patients by continuous intradermal membrane-free sampling.," Eur. J. Pharm. Biopharm., vol. 81, no. 3, pp. 635–41, Aug. **2012**. [10]

R. Holmgaard, E. Benfeldt, J. B. Nielsen, C. Gatschelhofer, J. A. Sorensen, C. Höfferer, M. Bodenlenz, T. R. Pieber, and F. Sinner, "Comparison of open-flow microperfusion and microdialysis methodologies when sampling topically applied fentanyl and benzoic acid in human dermis ex vivo.," Pharm. Res., vol. 29, no. 7, pp. 1808–20, Jul. **2012**. [11]

K. E. Pickl, C. Magnes, M. Bodenlenz, T. R. Pieber, and F. M. Sinner, "Rapid online-SPE-MS/MS method for ketoprofen determination in dermal interstitial fluid samples from rats obtained by microdialysis or open-flow microperfusion.," J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., vol. 850, no. 1–2, pp. 432–9, May **2007**. [12]

M. Bodenlenz, L. A. Schaupp, T. Druml, R. Sommer, A. Wutte, H. C. Schaller, F. Sinner, P. Wach, and T. R. Pieber, "Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access," Am.J.Physiol Endocrinol.Metab, vol. 289, no. 0193–1849, pp. E296–E300, Aug. **2005.** [13]

PROCEEDINGS

M. Bodenlenz, C. Dragatin, C. Hoefferer, T. Birngruber, J. Priedl, F. Feichtner, R. Schaller, B. Aigner, S. Korsatko, T. R. Pieber, and F. Sinner, "Certified dOFM sampling devices provide access to target tissue in pharmaceutical trials.," in Biomedizinische Technik. Biomedical engineering, **2013**. [14]

M. Bodenlenz, C. Hoefferer, F. Feichtner, C. Magnes, R. Schaller, J. Priedl, T. Birngruber, F. Sinner, L. Schaupp, S. Korsatko, and T. R. Pieber, "Novel catheters for in vivo research and pharmaceutical trials providing direct access to extracellular space of target tissues", in *IFMBE Proceedings, The 12th Mediterranean Conference on Medical and Biological Engineering and Computing – MEDICON 2010*, **2010**, vol. 29, pp. 268–269. [15]

M. Bodenlenz, R. Hellmich, H. Wedig, R. Schaller, C. Hoefferer, G. Koehler, B. Gruendig, C. Zaugg, T. Vering, C. Patte, P. Wach, and T. R. Pieber, "Tragbares Kontinuierliches Laktatmonitoring bei sportlicher Belastung", in *Biomed Tech - Proceedings Gemeinsame Jahrestagung der DGBMT, OEGBMT und SGBT*, ETH Zuerich, Schweiz, 6.-9.9.2006., **2006**, vol. CD ROM. Be, p. V93. [16]

M. Bodenlenz, H. C. Schaller, R. Sommer, A. Wutte, T. Druml, F. Sinner, W. Regittnig, L. Schaupp, T. R. Pieber, and P. Wach, "Hormonmessung in peripheren Geweben mittels Offener Mikroperfusion und der No-Net-Flux Technik", in *Biomedizinische Technik/Biomedical Engineering*, **2003**, vol. 48, no. s1, pp. 318–319. [17]

BOOK CHAPTERS

T. R. Pieber, T. Birngruber, M. Bodenlenz, C. Höfferer, S. Mautner, K. Tiffner, and F. Sinner, "Open Flow Microperfusion: An Alternative Method to Microdialysis?", in *Microdialysis in Drug Development(AAPS Advances in the Pharmaceutical Sciences Series)*, M. Müller, Ed. Springer New York, **2012**, pp. 283–302. [18]

AWARDS

for dermal OFM methodology & devices:

- Styrian Fast Forward Award, Graz, Austria 2011(1st)
- European EARTO Innovation Prize, Brussels, Belgium 2012 (2nd)

The key research papers for this thesis are summarized in the main chapter. Papers not protected by copyright are provided in full-text in the appendix of this thesis.

(ii) Further publications of the author:

- 13 peer-reviewed research papers as co-author [19–31]
- 4 proceedings in the field of Biomedical Engineering as co-author [32–35]
- 32 conference presentations as first author [36–67]
- 69 conference presentations as co-author [68–136]

Table 1 provides the number of citations and two common citation indices as measures of the author's research output and the impact of the above listed publications.

Table 1: Citation indices

Citation Indices	All	Since 2012
citations	958	384
h-index[1]	13	11
i10-index[2]	16	12

[1] h-index: Number of papers (h) with a citation number ≥ h. [2] i10-index: The number of publications with at least 10 citations. *Source:* Google Scholar, in August 2017.

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“When a man does not know what harbor he is making for, no wind is the right wind.”
(Original lat.: "Ignoranti quem portum petat nullus suus ventus est.")
Marcus Lucius Annaeus Seneca, 4 v.Chr.- 65 n.Chr.

Proven, well-known and repeatedly cited. Unfortunately, the significance of this insight is often forgotten in every day work. The author of this thesis has experienced how important it is to formulate a clear vision and long-term goals in a research team.

“It is not because things are difficult that we do not dare, it is because we do not dare that they are difficult.”
(Original lat.: Non quia difficilia sunt non audemus, sed quia non audemus difficilia sunt”)
The same – i.e. Marcus Lucius Annaeus Seneca

Successful research and innovation is associated with taking risks, controversial debates and conflicts. I thank all those persons, funding organisations and study sponsors taking a risk with me to invest in open-flow microperfusion research methodology.

“Slow and steady wins the race”
… once said by a foreign PhD-student working on other sampling devices

This student has been criticized for saying this, but he spoke from my own experience. Well, I did a number of spontaneous experiments for the fast knowledge gain, and some of those which I did on my own body in the early years would not be acceptable these days. However, I’m convinced that the foreign student was right: It is still most efficient in our fast (research) world to take the time to think and to recap and thus to create a sound scientific basis ensuring sustainable progress in your R&D. Your endurance and the strong scientific basis will finally be key when you need to convince funding organisations and investors to provide the significant financial resources which are always required for a breakthrough in medicine and pharmaceutical sciences.

DERMAL OPEN FLOW MICROPERFUSION (dOFM)

DESIGN, EVALUATION, RESEARCH

DI Manfred Bodenlenz

Doctoral Thesis

Abstract

This thesis summarizes (i) the design of novel sampling devices for continuous sampling from the skin and subcutaneous tissue, (ii) the evaluation of the devices at the clinic on volunteers and patients and (iii) the utilization of the devices in clinical pharmaceutical trials proving the devices' utility for clinical pharmacokinetics and -dynamics (PK-PD) as well as topical bioequivalence. The thesis has been motivated by the needs in basic medical research and pharmaceutical industry for an alternative clinical sampling method which can provide direct access to human tissues for the investigation of a wider range of biomolecules and drugs than is currently feasible by state-of-the-art microdialysis. The work focused on device applicability in the field of dermatology as the requirements in dermatological PK, PD and bioequivalence trials were most challenging considering the physico-chemical properties of the topical drugs designed for skin penetration and considering the specific needs of efficient and reliable clinical test settings. Consequently, this led to CE-certified medical devices for open-flow microperfusion (OFM) and specifically for dermal open-flow microperfusion (dOFM) which proved to be safe, tolerable, precise and versatile in clinical research supporting very long and data-rich multi-probe research protocols on virtually any molecule of interest in the interstitial fluid space. The devices enabled a number of clinical studies on healthy volunteers and patients providing important insights into the barrier function of psoriatic skin for topical drug penetration, on biomarkers in psoriasis, on therapeutic antibody concentrations within the skin as well as topical bioequivalence of generic drugs. This thesis summarizes the peer-reviewed publications in renowned medical and pharmaceutical journals along with information on the device design and discusses the vast opportunities this novel clinical research tool will provide in future pharmaceutical research.

Key words:

Open-flow microperfusion, microdialysis, pharmacokinetics, pharmacodynamics, topical bioequivalence, biomarkers, sampling, topical, drug, insulin, cytokine, relative recovery, adsorption, tolerability, skin, dermal, subcutaneous, muscle, in vitro, in vivo, ex vivo, clinical trial, medical device safety, risk management, accuracy, precision, reproducibility

Zusammenfassung

Diese Dissertationsschrift fasst zusammen: (i) Die Entwicklung von neuen Instrumenten für die kontinuierliche Probengewinnung aus Haut und Unterhautfettgewebe, (ii) die Evaluierung der Instrumente in klinischen Studien an freiwilligen Studienteilnehmern und Patienten und (iii) die erfolgreiche Nutzung dieser Instrumente für die klinische pharmazeutischen Forschung, welche die Tauglichkeit der Instrumente für klinische pharmakokinetische u. -dynamische (PK-PD) Studien sowie für topische Bioäquivalenzstudien belegen. Die Dissertation schöpfte ihre Motivation aus dem Bedarf in der Medizinischen Grundlagenforschung und der pharmazeutischen Industrie nach einer alternativen Probensammelmethode, welche einen direkten Zugang zu menschlichen Geweben ermöglicht, welche aber weniger Einschränkungen hinsichtlich der untersuchbaren Biomoleküle und Wirkstoffe aufweist als das bestehende Verfahren der Mikrodialyse. Die Arbeit hat sich auf die Anwendbarkeit der Instrumente im Bereich der Dermatologie konzentriert, weil die Anforderungen der dermalen PK-PD und Bioäquivalenz höchst herausfordernd waren in Anbetracht der physikalisch-chemischen Eigenschaften der für die Hautpenetration eingesetzten topischen Wirkstoffe und in Anbetracht der spezifischen Anforderungen für verlässliche klinische Studien zu topischen Produkten. Das führte letztlich zu CE-zertifizierten Medizinprodukten für die offene Mikroperfusion (OFM) und die dermale offene Mikroperfusion (dOFM). Diese haben sich in der klinischen Forschung als sicher, tolerabel, präzis und vielseitig einsetzbar gezeigt. Die Instrumente ermöglichen zeitlich ausgedehnte und datenreiche Studienprotokolle mit einer Vielzahl an Gewebekathetern zu praktisch allen Biomolekülen im interstitiellen Flüssigkeitsraum. Die Instrumente ermöglichten eine Anzahl von klinischen Studien in Gesunden und Patienten. Diese Studien lieferten Aufschlüsse zur Rolle der psoriatischen Haut bei der topischen Wirkstoffpenetration, zu Biomarkern bei Psoriasis, zur Konzentration von therapeutisch eingesetzten Antikörpern in der Haut sowie zur topischen Bioäquivalenz von generischen Produkten. Die Dissertationsschrift fasst die entsprechenden Publikationen in medizinischen und pharmazeutischen internationalen Journalen zusammen. Sie gibt zudem Einblick in die Entwicklungsarbeit und diskutiert die zahlreichen Möglichkeiten, welche dieses klinische Forschungswerkzeug der zukünftigen pharmazeutischen Forschung bietet.

Schlüsselwörter:

Open-flow microperfusion, microdialysis, pharmacokinetics, pharmacodynamics, topical bioequivalence, biomarkers, sampling, topical, drug, insulin, cytokine, relative recovery, adsorption, tolerability, skin, dermal, subcutaneous, muscle, in vitro, in vivo, ex vivo, clinical trial, medical device safety, risk management, accuracy, precision, reproducibility

Abbreviations

AGES	Austrian Agency for Health and Food Safety
BA	Bioavailability
BE	Bioequivalence
CE	„Communauté Européenne“, used to indicate the free movement of products within the EU
Da	Dalton
dOFM	Dermal Open Flow Microperfusion
ECF	Extracellular fluid
EMEA/EMA	European Medicines Agency as of 12/2009 „EMA“
EN ISO	European Standard (or “Norm”) taken from an ISO Standard
FEP	Fluorinated ethylene propylene, a copolymer of hexafluoropropylene and tetrafluoroethylene
FDA	American Food and Drug Association
GCP	Good Clinical Practice
ID	Inner Diameter
IRT	Ionic Reference Technique
ISO	International Organisation for Standardisation
ISF	Interstitial fluid
IV	Intravenous, intravascular
IM	Intra-muscular
kDa	Kilo Dalton
Log P	Partition coefficient of the unionized species in octanol-water
MD	Microdialysis
MWCO	Molecular weight cut off
NNF	No-Net-Flux
OD	Outer diameter
OFM	Open-Flow Microperfusion
PASI	Psoriasis Area Severity Index, a score used to rate psoriasis severity
PD	Pharmacodynamics
PK	Pharmacokinetics
PVC	Polyvinylchloride
PTFE	Polytetrafuoroethylene
RLD	Reference Listed Drug, the originator/innovator product
RR	Relative recovery
SC	Subcutaneous
SoA	State of the Art
TEWL	Transepidermal Water Loss
TSS	Total Sum Score, a score used to rate psoriasis severity

Structure of this thesis

Within this thesis medical devices for clinical research ("design") were created to have adequate tools for the subsequent task of pharmacological or (patho)physiology research on human subjects *in vivo* ("research"). A number of preclinical experiments and the use of the devices in clinical research provided valuable feedback, and virtually any larger clinical study was followed by a redesign of the materials to increase the output of subsequent clinical research. The structure of this thesis, in particular the structure of the main chapter 2, reflects this iterative process of design and research application, which step by step led to advanced sampling devices enabling the advanced clinical research protocols presented at the end of the thesis. In concluding chapters the achievements regarding methodology and research are discussed, current ongoing research is outlined and the future challenges in the field are outlined.

Chapter **1 Introduction** provides the state of the art of the methodology and research as the starting point of the design and research work summarized in this thesis. The principles of *open-flow microperfusion* methodology are outlined and the devices used until the year 2000 are described. A summary is provided of the research which those devices had enabled in the initial years of OFM. Moreover, the state of the art in related research using *microdialysis* is provided, as this thesis aimed to overcome the limitations of both techniques to enable the clinical pharmaceutical in vivo research as presented at the end of this thesis.

Chapter **2 OFM DESIGN AND PHARMACEUTICAL RESEARCH** is the main chapter of this thesis summarizing the design and research results. This chapter is composed as a chronological design and research report, thus providing the link between device design, clinical research and the publications. Each subchapter initially informs about devices/innovation and thereafter summarizes the published and unpublished clinical research each device generation had enabled. As the full-texts of the research papers are provided in the appendix or are publicly accessible anyway, the focus of these brief summaries has been placed on the particular methodological challenges and insights, which usually were not in the focus of the clinical research papers.
Readers who are exclusively interested in clinical research can easily identify those research sections within the main chapter by the headings "***Clinical Research**…***"**. All other research (studies on animals and human donor skin, feasibility studies on humans), which largely remained unpublished but were essential for the methodological advance, are identifiable by the headings "***Experiments** ...*". The summaries also provide information on the author's personal contributions to the research studies and the publications.

Chapter **3 OFM today – a safe, tolerable and reproducible research tool?** discusses whether or to what degree the goal of this thesis has been achieved.

Chapter **4 Current research** briefly outlines the current research applications. The new devices can be considered today's state–of-the-art tools for some clinical research applications, but there are also new applications in clinical and in preclinical research.

Chapter **5 Future challenges** tries to identify where further effort is needed. Methodology or devices will never be perfect or satisfying as the demands of users and health authorities regarding quality and reproducibility of clinical research will continue to rise as observed during the past decade.

Chapter **6 Summary and conclusions** summarizes the advances achieved by this thesis in two areas, those in clinical sampling methodology and those in clinical pharmaceutical research.

Chapter **7 Acknowledgements** mentions a number of important persons whose inputs were essential for design and whose support enabled this work to continue over the extended period of time.

Chapter **8 Bibliography** provides all peer-reviewed publications of the author and also lists most of the poster and oral presentations at conferences. The author's peer-reviewed publications cite all references relevant for the research published. Therefore, the citations within the thesis text were restricted to the most essential references. These essential references (not authored or co-authored) have been simply added to the bibliography to ease compilation of the thesis and the look-up of references.

Chapter **9 Appendix - research papers in copy** provides the main papers in full text, or at least provides the first page and the introduction of the paper if the copyright policy of the journal did not allow a full reprint of the text.

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1 Introduction

Within this thesis medical devices for clinical research were created implementing the approach of open-flow microperfusion (OFM). This chapter provides the state of the art of the methodology and research as the starting point for the design and research work summarized in this thesis. The principles of open-flow microperfusion methodology are outlined and the devices used until the year 2000 are described. A summary is provided of the research which those devices had enabled in the initial years of OFM. Moreover, the state of the art in the related method of *microdialysis* is provided, as this thesis aimed to overcome the limitations of both techniques to enable the clinical pharmaceutical in vivo research as presented at the end of this thesis.

1.1 Open-Flow Microperfusion (OFM)

Open-flow microperfusion is a method for the sampling of interstitial fluid in organisms in vivo. The interstitial fluid (ISF) resides in the interstitial fluid space (the 'interstitium') between the cells of living tissue, therefore the fluid has also been termed "extracellular fluid" (ECF). The method of OFM provides a direct access to the ISF in tissues for continuous ISF sampling by insertion of a small, minimally invasive sampling probe (also termed "catheter"). When the OFM probe is inserted into tissue of a human subject and ISF is sampled, the analysis of these ISF samples provides a valuable insight into local tissue biochemistry during states of health and disease as well as states in between during healing and drug therapy.

The OFM sampling approach has been developed in the 1990s based on the ideas of microdialysis. In brief, performing microdialysis means to implant a semi-permeable membrane into a living organism and to continuously sample (i.e. remove, 'recover') soluble analytes from the surrounding milieu by perfusing the membrane with a perfusion fluid, in analogy to haemodialysis. The recollected perfusion fluid, the dialysate, represents diluted and filtered ISF and contains the soluble analytes which can subsequently be measured by bioanalytical methods. If the membrane is inserted into tissue of a human subject and microdialysis is performed, the analysis of dialysate samples provides information on the tissues' interstitial bio-milieu, or at least on its soluble components which are small enough to pass the semi-permeable membrane. Microdialysis has been marketed for clinical (human) use and preclinical use and since the first publication in 1974 *(*Ungerstedt U. & Pycock C.) more than 16,000 papers have documented the utility of microdialysis for basic research in physiology/pathophysiology, for continuous metabolic monitoring as well as for research on the distribution of drugs to their target tissues.

The limitations of the microdialysis method have been vastly described in literature. It is in particular the semi-permeability of the membrane and the materials involved that limits the range of analytes well accessible for investigation by microdialysis. The molecular weight cut off (MWCO) of the membrane, usually between 5.000 to 100.000 Dalton, means that solely molecules significantly smaller than the MWCO can be recovered and will achieve quantifiable concentrations in the dialysate, while larger biological molecules of interest such as peptides or proteins are not sufficiently recovered. But also the sampling of small molecules may be

restricted in case the molecule is of rather lipophilic nature (‘sticky molecules’) and these are then lost for subsequent analysis due to unspecific binding to the membrane.
A unique strength of microdialysis in drug studies is that microdialysis solely recovers the free (i.e. not protein-bound) fraction of the drug. Thus, microdialysis may provide information on the pharmaceutically active drug concentration in the target tissue. In case of highly protein-bound drugs this may be a drawback and leads to difficulties in microdialysis data interpretation. The protein binding at tissue level may be higher than expected and the free ISF concentration may therefore be too low for a successful quantification in the microdialysis dialysate. An alternative explanation could be that the drug does not reach the investigated tissue at all because of inappropriate dosing, an inappropriate dosing regime, or an inappropriate formulation (‘galenics’) of the drug that does not release sufficient amount of drug into the body. In such cases microdialysis may not deliver conclusive results to support the clinical development of the new drug product.

OFM shares many methodological features, applications and advantages with microdialysis. OFM, like microdialysis, provides tissue-specific information on local biochemistry with time resolution by using minimally invasive sampling probes. However, OFM probes do not include any semi-permeable (dialysing) membrane. Instead, OFM probes show macroscopic fenestrations (or ‘openings’) usually of a size well visible to the naked eye. The first generation OFM probes showed macroscopic openings in the form of large circular holes of up to 0.5 mm in diameter. Such macroscopically perforated sampling probes deliver a diluted but otherwise unfiltered ISF. Any large molecule will pass the probe’s fenestration; there is no limit or MWCO like in microdialysis. This structural feature of OFM probes facilitates the recovery and investigation of large biological molecules of endogenous and exogenous origin from proteins to antibodies and larger entities. In contrast to microdialysis, OFM recovers the total concentration of a drug, i.e. the total concentration of the unbound and the protein-bound drug fraction. Therefore, the concentration of drugs with significant protein binding is significantly higher in OFM samples compared to microdialysis samples. This eases the detection or quantification of the drug in the ISF and can more reliably provide information on whether the drug reached the target tissue, regardless if present as free or protein-bound drug. OFM probes are rather robust compared to microdialysis membranes and while different biocompatible materials may be used for the manufacture of OFM probes the choice of materials for a semi-permeable membrane is rather restricted to polymers. Those polymers offer binding sites for unspecific binding of molecules which repeatedly poses a problem for a reliable sampling of lipophilic (sticky) drugs. OFM probes avoid the use of such polymers and OFM offers a small surface to lipophilic drugs for unspecific binding to the probe when compared to the microporous structure of a microdialysis membrane. This significantly reduces the risk for a misinterpretation of study results for this class of drugs. Consequently, the use of OFM is in particular indicated in research areas dealing with such difficult drugs, like the mostly lipophilic drugs used for topical application to the skin.
The beneficial properties of macroscopically fenestrated OFM probes may help to overcome those well-known limitations of microdialysis in drug research. However, the macroscopically open probes mean a challenge in fluid handling. A loss of perfusate into the tissue is observed, an observation quite similar to microdialysis when using high MWCO membranes that are also

called “large pore membranes”. Therefore, the outflow of fluid from the probe has to be actively supported by the sampling device, or ideally actively controlled, in order to achieve a stable and defined perfusion flow rate.

1.2 The state of the art of OFM until 2000

1.2.1 The invention in 1987

The first “open-flow microperfusion” sampling probe was described in an Austrian patent application filed by Falko Skrabal in the year 1987. In the US version of his patent granted in 1992 [137], he described his idea of sampling: “*In order to determine at least one parameter of interest in a living organism, a perfusion fluid is directly introduced in the tissues. After its partial balancing by the tissue parameter of interest, the perfusion fluid is collected and analysed for the parameter of interest,…” and further “Contrary to usual processes, used exclusively inside blood vessels or other body cavities filled with liquid, this process creates in the tissues, i.e. in the closed cellular structure, a previously inexistent cavity, in which the perfusion fluid introduced in the tissue interacts directly with the organic tissue, with no intervening membranes*”.
Thus, the patent clearly specified a perfusion process for sampling from tissues without involvement of a semi-permeable membrane and with a direct contact of the perfusion fluid with the tissue. This description demarked the novel approach clearly from state-of-the-art microdialysis. The figures in that patent disclosed the sampling probe as a concentric type of probe with macroscopic circular openings, i.e. round holes, in the outer lumen [137]. In a later patent by Helmut Masoner, Falko Skrabal and Helmut List also a linear type of sampling probe with macroscopic circular holes was disclosed [138].

1.2.2 The State-of-the-Art devices until 2000

The invented probe was implemented in the 1990s as clinically applicable probes by a group of biomedical engineers at the Technical University of Graz. Because the invented sampling process was meant for use in continuous glucose monitoring and glycaemic control, the Technical University Graz started collaboration with the Medical University Graz (formerly the Medical Faculty of the Karl-Franzens University Graz). Collaborative projects with the industry which had an interest in the use of OFM for purposes of glucose monitoring also led to the first types of specialized pumps.

The first OFM probe was put into practice as a concentric type of probe with circular holes close to the description in the patents. The probe was implemented by perforating standard intravenous sampling catheters (‘venflons’, see Figure 1-1). The holes in those venflons were created by a lasering process, which was a rather laborious and expensive process because only excimer lasers, featuring a sufficiently low wavelength below 200 nm, were adequate to form holes into fluoropolymer venflon material (PTFE, FEP) by a pulsed ablation process. The quality of the lasering result, i.e. the quality of the holes, varied at that time as it depended on the laser type as well as on the effort done for the process to find the optimal pulse energy, pulse lengths and

pulse frequencies. Two different dimensions of the probe were implemented, one large probe based on a large venflon (50 x 1.2 mm, length x outer diameter) and a small one based on a venflon designed for babies (30 x 0.7 mm). While the larger probe was meant for insertion into subcutaneous adipose tissue as well as deep skeletal muscle tissue for research on tissue metabolism, the smaller probe was meant for self-application by diabetic patients into subcutaneous tissue for continuous glucose monitoring. In order to allow fluid circulation within the probe by Pull or Push-Pull operation, an inner lumen part was manufactured which reached to the tip of the venflon. It was tightly connectable to the venflon screw at the basis, and connectable to the perfusate supply tubing as well as the sample withdrawal tubing which were both connected to a pump (Figure 1-2). This set of tubings ('tubing set') had an inner diameter of 0.19 mm to reduce the dead volume in the sampling process and it had a length of 80 cm in order to bridge the distance between probes and stationary pumps placed next to the bed. The perfusion fluid (perfusate) was filled into a custom made 20 ml perfusate bag. The ready probes, the inner lumen including the tubing sets and the perfusate bags were subjected to visual inspection, and a test of tightness for water was performed with the probe combined with the inner lumen and tubing. Finally, when the test was passed, all these components were packed into double sterilization packing, sealed and sterilized by formaldehyde-vapor sterilization.

The first OFM pumps, or those used as such, were of either a peristaltic type or of a combination of peristaltic and syringe type. The type differed depending on the application of OFM.
Standard heavy-weight peristaltic pumps with power line connection were used when OFM sampling was performed in clinical studies with study volunteers mainly lying in bed during the study. Due to the weight of those standard laboratory pumps (5 kg) the pumps were placed next to the bed of the study volunteer on a very stable wagon. After probe insertion into the tissue, the inner lumen was screwed into the probe, thus connecting probe and pump for push-pull perfusion over a distance of at least 50 cm. The sample was pumped the long way through the 80 cm pull-tubing to reach the cooled sampling container. As a flow rate of 0.5 µL/min was used, this meant a passage time for the sample of 80 min.
A much smaller and lighter pump had been designed for the purpose of a metabolic monitoring approach using the OFM probe as a body interface. For active metabolic control by a combination of glucose monitoring and insulin infusion, a box had been designed (the GI-Box, the Glucose-Insulin Box) that included several reservoirs, one for perfusate, one for insulin and one for interstitial fluid that has passed through the glucose sensor. This box used a combined approach for pumping, using a combination of syringe and peristaltic finger pumps to manage the fluidic challenges. This pump was designed in cooperation with AVL List GmbH and already much smaller, having a weight of less than 1 kg. As the GI-box could be placed within the bed, the length of tubing was reduced to approximately 30 cm.

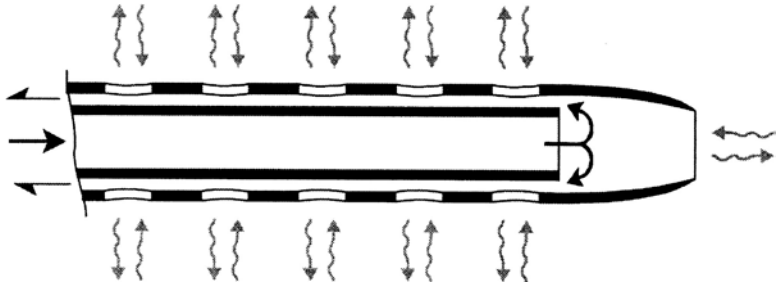

Figure 1-1: Schematic representation of the OFM double lumen probe. After insertion into the tissue, the OFM probe is continuously perfused with a sterile isotonic perfusate via the inner lumen and the sample is simultaneously withdrawn via the perforated outer lumen. The passage of the perfusate by the macroscopic openings leads to partial equilibration with the surrounding interstitial fluid in the tissue (schematics taken from Ellmerer and Schaupp et al. [139]).

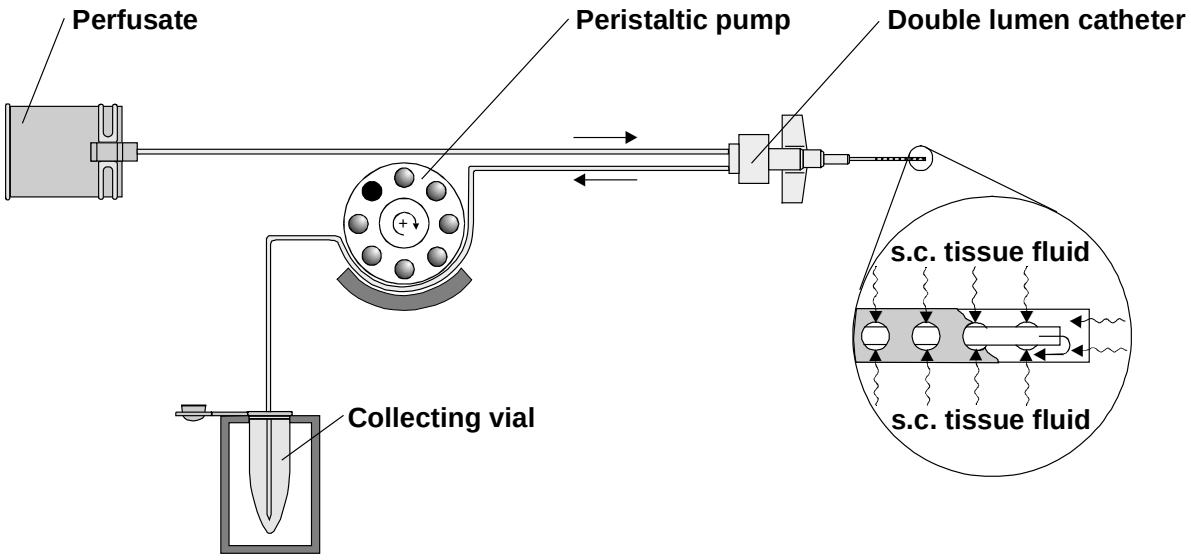

Figure 1-2: Schematic representation of the sampling system and its basic components (underlined in text above). The perfusate is “pulled” through the catheter by the peristaltic pump. Later, the perfusate was also actively pumped (“pushed”) to the catheter by the same pump in order to assure the same flow in and out of the catheter (push-pull mode). The schematic was taken from Schaupp et al.

1.2.3 The initial years of OFM research

In the initial years of OFM, the research group in Graz performed fundamental studies on the utility of this novel minimally invasive approach for purposes of continuous metabolic monitoring as well as metabolic research. The first scientific paper on “open-flow microperfusion” was published in 1995 by Skrabal et al. [140] and dealt with a portable system for on-line continuous monitoring of s.c. adipose tissue glucose using “open tissue perfusion”. Trajanoski et al. [141] also used OFM for on-line glucose monitoring in s.c. adipose tissue. Subsequent papers by Ellmerer et al. [142,143] also reported on-line monitoring applications using s.c. OFM, but now of lactate monitoring under exercise conditions. Cline et al. [144,145] was the first to report the use of OFM in skeletal muscle tissue both in rats and humans, the first to report the use in insulin-resistant type 2 diabetes mellitus patients and the first to use OFM for investigation of larger

analytes (insulin, 5.9 kDa). Orban et al. [146] reported the utility of s.c. OFM for research into fat metabolism and the role of polypeptides for metabolic signalling (leptin, cytokine IL-6; and TNFα, a trimer of 51 kDa). Schaupp et al. [147] introduced different calibration approaches for quantitative sampling into OFM studies which were known from microdialysis (zero-flow rate method, suction, recirculation, no-net-flux calibration) and introduced the “ionic reference technique” as a very convenient calibration technique in metabolic monitoring that was subsequently also implemented in wearable glucose monitoring devices.
Ellmerer et al. [139] showed in 2000 that OFM, when combined with no-net-flux calibration, enables the quantification of a protein (albumin, 68 kDa) in s.c. adipose tissue as well as in skeletal muscle tissue, and thus opened up the possibility to investigate protein-bound drugs directly in peripheral target tissues, such as highly protein-bound insulin analogues designed for a prolonged, retarded insulin action. While a convenient method for calibration of small metabolic molecules such as glucose and lactate was found with the ionic reference technique, a convenient method for the calibration of larger molecules like human insulin was missing. Therefore, Ellmerer et al. studied the work of the group of Lönnroth et al. [148] and successfully verified the use of the metabolically inert polysaccharide inulin (also known as ‘sinistrin’, MW 3-5 kDa) as a interstitial reference substance, which under equilibrium conditions following constant intravenous infusion enables the determination of the relative recovery of larger analytes.

The state-of-the-art devices are described in more technical detail within the design chapter 2.1, as their limitations motivated further work and initially also defined the aims of this thesis.

1.3 The state of the art of (dermal) microdialysis

The research published by the pioneers of clinical microdialysis has served as an ideal for the research done with OFM and it has repeatedly stimulated methodological innovation and new applications for OFM. For instance, the pioneers’ protocols for calibration and quantification of analytes to access the true (i.e. undiluted) interstitial concentrations were evaluated for use in OFM trials and were subsequently adapted and improved further. Similarly, the application of microdialysis to new tissues or organs and to new drugs was observed closely by following the literature. Consequently, when researchers reported on applications of microdialysis in pharmaceutical research and therein discussed possible pitfalls and limitations associated with the use of semi-permeable microdialysis membranes, this prompted discussion among OFM users about the potential advantages of using membrane-free OFM for such research applications. Hence, it became obvious for OFM users already during the 1990s that OFM might have a potential for a future use in pharmaceutical research, in particular in the research on large endogenous molecules (peptides, proteins) and exogenous hormones (e.g. insulin and analogues). At that time, from the point of view of OFM which was used in subcutaneous adipose tissue and muscle only, the objective evolved to improve the production and quality of the OFM probe to enable the safe use of an increased number of probes per year in clinical research on new types of insulins and other signaling hormones. Shortly after, around the year 2000, the objective was redefined to also find or develop a small pump that can be worn on the body, but

predominantly for purposes of glucose monitoring using an OFM or microdialysis probe as the body interface.

The state of the art of continuous interstitial sampling for questions of pharmaceutical research at that time, however, was defined by microdialysis. CMA Microdialysis (Solna, Sweden) offered different approved clinical probes as well as a wearable pump (CMA106/CMA107) to the research community. Still, dermatologists manufactured their own probes taking membrane fibers from hemodialysis devices, because a linear type of probe for cutaneous insertion was not yet available on the market for ready use. The increasing number of papers on the use of cutaneous microdialysis for the investigation of topical drugs created awareness among the OFM users for this attractive field of application, as the number of drugs in the pipeline for treatment of skin diseases was obviously much higher than that of e.g. for metabolic diseases. While researchers like Christoph Anderson thoroughly investigated skin physiology and pathophysiology publishing numerous papers starting from 1992, others focused on the penetration of drugs into the skin after topical application. Starting from 1998, Lotte Groth and later Eva Benfeldt in Copenhagen dedicated research to the use of cutaneous microdialysis for the investigation of topical penetration. They performed in-depth evaluation for the use of cutaneous microdialysis for the comparison of topical formulations and products regarding their topical bioequivalence. Interestingly, they used a series of linear cutaneous probes for the simultaneous investigation of different products, and published the evaluation of topical bioequivalence in the renowned Journal of Investigative Dermatology as well as in a White Paper written in collaboration with the American Food and Drug Association FDA [12, 13]. In brief, Benfeldt et al. described statistical powerful study settings using several test areas on the skin for the parallel application and head-to-head comparison of topical drug penetration from the generic products (test products) versus the originator product (reference product). The authors convincingly outlined the utility of the continuous dermal sampling approach and the relevance of using multiple test areas and multiple probes in parallel for efficient bioequivalence studies in a relatively low number of subjects. Their statistical calculations indicated that with intradermal sampling methodology, bioequivalence studies with 90 % confidence intervals and 80 to 125 % bioequivalence limits can be conducted in 27 subjects when using two probes in each test area, or 18 subjects using three probes per test area [12]. According to those publications large settings with multiple probes per volunteer were ideal and the way to go for the future of microdialysis and OFM. However, the applicability of the microdialysis approach for topical PK and bioequivalence was limited by the microdialysis materials, as the probes were poorly suitable for sampling of more lipophilic topical drugs and as there were no wearable pumps supporting multi-probe settings over a sufficient long study duration. It was obvious that the use of OFM may overcome some of those limitations. Thus, those publications of Eva Benfeldt et al. decisively influenced the aims of this thesis and the design of current OFM materials which are now successfully used for topical bioequivalence.

1.4 Aims of this thesis

The overall objective was to overcome the limitations of state-of-the-art continuous sampling methodology to enable the study of all drugs and biomolecules directly at tissue level irrespective of the drugs' or biomolecules' molecular size or lipophilicity.
In particular, this thesis aimed to create devices for continuous sampling from the dermis of human skin and to demonstrate the devices' utility for basic dermatological research, pharmacokinetics and -dynamics (PK-PD) of novel anti-inflammatory drugs, and for bioequivalence testing as the most challenging future application of continuous sampling methodology.

Time has repeatedly refined the objectives and complicated the introduction of novel devices for clinical research. Specifically, the international regulations on the design of devices for human use (Medical Device Directives) and the guidance on the performance of studies involving human subjects (Good Clinical Practice & ethics guidelines) have changed significantly during the last decade and impacted the design process. Furthermore, the continuous examination of the needs in pharmaceutical R&D (pharmaceutical industry, health authorities, clinical investigators) and the growing personal awareness about future research opportunities provided by the OFM approach have modified the requirements regarding the applicability and performance of future sampling devices. That is, the work on a highly advanced clinical sampling approach took longer and this thesis summarizes more than a decade of clinical research and stepwise device optimization.

2 OFM DESIGN AND PHARMACEUTICAL RESEARCH

This chapter provides a chronology of OFM design during the past 15 years. The evolution of OFM is described from the *state-of-the-art* OFM materials existing in 2001 to today's CE-certified medical OFM products that fulfil the latest regulatory standards and the current needs of efficient and informative clinical in vivo research on pharmaceutical products.
The steps of clinical evaluation of OFM methodology as well as the clinical research applications are described in more technical detail than in the peer-reviewed papers which the author published in the dermatological and pharmacological journals. Thus, this chapter provides the links between the device design outcomes (i.e. the novel devices) and the pharmaceutical studies the author was able to perform utilizing those devices.

2.1 Research with *state-of-the-art OFM devices*

The state-of-the-art materials used for OFM studies in 2001 essentially comprised (i) a standard heavy-weight laboratory pump, (ii) a set of self-tailored tubings and (iii) a laser-perforated intravenous cannula (a 'venflon') serving as the OFM probe. These *state-of-the-art* materials are briefly described in the subsequent sections of this chapter also stating their main limitations in order to layout the methodological basis and motivation for the design of new OFM materials during the subsequent years. This section at the end also summarizes the first pharmaceutical clinical studies performed by the author of this thesis using the existing OFM materials.

2.1.1 *SoA laboratory pumps*

The s*tate-of-the-art* pump for OFM trials was a standard heavy-weight peristaltic pump for laboratory use (Type "Minipuls® 3", Gilson Inc., Middleton, WI 53562-0027, USA; Figure 2-1). The pump was driven by a high-torque stepper motor that drove a pump with 10 stainless steel rollers. The pump was equipped with an interchangeable top providing bearings for 2 or 4 tubings (pump channels). Thus, the pump enabled the operation of 1 or 2 OFM probes in push-pull mode. By the creation of new tubing sets with special stoppers (each accommodating 2 tubings) the 4-channel pump accommodated 8 channels operating 4 OFM probes, which was the maximum number of probes used on one study volunteer at that time.

Figure 2-1: 4-channel peristaltic laboratory pump (Minipuls® 3, Gilson Inc, Middleton, WI, USA). Picture taken from Gilson Inc.

The main drawbacks in using those pumps in OFM trials were:

- A massive table top was needed added to safely support the pump (pump weight 5 kg). In addition a medical safety transformer (another 10 kg) was needed to safely power the pump via a 230 Volts network. Thus, the setting required space.
- Long tubings were required for perfusate supply to the probe and sample collection from the probe (each 80 cm). This was associated with considerable time delays (up to 60 min).
- The study volunteers were not able to leave the bed for the toilet. Thus, the trials were limited to 8 h and only male volunteers were included as they were able to use the flask for urination.

While the use of heavy-weight stationary pumps was well acceptable at that time for pharmaceutical trials with volunteers resting in bed, those pumps were not a viable solution to operate OFM probes in diabetic patients for the purpose of continuous glucose monitoring in everyday life. For the latter purpose a wearable light-weight pump was needed. The idea of an artificial pancreas, consisting of a glucose monitoring unit based on an OFM interface providing the glucose signal to an insulin pump, was funded in the EU-Project “ADICOL – ADvanced Insulin infusion using a COntrol Loop”. Thus, ADICOL drove the design of the first wearable OFM pump.

2.1.2 SoA OFM probes

OFM probes had been manufactured from catheters used for venous blood sampling (“venflons”). Those venflons were available as sterile CE-certified medical devices for human use and modified by perforation of the tubular catheter material to form a number of macroscopic holes. The perforated catheter (OFM probe) was repacked and resterilized before human use. The probe was inserted by the needle which was an integral part of the venflon.

The OFM probe was completed by a custom-made sterile inner lumen system, which connected the probe to a pump for both the delivery of perfusate to the probe and collection of equilibrated perfusate (‘sample’) from the OFM probe. This inner lumen system was inserted into the perforated venflon after the insertion needle had been removed. The inner lumen system allowed the operation of the perforated venflon as the OFM probe such that the perfusate delivered to the probe could pass by the perforations in a uniform direction and thereafter recollected at the second end of the probe.

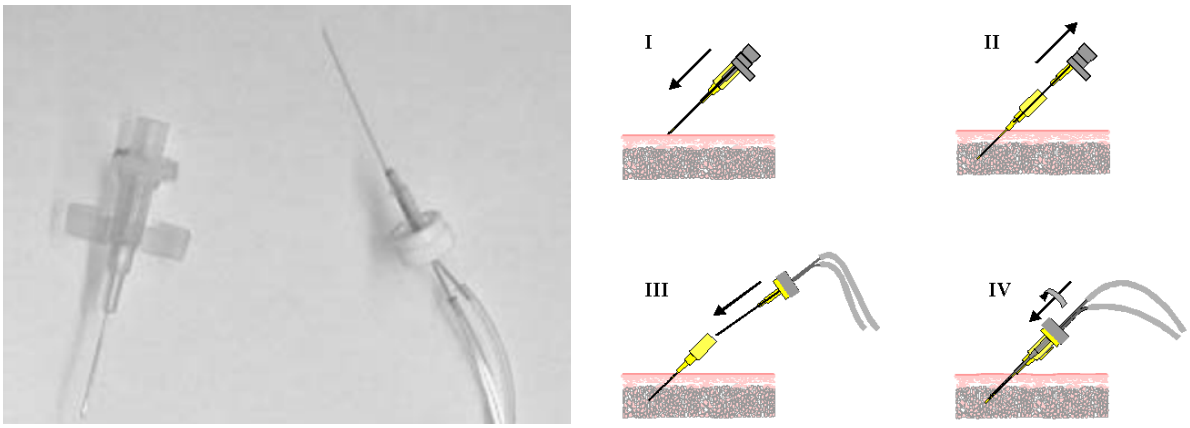

Figure 2-2: Left: An OFM probe consisting of a perforated 24-gauge venflon and the inner lumen. Right: The insertion of the probe into the tissue with the help of the integrated steel mandrel, which is subsequently removed and replaced by the inner lumen of the double lumen probe. (Schematics taken from Ellmerer and Schaupp et al.)

These probes were well usable for sampling from subcutaneous adipose tissue as well as skeletal muscle tissue (see research use for monitoring purposes in 0 and 2.2.2, and insulin research in 1.2.3 and 0). However, the perforation of the venflons (fluoropolymeric materials PTFE, FEP) required processing by lasers with extremely low wavelengths below 200 nm. Such lasers needed to be operated in vacuum and thus made probe production costly. The result of a non-satisfactory lasering result with inappropriate high wavelengths is shown below in Figure 2-3.

Moreover, the perforation of polymeric materials by lasering reduced the mechanical robustness (tensile strength) of the original venflon resulting in safety concerns in case of mass production. Other methods for probe perforation (high-speed cutting, punching techniques) were tested but did not result in a significant improvement regarding costs or mechanical stability. Notably, these OFM probes were more robust than commercial microdialysis probes at that time (tensile

strength of 6.7 N for OFM vs. <5 N for microdialysis, results obtained at the facility for material testing at the Technical University Graz).

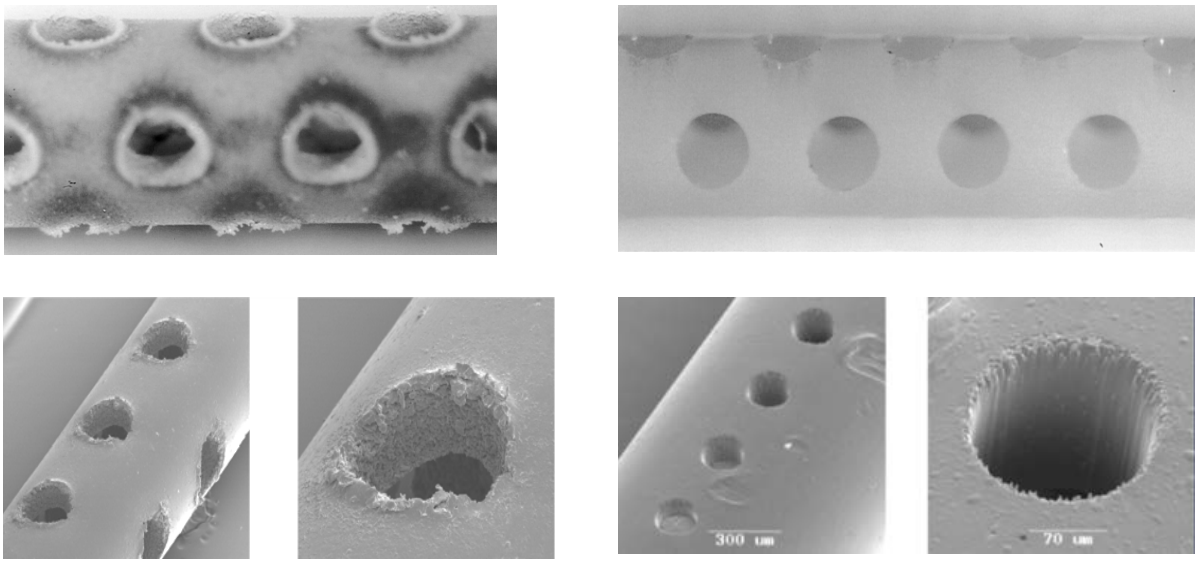

Figure 2-3: Surface of the OFM probe using different methods for production of perforations. Top Left and Right: 280 nm laser process not adequately adjusted vs. high-speed cutting. Bottom Left and Right: Electron microscope evaluation of suboptimal (left) and optimal (right) adjusted lasering process.

The subsequent information summarizes the essential details of the SoA OFM probes.

Types of OFM probes (sterile, single use):

- 18-gauge OFM probe (large)
  - concentric type based on 18-gauge venflon (50 x 1.2mm, PTFE)
  - 100 holes, each 0.5 mm diameter
  - inner lumen system using 0.5 mm PTFE tubing
  - *applicability*: s.c. adipose tissue and i.m. in skeletal muscle
- 24-gauge OFM probe (small)
  - concentric type based on 24-gauge venflon (30 x 0.7 mm, FEP)
  - 27 holes, each 0.3 mm diameter
  - inner lumen system using 0.2 mm steel tubing
  - *applicability*: s.c. adipose tissue

Other fluidics parts (sterile, single use):

- Peristaltic pump tubing
  - PVC-based tubing equal for 18-gauge and 24-gauge OFM probe (Type “Tygon tubing”, 80 cm, ID 0.25 or 0.19 mm, OD 2 mm)
  - Included bearings (“stoppers”) for use with *Gilson Minipuls Pumps* (see 2.1.1)
  - Peristaltic tubing was attached to inner lumen before packing
- Perfusate reservoirs: Perfusate bags 20 ml (PVC, custom-made)

Packing and sterilization:

- Double packing in sealed paper-film bags
- Sterilization by formaldehyde-vapor, later with ethylene-oxide

Advantages & disadvantages:

- In case of occlusion the inner lumen could be exchanged
- Considerable costs for laser perforation
- Concerns regarding mechanical stability after laser processing
- Not applicable for sampling in human skin

The main drawback of this concept of venflon-derived concentric probes was that these probes were not robust and thin enough for insertion into human skin. This limitation prevented the continuation of this concentric probe concept when the application of OFM in human skin was prioritized over s.c. and i.m. applications and when limited resources prevented the implementation of more than one probe type to achieve CE-certification as a regular medical device for human use. The design and evaluation of novel probe types for intradermal use are described in 2.3.

The subsequent sections summarize two clinical studies which were performed with the SoA OFM materials as described above. These studies were relevant for the further development of OFM materials: These studies were the first using OFM for the investigation of drug concentrations at the level of interstitial level (pharmaceutical application), and these studies demanded the study of the SoA materials and the challenges related to their production and research use in humans. Both studies have been published by the author in peer-reviewed journals and are summarized in the subsequent sections.

2.1.3 Clinical research on human insulin – Bodenlenz et al. 2005

Am J Physiol Endocrinol Metab 289: E296–E300, 2005.
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Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access

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The results of this study have been published in the American Journal of Physiology as a publicly accessible full-paper [13]. The scientific content is not fully repeated here again (neither are the references), but a summary is provided below which reuses parts of the abstract of the published paper. As published in a medical journal, the paper focused on the bioavailability of human insulin but did not provide the wider context. Therefore, the summary below includes some extra information on the methodology and discusses the significance of the study in view of the subsequent research in the field of diabetes and dermatology research. The synopsis table (Table 2) at the end of this section provides condensed information on study design and methodology.

This study was conceived to support and to complement the ongoing research on insulin and human insulin analogues using OFM. The quantification of insulin in dermal interstitial fluid in situations with dynamic insulin tissue concentrations required a convenient but still reliable method for the calibration of the insulin concentration in each OFM sample. For a convenient calibration a reference substance was needed, and the reference finally needed to be validated for the purpose by comparison of the calibration results to results which were obtained differently and independently from the reference substance. The envisaged reference substance for human insulin was the inert polysaccharide inulin (sinistrin), and for its validation and subsequent use human insulin needed to be most reliably quantified in the tissues without involvement of any (non-validated) reference.
For this purpose we utilized the no-net-flux (NNF) approach, an approach successfully established in microdialysis by Lönnroth et al. and later successfully applied in OFM by Ellmerer et al. for albumin [139,149]. The NNF approach has the drawback that the quantification of one concentration of the analyte takes several hours, as different concentrations of the analyte have to be presented to the tissue (subcutaneous adipose and muscle tissue) to evaluate at which concentration no loss and no gain (no net-flux) occurs. Therefore, the tissue concentration of human insulin needed to be stable at an elevated (quantifiable) level for more than 8 hours, which was achieved by a primed-continuous intravenous infusion of human insulin. The effect of the slight hyperinsulinemia (stimulated glucose consumption) was compensated by variable (repeatedly adjusted) infusion rate of intravenous glucose. Both primed-continuous infusion to

achieve steady-state insulin levels and the glucose clamp technique using variable infusion had been well established in the research group and was not a challenge. The challenge was to find the adequate sequence of insulin concentrations and the time schedule for the change of perfusate concentrations at the inflow of the probes and the change of samples at their outflows, considering the long tubings on both ends and the considerable time delays and effects of diffusion. Two small studies (unpublished) were performed for the optimization of the non-net-flux procedures in order to obtain samples for each concentration which were clearly attributable to a known verifiable inflow concentration. For the study published, a NNF procedure was developed which has been successfully reused 10 years later when similar validation work was done in the field of dermatology for quantification of monoclonal antibodies.

We investigated nine healthy subjects. Each subject received a primed-constant intravenous insulin infusion of 1 mU kg^{-1} min^{-1} and euglycemia was maintained using variable glucose infusion rates. State-of-the-art OFM probes (type based on 18-gauge venflon) were inserted for a direct access to interstitial fluid in adipose and muscle tissue. During steady-state conditions of human insulin in plasma (9.5 h), interstitial fluid samples were collected in 30-min fractions using five different insulin concentrations in the inflowing perfusates. A regression analysis of insulin concentrations in perfusates and effluents was performed to assess the insulin concentration which was in equilibrium with the surrounding tissue (i.e. the tissue concentration). The slope of the linear regression lines reflected the relative recovery of human insulin. Thus, in subcutaneous adipose tissue and skeletal muscle, the mean ISF-to-serum insulin level was calculated as 21.0 % [95 % confidence interval (CI) 17.5–24.5] and 26.0 % (95 % CI 19.1–32.8; P = 0.14), respectively. Recoveries for insulin averaged 51 % and 64 %, respectively. These sampling recoveries for human insulin were well above the recoveries of 3 % to 11 % published from microdialysis studies. This means that the quantification of human insulin in those microdialysis studies was based on small effluent concentrations resulting from the microdialysis probes' low insulin recovery, which may have added a significant error to their insulin results. We hypothesized in our paper that these facts may explain some of the variation in the results in microdialysis studies, which ranged from 12 % to 54 % in muscle tissue of healthy subjects.
The study was the first to compare interstitial insulin levels in human subcutaneous adipose and skeletal muscle tissue. The paired measurements during steady-state conditions demonstrated that there was no statistically significant difference in the insulin concentrations between the two different insulin-sensitive peripheral tissues. The low interstitial insulin fractions confirmed reports of low peripheral insulin levels during moderate insulin clamps.

This study demonstrated the strengths of OFM. The probes' macroscopic openings assured direct access to ISF insulin without interference of a membrane. This led to recoveries of insulin 50 % on average, as indicated by the pronounced slopes of the regression lines. The high recovery was fundamental for the reliability of the NNF quantification approach. As had been expected, recoveries varied between catheters; however, the recovery was not a determinant for ISF insulin. Although the mean insulin recovery in the two investigated tissue beds was not equal (12 % higher in muscle; maybe because of the different vascularization), the ISF insulin fraction was the same for both tissues. It has been repeatedly experienced in subsequent studies using

OFM methodology, and in particular in studies using OFM for metabolic monitoring in during non-steady-state conditions, that the sampling results were highly reproducible within the same tissue or between different tissues if the relative recovery was properly monitored and used for calibration. Examples of such results are shown in later sections for glucose monitoring and lactate monitoring during heavy exercise.

To summarize, this study fulfilled its purpose to provide a reference concentration for human insulin in healthy subjects under moderate hyperinsulinemia. As will be shown in the subsequent section, inulin could be successfully validated as a convenient reference based on the results of this insulin no-net-flux study.

Author's contribution to research and publication: The author of this thesis conceived the study design, prepared the study, conducted the study with the clinical research team, analyzed and interpreted the data, and wrote the manuscript.

Table 2: Synopsis of the human insulin study

Description	
Type of study	Kinetic Study of steady-state concentration of human insulin (HI) in tissues under moderate hyperinsulinemia following continuous i.v. HI administration
Subjects	8 healthy volunteers (not including pre-study to optimize no-net-flux schedule)
OFM material	OFM Probe: 18-G concentric probe, Pump: Gilson-Minipuls 3
Design	Primed-continuous human insulin (HI) infusion at 1 pmol kg^{-1} min^{-1} No-Net-Flux Protocol with OFM to quantify HI in tissues 4 probes, 2 in adipose tissue, 2 in muscle tissue, 0.5 µL/min sampling 10 hours of OFM sampling in 1 hour intervals
Analyses	OFM analytes: HI
Outcomes/ Significance	The study showed the bioavailability of regular human insulin at the level of interstitial fluid at moderate hyperinsulinemia. The study was essential for further research on insulin kinetics and served as reference. The results for human insulin could be verified in a study using a different calibration methodology [8]. Moreover, this study shows a comprehensive and robust no-net-flux protocol using 5 different drug concentrations in each probe, a protocol which was successfully utilized 10 years later when validating methodology for the quantification of IL-17 antibody in the skin of psoriatic patients [7].
Publications	M. Bodenlenz, L. A. Schaupp, T. Druml, R. Sommer, A. Wutte, H. C. Schaller, F. Sinner, P. Wach, and T. R. Pieber, "Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access.," Am. J. Physiol. Endocrinol. Metab., vol. 289, no. 2, pp. E296-300, Aug. 2005. **[13]**

2.1.4 Clinical research on insulin detemir – Bodenlenz et al. 2015

original article

Diabetes, Obesity and Metabolism 17: 1166–1172, 2015.

ORIGINAL ARTICLE

Bioavailability of insulin detemir and human insulin at the level of peripheral interstitial fluid in humans, assessed by open-flow microperfusion

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This study has been published 15 years after its performance. The acceptance as a full-paper by the renowned *Journal of Diabetes, Obesity and Metabolism* may be considered as proof that the findings and methodology reported in the paper are still relevant for pharmaceutical research and drug development in the field of diabetology. The paper describes the bioavailability of two very different types of insulin at the level of insulin-sensitive peripheral tissues. The topic was still relevant for (late) publication because the designers of novel drugs continue to try to control the pharmaceutically active (unbound) drug concentration and the drug's duration of action by using the mechanism of protein binding. Moreover, there was not yet and is not yet another methodology than OFM to investigate reasonably reliably the concentration of larger molecules at tissue level in humans in vivo.

Actually, this was the first study using OFM for pharmaceutical research for the pharmaceutical industry. The preparation required a critical view on the SoA materials and their production. Its performance meant working with the study volunteers for many hours and monitoring the continuous sampling process with all its difficulties and limitations, mainly caused by stationary pumps equipped with long tubing requiring the volunteers to leave the bed.

The full-paper is provided in the appendix and publicly accessible [8]. Therefore, most of its scientific content is not repeated or discussed here again (neither are the references), but a summary is provided below which reuses parts of the abstract of the paper. As published in a medical journal, the paper focused on the kinetics of insulins and the mechanism and effects of protein binding. Therefore, the summary below includes some extra information, discussing the results and methodology then with the knowledge of later (pharmaceutical) research in the field of dermatology. The synopsis table (Table 3) at the end of this section provides condensed information on study design and methodology.

Summary

In human trials the soluble long-acting insulin analogue insulin detemir (later marketed as "Levemir" by Novo Nordisk AB, Denmark) had shown lower potency in humans compared to regular human insulin. The clinical study in Graz investigated whether lower detemir

concentrations at the site of action in interstitial fluid (ISF) of peripheral tissue might provide an explanation for the observed lower potency of detemir in humans.

We utilized OFM to sample detemir and human insulin in the ISF of s.c. adipose and skeletal muscle tissue during steady-state of euglycaemic-hyperinsulinemic clamp experiments on healthy volunteers. Human insulin was administered as intravenous infusion (6 pmol $kg^{-1}min^{-1}$). In another study visit insulin detemir was administered in analogous manner but as a tenfold dose (60 pmol kg^{-1} min^{-1}) in order to achieve similar steady-state glucose infusion rates relative to the human insulin dose. In a third visit, the detemir dose was doubled in order to assess dose proportionality at the interstitial level. The sampled insulin concentrations were calibrated (corrected) using inulin (also “sinistrin”) as a marker for the interstitial fluid recovery. To verify that inulin distributes evenly between serum and ISF, Ellmerer et al. (unpublished) had performed an inulin validation study using the no-net-flux approach [139,149]. Due to the lack of interest of journals in validation studies without direct application, the results of that study of Ellmerer et al. were reported within this publication.

Importantly, inulin calibration experiments using the no-net-flux approach verified inulin as a suitable reference substance for OFM in adipose and muscle tissue. Both the no-net-flux procedure and inulin as reference molecule repeatedly played an important role in later studies when CE-certified OFM already existed and was applied for dermal studies for quantification of analytes (see the antibody study in 2.5.3). The concentration of inulin as a marker in serum and its diluted concentration in the OFM samples (i.e. the relative recovery) was well comparable throughout the three study visits, thus providing further confidence in the utility of the inulin reference.

The concentration of detemir in interstitial fluid was found to be 2 % of the serum concentration, while for human insulin this percentage was 25 %. These relative concentrations (or gradients over capillary membrane) were in line with the free drug theory, saying that only the unbound fraction in plasma (for detemir ~2 %) would be able to equilibrate with the interstitial compartment across the capillary epithelium. The absolute detemir interstitial levels were 5 to 6 times higher than human insulin interstitial levels. At the same time the metabolic clearance of detemir from serum was substantially reduced compared to human insulin. Doubling the detemir dose resulted in almost doubled interstitial concentrations, confirming dose proportionality and indicating non-saturable diffusion of unbound insulin detemir from serum to the ISF of peripheral tissues. The double dose led to a statistically significant increase but did not result in a proportional increase in glucose uptake, which we explained by the extraordinarily high detemir concentrations usually not obtained in treatment trials in which insulin is given as subcutaneous bolus.

We concluded that our comparative study of insulin detemir and human insulin demonstrated that increased albumin binding results in a reduced interstitial concentration in peripheral tissues, an effect which should contribute to the previously reported reduced potency in humans.

This trial was of utmost importance for the further use and further development of OFM for use in pharmaceutic trials. It demonstrated the reliability and wide applicability of the OFM sampling principle and pointed at the need to measure drugs at the target level, rather than in plasma where hardly any drugs have their target. An important observation in this study supporting this was that the time to steady-state of insulin action (glucose infusion rate) did not agree with the

time to insulin steady-state in serum. This indicated that the concentration in peripheral ISF could be more relevant for pharmacologic action, and that peripheral measurement (by e.g. OFM, microdialysis, etc.) could be more relevant than measurements in plasma or serum samples. In later trials the relevance of tissue concentrations was already taken as a fact by the industry, which designed or manipulated molecules such to achieve a certain exposure at the target at tissue level. This was in particular true for trials on drug concentrations in the skin, as the drug target was clearly and exclusively within the skin, and the drugs hardy detectable in blood if applied topically directly onto the skin.

Author's contribution to research and publication: The author of this thesis contributed to the preparation, conducting of the study, acquisition of data, data analysis and data interpretation. The author of this thesis wrote the manuscript.

Table 3: Synopsis of the Insulin Detemir studies

Description	
Type of study	Kinetic Study of steady-state concentration of human insulin (HI) and insulin detemir (ID) in tissues under hyperinsulinemia following continuous i.v. administration of HI or different doses of ID
Subjects	10 healthy volunteers; thereafter repeated/reproduced in the target patient population involving 10 patients with type 1 and 10 patients with type 2 diabetes
OFM material	OFM Probe: 18-gauge concentric probe, Pump: Gilson-Minipuls 3
Design	Primed-continuous i.v. infusions of inulin and HI or ID 4 probes, 2 in adipose tissue, 2 in muscle tissue, 0.5 µL/min sampling 10 hours of OFM sampling in 80 min intervals
Analyses	OFM analytes: inulin, Human Insulin (HI) or Insulin Detemir (ID)
Outcomes/ Significance	Inulin (sinistrin) was validated as an interstitial marker and successfully applied for calibration. The results for human insulin, obtained by an insulin no-net-flux could be reproduced in this study using the more convenient inulin calibration. The effect of plasma protein binding on bioavailability at tissue level was demonstrated, contributing to the reduced potency of the investigated analogue. This first pharmacological trial demonstrated the potential of OFM in this field and was key for subsequent efforts to establish OFM as a tool for clinical in vivo drug research.
Publications	M. Bodenlenz, M. Ellmerer, L. Schaupp, L. V Jacobsen, J. Plank, G. A. Brunner, A. Wutte, B. Aigner, S. I. Mautner, and T. R. Pieber, "**Bioavailability of insulin detemir and human insulin at the level of peripheral interstitial fluid in humans, assessed by open-flow microperfusion**.," Diabetes. Obes. Metab., vol. 17, no. 12, pp. 1166–72, Dec. 2015. **[8]**

2.2 Designing a wearable OFM pump

2.2.1 Motivation

The initial motivation for the design of a wearable OFM pump was the implementation of a wearable device for glucose control in diabetic patients (“artificial pancreas”) in international collaborative research projects. The details of the design of this first *small pump for monitoring* are described in the subsequent section 2.2.2.

Later the team in Graz realized the potential of the OFM approach in pharmaceutical research. This field of application meant to consider different or additional requirements. The details on the design of a *versatile pump for drug research* are described in the subsequent section 2.2.3.

2.2.2 Pump 1 – A small pump for monitoring

In the initial years of OFM the method was used for metabolic monitoring purposes only. The long-term goal was the creation of a wearable artificial pancreas including (i) a glucose monitoring unit using a glucose sensor with an OFM probe serving as the subcutaneous body interface, (ii) an insulin pump and (iii) an algorithm which controls insulin infusion based on the glucose signal. Initially, immobile laboratory pumps had been used which limited experimental monitoring studies of volunteers to approximately 10 hours. A first experimental pump device had been developed which established a flow using the peristaltic principle of a finger pump. However, the pump required power supply and the finger-pump principle did not fulfill the requirements of a stable push-pull operation.
Therefore, there was a need for the creation of a small, lightweight wearable pump to enable stable operation of a single OFM probe for prolonged duration.

2.2.2.1 Requirements

The design of an OFM pump as part of the monitoring unit of the artificial pancreas was associated with distinct challenges. The main requirements for the pump were:

- wearability, weight <300 g, size of a cigarette pack
- constant and equal flow in 2 channels for push-pull operation
- able to create two flow rates, a low flow for monitoring and a high for initial filling
- low power consumption to enable >48 hours continuous operation with one battery
- sterile exchangeable fluidics

2.2.2.2 Pump design

The use of OFM probes which required push-pull operation (i.e. with active suction functionality) favoured the implementation of a pump using the peristaltic principle over the syringe principle.

The pump design using the peristaltic principle was associated with two major challenges:

- Flow stability, as pump tubing is deformed and stretches over time
- Energy consumption, due to high deformation energy required for 2 channels

The pump head was designed as a 16 mm pump-head with 4 rolls (brass rolls on polished steel bearings), and an adjustable clamping bar (plastics) with optimized geometry (Figure 2-4). The challenge of flow stability could be solved by pre-treatment of the tubings (no details disclosed). The flow stability test results are shown in Figure 2-5.
A drive was found which was strong enough to exert the deforming forces onto 2 pump tubings with the lowest inner lumen diameter and therefore high stiffness (PVC-tubing, OD 2 mm, ID 0.19 mm) and which at the same time would consume such low power to enable >48 h operation with a single standard battery. A standard lithium cell (3 Volts, 2300 mAh) was selected as those cells were of the highest capacity and available for use with cameras.
Experiments were performed with different DC-motors (different nominal voltages) which were coupled with different set of gears (e.g. 1:19,000) to reduce the 17,000 rpm of the motor to the 0.25 rpm required for the pump head. The motors were chosen such to operate most efficiently at an input voltage lower than the nominal voltage which could be provided by the 3 Volts lithium cell. Thus, the drive consumed 3 mA at 2 Volts (60 mW) at the nominal flow of 0.5 µL/min push-pull and was able to operate the OFM probe for 7 days continuously. To allow a higher initial flow for flushing (1.75 µL/min) and a fine adjustment of the nominal flow, a converting circuit was designed which enabled supply of the pump with significantly higher voltages than the battery voltage.

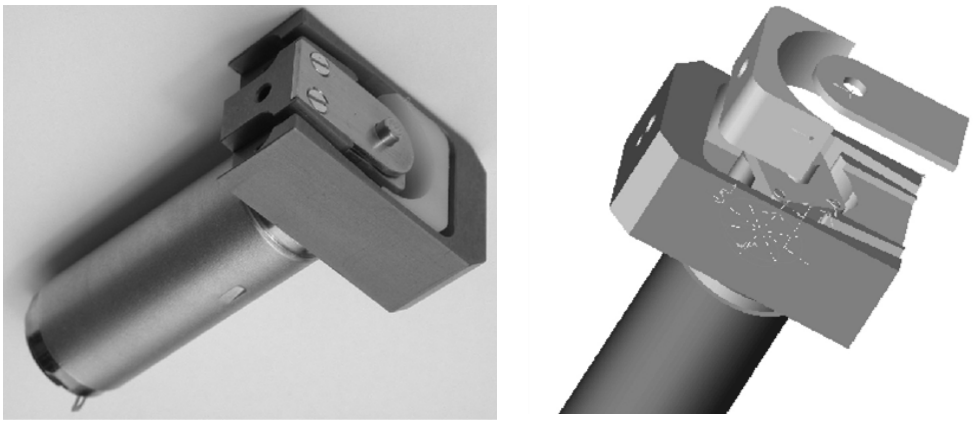


Figure 2-4: OFM push-pull micropump (16 mm 4-roll pump-head, DC drive, 1:19,000 gear reduction).

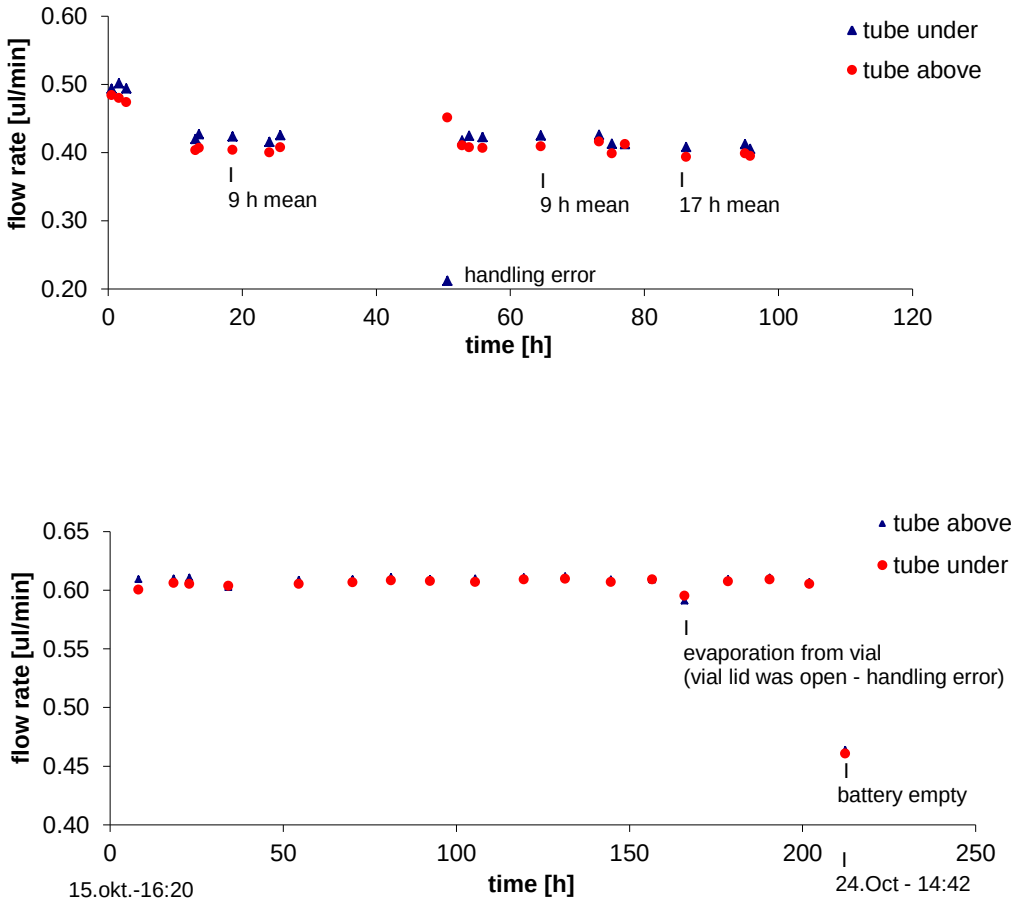


Figure 2-5: Results of flow stability testing over 100 hours (top panel) and 214 hours (bottom) showing that flow stability could be achieved for 200 hours. The results for “tube above/below” indicate that the flow was not dependent on the hydrostatic pressure. Each point represents one measurement (N=1).

2.2.2.3 Evaluation for long-term s.c. sampling (100 h OFM)

The pump was evaluated in three prolonged sampling experiments wearing the pump in combination with a subcutaneously implanted OFM probe (24 G version) for 72 to 100 hours. Subcutaneous OFM samples were collected continuously and used to assess the sample volume, the relative sampling recovery as well as the subcutaneous glucose concentration. A 5 % mannitol solution (ion-free, isotone, sterile) was used as perfusate, in order to utilize the ionic reference technique (IRT, [147]) for monitoring the relative recovery (RR) based on the recovered sodium and potassium concentration.

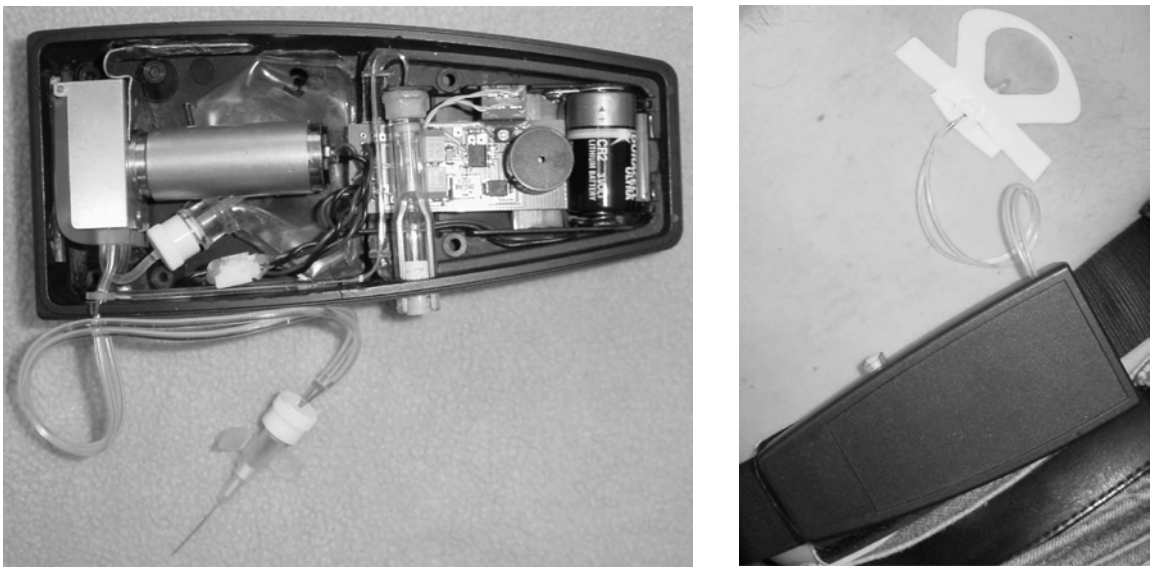


Figure 2-6: First version of the wearable 2-channel OFM pump for the sampling from a subcutaneous OFM probe (Year 2002)

The relative recoveries derived by sodium and potassium correlated well (Figure 2-7). The relative recovery typically ranged within 40 % to 60 % in the three sampling experiments. Increased recoveries were usually found during active phases (days), while lower recoveries correlated with phases of low or no activity (night). The mean flow rates in the three experiments ranged from 0.47 µL/min to 0.54 µL/min and were thus close to the nominal rate of 0.5 µL/min.

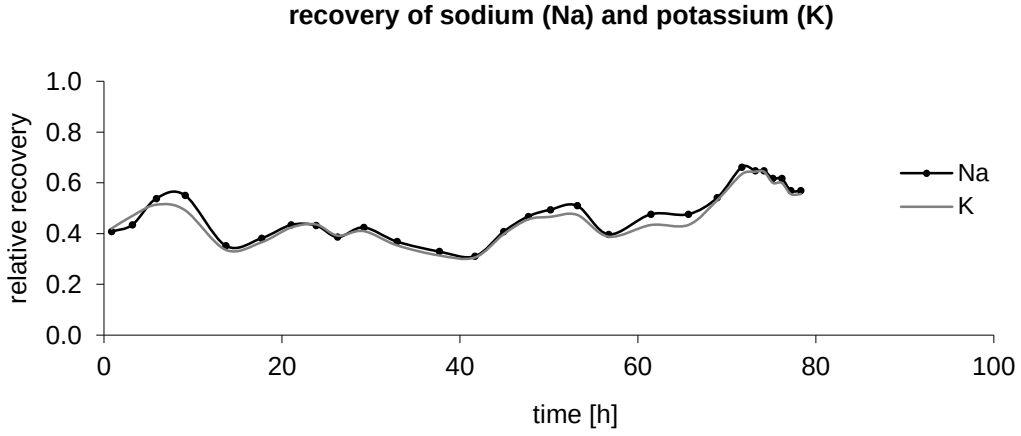


Figure 2-7: Relative recovery of sodium (Na) and potassium (K) during 80 hours of subcutaneous OFM sampling using the wearable pump (N=1).

The glucose and lactate concentration in the OFM samples were stable during the prolonged period (Figure 2-8). The interstitial glucose concentration – obtained by calibration with sodium (IRT-technique, [147]) – was approximately 70 % (60 % to 90 %) compared to glucose in capillary blood which is in agreement with prior glucose sampling studies of shorter duration [147]. Thus, this experiment demonstrated that the adipose tissue/blood relationship for the concentration of

glucose sampled by OFM was stable for extended periods. This stable relationship meant that a single-point calibration to blood concentrations was feasible in order to directly follow systemic concentrations for glucose monitoring purposes. The data of this experiment, however, did not allow an assessment of the correlation between adipose tissue glucose and blood glucose. The author had clearly focused on the long-term stability of sampling using a wearable OFM pump and used convenient finger prick glucose measurements which could also been performed at home.

Importantly, the sampling probe did not cause any visible irritation. Local skin irritation was caused by the adhesive material of the plasters.

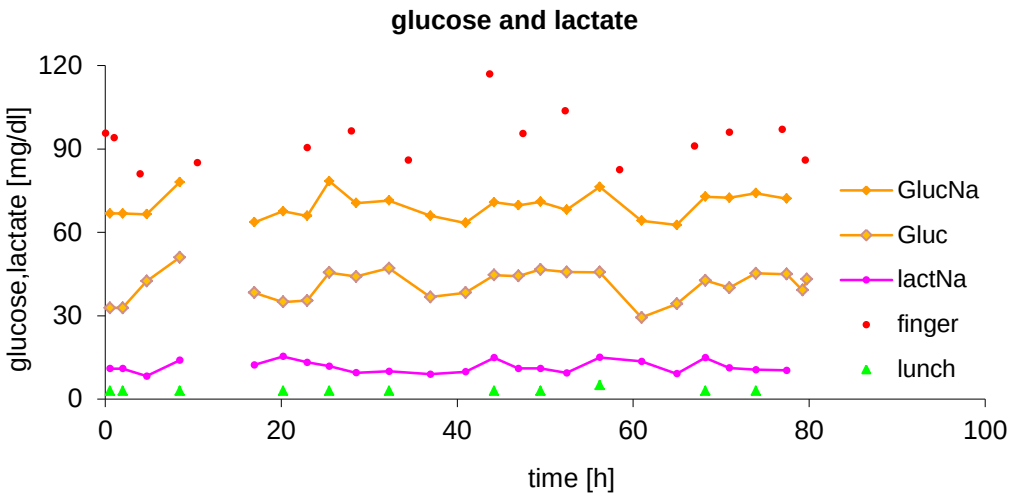


Figure 2-8: Glucose and lactate concentrations as monitored by OFM. Glucose was assessed from capillary finger blood. Glucose and lactate were corrected for the relative recovery (GlucNa, lactNa) to show interstitial fluid concentrations. Each point represents one measurement (N=1).

This proof-of-concept experiment confirmed that such a wearable peristaltic pump would be appropriate for the use in a continuous glucose monitoring device. Moreover, it confirmed that an OFM probe can provide reliable access to subcutaneous adipose tissue for metabolic monitoring purposes for more than 3 days.

2.2.2.4 Research on s.c. glucose monitoring

Following the successful proof-of-concept, the peristaltic pump unit including battery-driven power supply and tubing set was refined slightly and integrated into a wearable glucose monitoring device developed within the EC-funded research project “ADICOL” (ADvanced Insulin infusion using a COntrol Loop; Partners: Disetronic Medical Systems AG, Burgdorf, Switzerland; SensLab GmbH, Leipzig, Germany; FhG ISIT, Hamburg, Germany). Figure 2-9 shows the ADICOL monitoring device including its components.

In this project a series of experiments involving healthy human volunteers and diabetic individuals were performed confirming that the pump is appropriate for use in combination with glucose sensors. The wearable monitoring unit was able to monitor the subcutaneous glucose concentration and, when properly calibrated to blood glucose levels to reliably reflect the volunteers blood glucose profiles. Figure 2-10 shows the results of such a collaborative monitoring experiment of all project partners performed in Graz, Austria. Notably, the sensor unit also continuously sensed the conductivity of the interstitial fluid collected from the probe. Thus, the monitoring unit utilized the "Ionic Reference Technique – IRT [147]" which informs about the relative recovery of small molecules from the tissue by the OFM probe. The IRT technique enabled the "online-correction" of the glucose signal and provided the information that subcutaneous sampling using OFM probe and OFM pump was reliable and stable for prolonged duration.

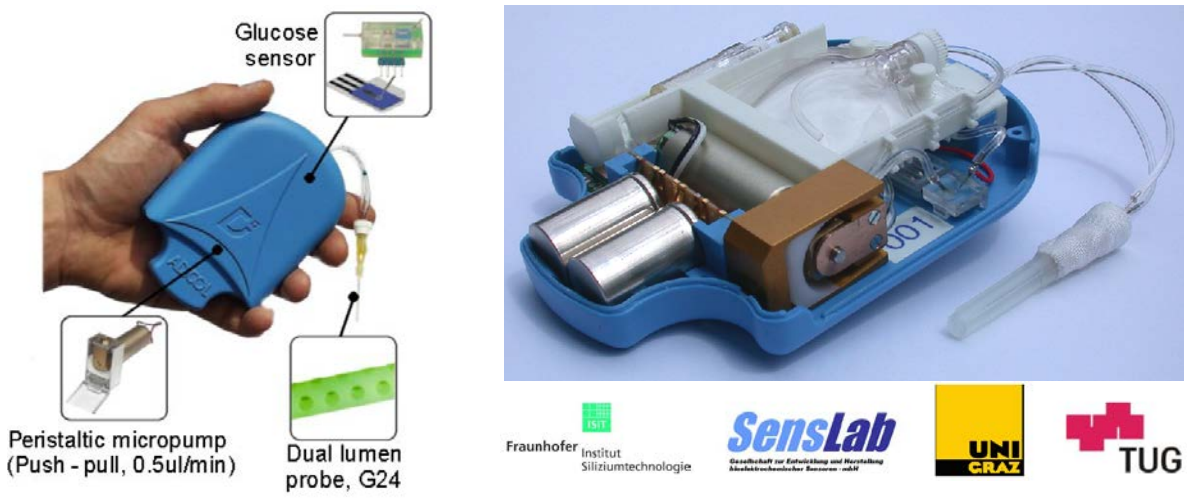


Figure 2-9: The ADICOL glucose monitoring device including OFM pump, OFM probe and a thick-film or thin-film sensor. Partners were Disetronic Medical Systems AG, Burgdorf, Switzerland; SensLab GmbH, Leipzig, Germany; Fraunhofer - Institut für Siliziumtechnologie FhG ISIT, Hamburg, Germany; and OFM R&D team at Technical University Graz and the Medical University Graz, Austria.

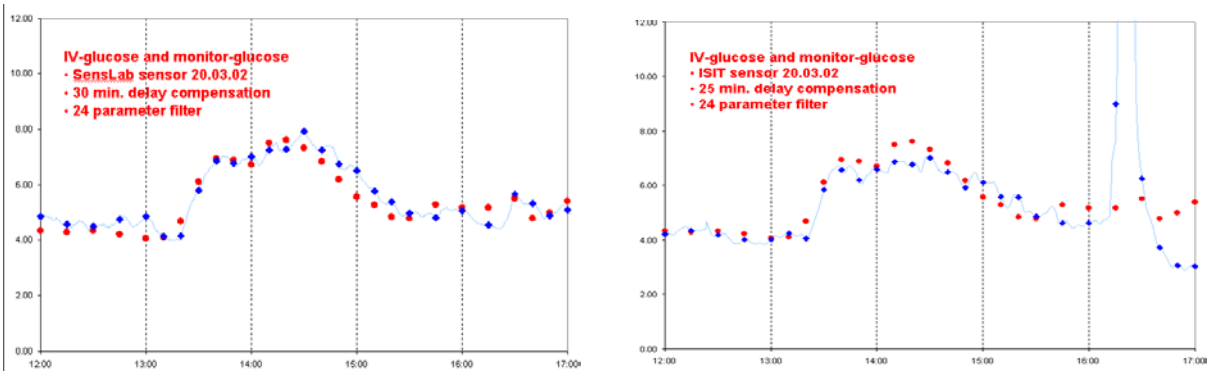


Figure 2-10: Illustration of online glucose monitoring results (blue line) following an oral glucose load. Two ADICOL monitoring devices were used in parallel to compare two different sensors. Red dots indicate plasma glucose levels. Each dot represents one measurement (N=1).

2.2.3 Pump 2 – A versatile pump for drug research

A pump for clinical pharmaceutical research needs to be more versatile than a pump for metabolic monitoring. Versatility is required to support the different settings and protocols used in drugs research. The pump needed to support exploratory and basic research which is characterized by experimenting with different probe types, flow rates and perfusates until the appropriate parameters are identified for subsequent more standardized exploratory studies. In addition the pump needed to support highly standardized research settings as required in clinical trials generating data for the market release of a novel medicine.

The most challenging future use case for OFM was chosen as a goal for the design of the wearable pump. This most challenging use case had been outlined by Dr. Eva Benfeldt et al. in publications on the use of microdialysis for the evaluation of topical bioequivalence in the renowned Journal of Investigative Dermatology and in a White Paper written in collaboration with the American Food and Drug Association FDA [12, 13]. In brief, Benfeldt et al. described statistically powerful study settings using several test areas on the skin for the parallel application and head-to-head comparison of topical drug penetration from the generic products (test products) versus the originator product (reference product). The authors convincingly outlined the utility of the continuous dermal sampling approach and the relevance of using multiple test areas and multiple probes in parallel for efficient bioequivalence studies in a relatively low number of subjects. Their statistical calculations indicated that with intradermal sampling methodology, bioequivalence studies with 90 % confidence intervals and 80 % to 125 % bioequivalence limits can be conducted on 27 subjects when using two probes in each test area, or 18 subjects using three probes per test area [150]. According to these considerations large settings with multiple probes per volunteer were ideal and the way to go for the future of microdialysis and OFM. According to this a wearable pump was ideal if it could operate all probes in a topical application area or even all probes of two or three neighboring test areas. Thus, the number of pumps (and sterile tubing kits) required per volunteer would be low and the setting would more likely be tolerated by the volunteer and the clinical investigators.

2.2.3.1 Requirements

The different use cases described above for exploratory research and highly standardized drug product evaluation such as topical bioequivalence resulted in different requirements and different priorities regarding the degree of fulfillment of those requirements.

The main requirements for the pump for drug research were:

- *wearability*
- *low power consumption to enable >48 h continuous operation with one battery*
- *sterile exchangeable fluidic components*

Specific requirements for pharmaceutic research:

- operation of 3 OFM probes in push-pull or 6 MD probes in push mode,
  i.e. 6 channels for push-pull operation required

- flow rates 0.1 to 5 or 10 µL/min
- flow rates in push & pull independently adjustable
- single use sterile perfusate bags are easy to fill, easy to exchange
- single use sterile tubing kit is easy to insert
- pump settings are easily adjustable by the experimenters without tools
- pump settings are easily verifiable by the experimenters
- Support sampling of all molecules without restriction in size, lipophilicity or protein binding
- Supports all known protocols for quantification: Zero-flow-rate protocol, no-net-flux protocol, recirculation

These parameters were more important compared to a monitoring pump:

- Reproducibility of flow rates between channels and between pumps
- Adaptability for the needs in clinical research (flows, perfusates, modes) during the use by clinical research staff

These parameters were less of importance compared to a monitoring pump:

- Size, weight, i.e. acceptable as long as still wearable
- Ease of use, i.e. function & adaptability had priority over simplicity

2.2.3.2 Pump design 1

In an initial step the 2-channel peristaltic monitoring pump was adapted to a 6-channel pump.

In brief, this was achieved by adapting the pump head (pump bed) dimensions and by using a different type of tubing (smaller diameter outside, larger inner diameter). Due to the smaller tubing the pump bed could accommodate 6 tubes in parallel, and due to the reduced wall thickness of the tubing the deformation energy per tube was reduced such that the same pump was able to establish the flow in 6 channels.

In addition, to enable the sampling of lipophilic analytes, a sample trap (‘collection unit’) was designed which directly entraps the interstitial fluid at the probe outlet in capillaries, which can be emptied into regular sampling containers for analysis. The pump’s pull channel is connected to the other end of the capillaries and thus is able to actively control the sample flow rate and thereby ensures that inflow rate equals the outflow with fluid loss to the tissue across the probe’s exchange area.

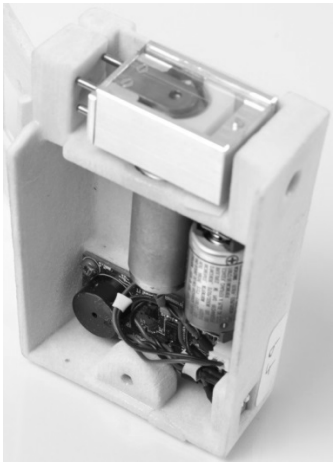


Figure 2-11: First version of a 6-channel microperfusion pump for the operation of 3 OFM probes in push-pull mode (size 10 x 5 cm)

A small series of this type of pump was produced for the performance of the first clinical pharmaceutical study with dermal open-flow microperfusion (dOFM) investigating a topical drug. The study investigated the penetration of a lipophilic topical drug candidate in the dermis of non-lesional and lesional skin of psoriatic patients over 25 h, and also investigated the modulation of the locally released biomarker TNFα. Multiple dOFM sampling was performed for 25 h from non-lesional and lesional skin on day 1 and day 8. Patients tolerated the 25 hour uninterrupted sampling protocol with several probes as the novel light-weight push–pull pump enabled mobility for normal bathroom visits.
This initial dOFM study was successful in terms of dOFM methodology and the scientific results. A summary of the study is provided in section 2.4.4., and the scientific paper provided in the appendix. Based on the success the decision was taken to intensify work on OFM and the devices in order to create a versatile tool for research which is accepted by authorities in Europe as a regular medical device for human use according to international regulations for medical devices. To enable this, the entire research group was educated and re-organized in a laborious 2-year process resulting in the institute's successful certification according to international guidelines for quality systems for medical device manufacturers (EN ISO 13485).

2.2.3.3 Pump design 2

In the second step all scientific requirements as outlined above in 2.2.3.1 were considered in the design of the pump.

In addition, as the standard of a medical device had been envisaged, the requirements were considered which arose from the international quality system standards for medical devices as issued by the International Organization for Standardization. These regulations cover the methods, facilities and controls used by the manufacturer in the design, manufacture, packaging, labelling, storage, installation, servicing and post-market handling.

In brief this meant for the design process:

- Certification of the Quality Management System according to EN ISO 13485 and EN ISO 14971:2007 (risk management process) by a notified body (TÜV Austria).
- Proof of conformity of medical device according to European Union Directives 93/42/EEC, including …
    - Evaluation of electrical safety (IEC 60601)
    - Evaluation of the sterility for sterile components of the pump (ISO 11737-2)
    - and several other standards

The requirements resulted in the implementation of a peristaltic pump again, with many similarities to its precursors. In contrast to its precursors the pump features 2 independent pump heads in order to control push and pull independently or, if used for microdialysis, in order to perfuse the probes in different test sites independently. Furthermore, the pump features different buttons for fast adjustment of parameters and a display providing information on the internal pump settings.

The re-usable pump was classified as a class I medical product, while the sterile components (tubing kit, perfusate and waste bag) were classified as class IIa product like the sterile dOFM probe. The standards for medical devices could be successfully implemented. The pump passed all tests at the notified body and was CE-certified as a medical device for human use in 2010. The pump has proven applicability and performance at the clinics in medical device evaluation trial on 17 volunteers.

Since 2010 the pump has enabled clinical and preclinical research on various drugs and products. Moreover, all functions of the pump have been required in the initial years of research use. This means the pump has been used with different settings between the two pump heads for netto delivery to the tissue, used for perfusion of probes with different flow rates, and used for quantification with no-net-flux protocols as well as recirculation protocols.

Figure 2-12: First CE-certified microperfusion pump

2.3 Designing dermal OFM probes

2.3.1 Motivation

The design of dermal OFM probes was motivated by the attractive research opportunities a dermal probe would provide, which does not limit dermatological pharmaceutical research to small and hydrophilic drugs but would extend research to very large and more lipophilic drugs.

Very large molecules appeared attractive in view of the large polypeptides (proteins, markers of inflammation like cytokines) which characterize states of skin inflammation in skin diseases like psoriasis or atopic dermatitis. Most of these cytokines feature a molecular mass above 10 kDa, with the pro-inflammatory cytokine TNFα appearing as a trimer (combination of three monomers) of 51 kDa. The access to very large molecules in the skin also appeared attractive in view of the increasing number of monoclonal human antibodies which were in the development pipeline of the pharmaceutical industry.

Lipophilic drugs appeared attractive due to the fact that most topically applied small molecules for skin penetration are predominantly of lipophilic nature in order to be able to penetrate the lipid-rich barrier of the skin, the stratum corneum. Also for this type of drug the development pipeline in the pharmaceutical industry is filled with many drug candidates which need to be characterized for their bioavailability at their dermal target.

Finally, the chance to enable clinical bioequivalence testing of new formulations and topical generic products regardless of the challenging properties of the drug appeared attractive too, and worthwhile to apply the OFM approach to dermal sampling.

A brief background on the history of dermal sampling by microdialysis including the SoA devices has been provided in 1.3.

2.3.2 Requirements & Challenges

The main requirements for the dermal sampling probe were:

- *minimal invasiveness for implantation into the dermal layer of the skin including an insertion needle*
- *large open exchange are for efficient recovery of analytes*
- *mechanical robustness (high tensile strength) to withstand forces during implantation and removal from the collagen-rich dermal layer of the skin*
- *accurate positioning of exchange area*
- *sterility*

Specific requirements for pharmaceutic research:

- Exchange area should allow sampling of all molecules without restriction in size, lipophilicity or protein binding
- Surfaces should prevent loss of analytes by unspecific adsorption

2.3.3 Overview – Probe designs & research

The subsequent sections provide details on the different probe versions and summarize the research these probes enabled, from initial dermal sampling experiments in animals with prototypes to clinical pharmaceutical research with CE-certified OFM products today.
In brief, the design of probes and introduction into clinical research followed a stepwise approach which took several years. The initial step towards dermal OFM was done with a probe type used in rat skin only (**Probe 1**, section 2.3.3 provides technical details including studies). The subsequent probe type (**Probe 2**, section 2.3.5) showed superior mechanical stability had been immediately carried forward to human experiments and characterized for its basic sampling performance, utilizing the knowledge from subcutaneous sampling of small molecules for glucose monitoring. When these first human experiments with the dermal OFM probe delivered dermal glucose profiles nicely tracking the capillary glucose concentration profile, patent protection has been envisaged, and sponsor in the industry for dermatological products found for a first dermal OFM drug study investigating a novel topical compound's pharmacokinetics and –dynamics (PK-PD) directly in inflamed and unaffected skin of patients (Summary in 2.4.4, paper provided in the appendix). The success of this first PK-PD study led to the invention of an advanced probe type (**Probe 3**, 2.3.6) which combined a maximum of robustness (safety) with a maximum of effective exchange area (minimal material intervening with exchange of solutes).
This dermal probe concept, together with the concept of a versatile wearable pump, prompted the implementation of the quality management system for medical device manufactures at Joanneum Research and the subsequent development of probe and pump as regular medical devices with CE-certification for human use in Europe. These medical devices have been thoroughly evaluated for safety and performance and subsequently used in several trials which proved the utility of dOFM devices for PK-PD studies of lipophilic compounds, large antibodies and for highly demanding trials of topical bioequivalence. The research performed with current certified medical devices is summarized in a separate chapter, chapter 2.5.

2.3.4 Probe 1 - Perforated steel cannula

The initial dermal probe version showed similarities to the SoA s.c. probes then and were used in animal skin (rat experiments) only. The probe was manufactured by lasering holes into the insertion needle of a clinical venflon device. Thus, an initial version of a membrane-free probe was created which could directly be inserted into the skin (dermal layer) of the animal. After insertion, the perforated needle was connected with peristaltic pump tubing on both ends for constant perfusion with the perfusate and rate-controlled withdrawal of sample.

This initial dermal OFM probe enabled studies investigating the penetration of topically applied ketoprofen into the skin (Pickl et al. 2007, see 2.4.1). dOFM data indicated that the Transfersomes® enhanced the penetration of the drug. Further studies were performed investigating the topical penetration of interferon-γ (unpublished).

Shortcomings of probe 1:

- High production costs (CO_2 lasering of 100 holes per probe)
- Poor mechanical stability with risk of breakage at perforated section
- High forces required during insertion
    - Needle-cut not suitable for dermal insertion
    - Increased friction of perforated area within tough dermis

Key learnings regarding probe design:

- Mechanical properties of the dermis of skin are demanding when inserting a probe horizontally within the collagen rich layer of the dermis (“leather”).
- The asymmetric cut of the needle leads to lateral forces and difficulties to maintain a certain depth.
- Any needle designed to penetrate the skin (venflon, normal insertion needles) is not ideal for the purposes of probe insertion.
- Surface properties of needles decide whether insertion over 30 mm is feasible or forces are too high to finalize insertion.

In conclusion, the laser-perforated needle was non-ideal for use in animal skin and not suitable for human application.

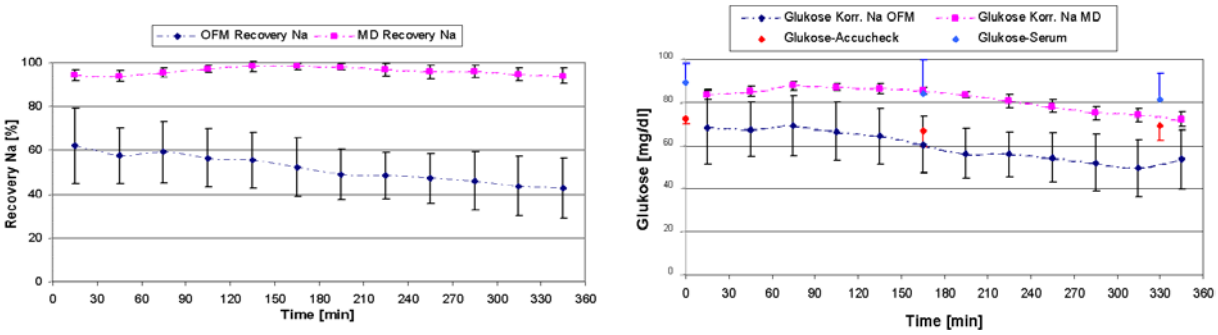


Figure 2-13: First dermal OFM probe version vs. microdialysis probe. Left: Relative recovery of sodium. Right: Glucose concentrations after relative recovery correction.

2.3.5 Probe 2 - Invention of Helixcath – Patent 1

The experience with probe 1 suggested that a dermal probe should be based on tough polymeric material whose mechanical (tensile) strength was not diminished by the production process. This ruled out any possibility to produce a safe probe for human use based on any known fluoropolymeric material if holes were to be produced by a lasering process. Similarly, the production of holes by punching or high-speed drilling would diminish the probe’s tensile strength in axial direction, and moreover, the production would have been too laborious (expensive) for mass production and pose the additional risk of particles released from the surface into human subjects.

Probe 2 was finally based on biocompatible polymeric tubing (a fluoropolymer) which, however, was not processed in such a way to form holes but in a way that resulted in a longitudinal slit, i.e. a slit in axial direction. The longitudinal slit did not diminish the tensile strength and provided an axial exchange area over a distinct length. The exchange area was maximized by twisting the tube under heat treatment resulting in a stable helical structure with sufficient exchange area towards the tissue. For insertion the probe was equipped with an insertion needle on one end by a crimping technique.

Advantages over probe 1:

- Highly flexible biocompatible material
- Clean production process
- High tensile strength (safety)
- Cost-effective production

Disadvantages:

- Exchange area was not perfectly reproducible as the width of the helical slit after processing and implantation varied.
- Slit design showed a low effective surface area for molecular exchange

A rat experiment showed that this probe had a sampling efficiency (relative recovery) for glucose which was stable and comparable to that of a microdialysis membrane (Figure 2-14).

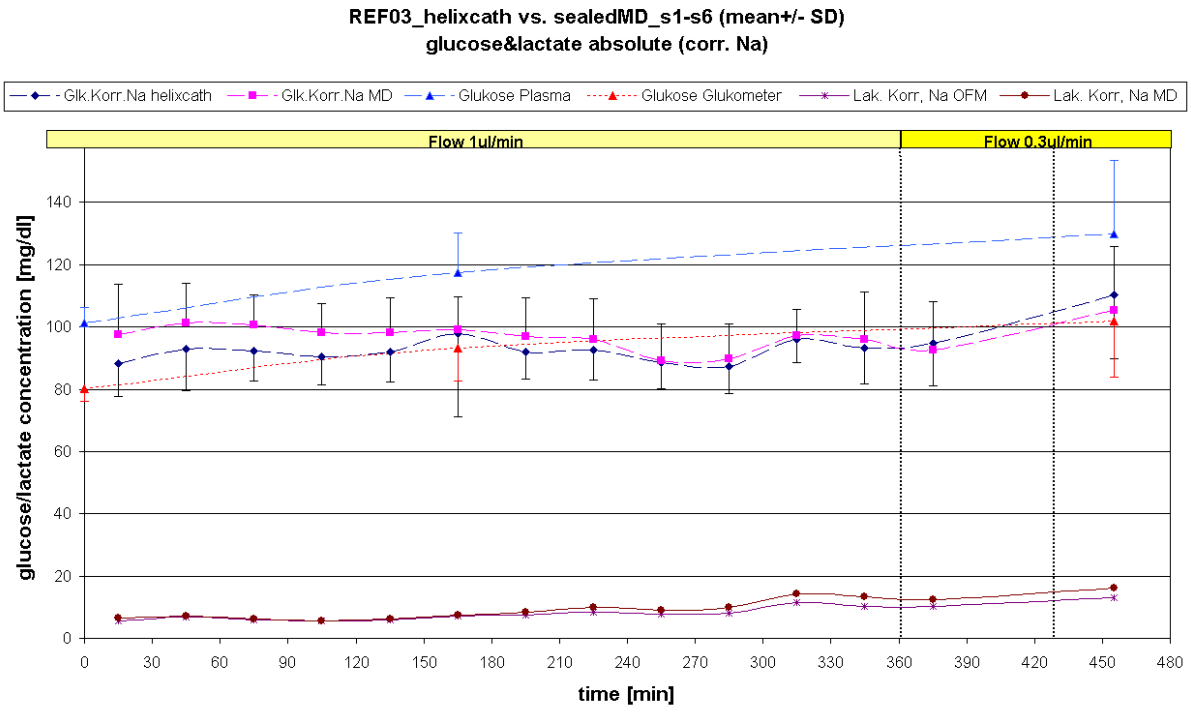


Figure 2-14: Comparison of Helixcath with a microdialysis membrane of equal length when sampling glucose and lactate in rat skin. Data are mean ± SD, n=6 rats.

This probe was used in rats to investigate the penetration of topically applied drug candidates, topical interferon-gamma and topical ketoprofen. This study on ketoprofen led to the first OFM publication in the field of dermatology. Research and paper are summarized in section 2.4.1.

From animal to human use

The superior safety properties of dermal probe no. 2 allowed human use and a characterization for its basic sampling performance, utilizing the knowledge from subcutaneous sampling of small molecules for glucose monitoring. This first feasibility study for human use and the use for metabolic monitoring are summarized in section 2.4.3.

Thereafter, this first dermal probe was successfully used in a feasibility study for lactate monitoring during sports. A parallel collaborative European project on continuous glucose monitoring had provided the opportunity for this feasibility study. The results of this feasibility have been shown in the section on the clinical use of the wearable pump (2.3.5.).

In consequence, patent protection has been obtained for this first clinical dermal OFM probe [1].

Moreover, a sponsor had been found to fund a **first clinical dermal OFM drug study**, which was the proof-of-concept for dermal OFM for clinical pharmaceutical research. This study investigated a novel topical compound's pharmacokinetics and –dynamics (PK-PD) directly in inflamed and unaffected skin of patients (summary in 2.4.3, paper provided in the appendix).

This first clinical dermal probe, however, was not been used in any further study beyond the successful proof-of-concept but was immediately substituted by the next invention (Probe No. 3, "A filament-based catheter", [2]) which is described in the subsequent section. Probe No. 3 was developed to a regular medical device right from the beginning and since then has been used in all clinical pharmaceutical research trials with OFM to this date.

To summarize, probe no. 2 (Helixcath) was the first dermal OFM probe used in humans. In its short period of utilization it enabled the following experiments/studies which step by step led to the introduction of OFM into clinical dermatological research:

- Dermal PK of carriers and topical drug candidates in rats - data not shown
- Dermal PK of topical ketoprofen in rats – see 2.4.1
- Dermal glucose/lactate in rats compared to microdialysis – see data above
- Clinical feasibility of human use for dermal and subcutaneous glucose/lactate sampling – see data above
- Clinical feasibility for dermal lactate monitoring during sports – see 2.4.3
- Clinical proof-of-concept for PK-PD of a topical drug using dermal OFM - see 2.4.4

2.3.6 Probe 3 - Invention of filament-based catheter – Patent 2

Probe 2 was not yet ideal regarding the size of the effective exchange area and its reproducibility. An increase of the exchange area did not seem feasible due to further reduction of the

mechanical resistance (tensile strengths) and further loss of the reproducibility of the exchange area.

The invention of probe 3 was the consequence of a reflection on the OFM principle and its ideal implementation with the perfusate being in direct contact with interstitial fluid over a maximum exchange area. In the ideal situation the perfusate flows from a (supply) point A in the tissue to a distant point B (collection) without any material in between limiting the exchange of molecules between perfusate and the surrounding tissue. However, even after a traumatic creation of such a channel, the channel would be closed immediately by a collapse of tissue followed by tissue repair mechanisms. In the dermis, immune response and tissue repair would be fast and would close such a fluidic pathway immediately. The probe structure therefore would have the minimum requirement to prevent the tissue from collapsing after creation of a channel between the two points of perfusate supply and collection. Such a stable supporting structure would be formable by thin metal wires forming a cylindrical grid. The metal wire(s) would feature the elasticity to withstand the radial forces and to maintain the cylindrical structure, and a few wires would show sufficient tensile strength to bear the axial forces during insertion and removal of the probe. But the problem of the mechanical bonding remained, as it would be hard to produce an acceptable safe probe for human use at acceptable costs by attaching such a structure between supply and collection tubing or by attaching it firmly to the surface of a continuous tube acting as a guide. The problem with the bonding and mechanical strengths could be avoided by forming a probe from a small tube of biocompatible polymeric material with a wall reinforced by very thin steel wires, a structure comparable to a garden hose with a wall reinforcement provided by a grid of glass fibres during the extrusion process. An exchange area could be created by removing the polymer on a section of the tube such that the thin steel grid (‘mesh’) remained providing an ideal exchange area. Such a probe would show favourable mechanical tensile strength and ideally be producible in an automated continuous industrial process with highly reproducible dimensions. The probe would need to include printed positioning markers for reproducible placement of the exchange area in the skin and be combined with a standard Luer for supply and an insertion needle for intradermal or subcutaneous placement.

The idea could be implemented when an industrial partner was identified who had expertise in the production of reinforced tubing in low dimensions.

Features of the probe:

- Exchange section 15 mm, composed of stainless steel mesh
- Inner diameter of 0.25 mm, coated with an anti-adoptive layer
- Outer diameter of 0.40 mm, printed positioning marks
- Standard Luer connector for perfusate supply
- 0.5 mm insertion needle attached, alternative insertion through a larger hollow standard needle like microdialysis

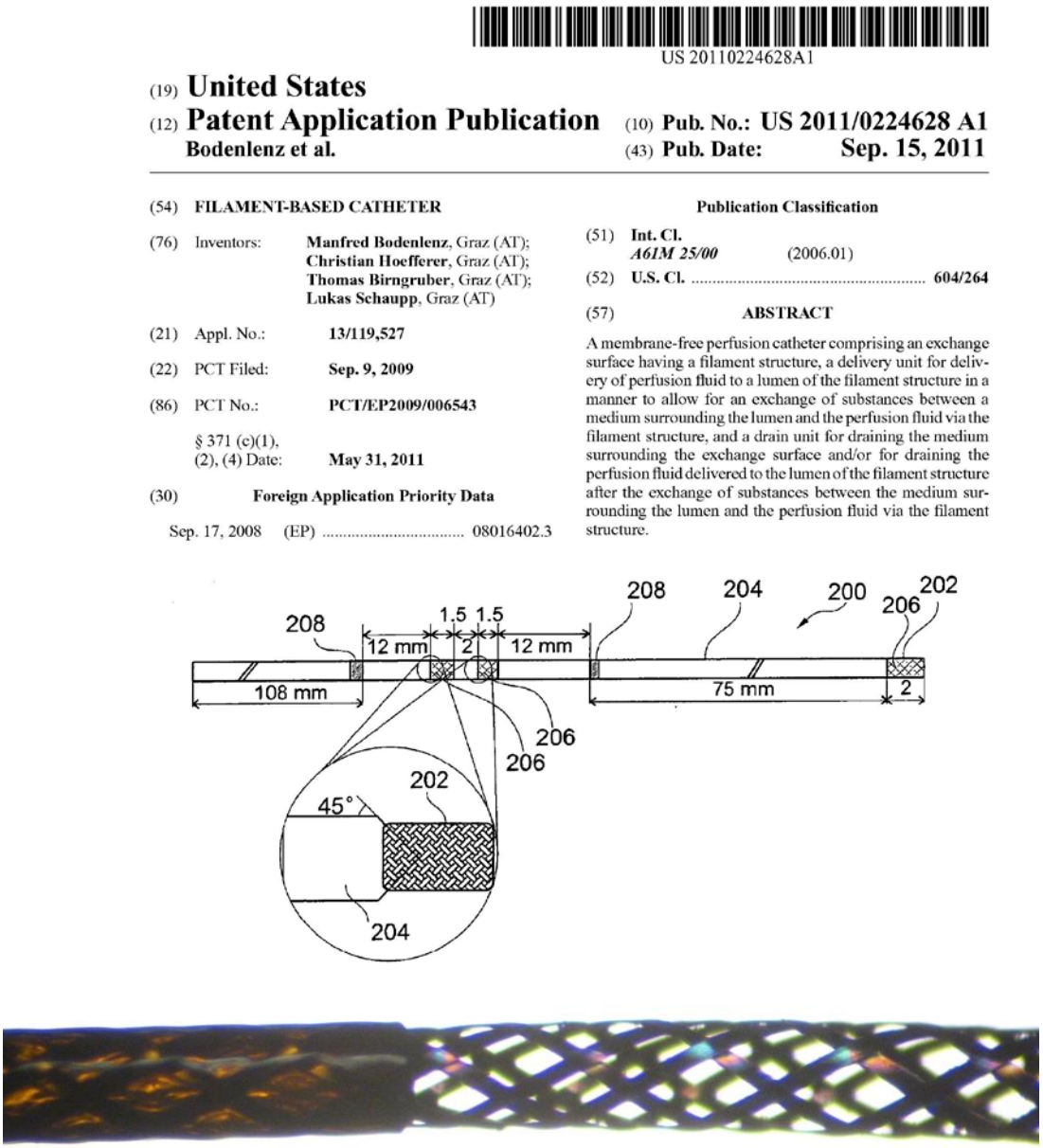
Advantages over probe 2:

- Maximum exchange area

- Very high tensile strength, i.e. safety
- Reproducible dimensions of exchange area
- Reproducible positioning by position markers

Probe 3 has been patented as well (Figure 2-15). However, and this was an essential difference compared to the prior probe, probe 3 was developed from the beginning as a regular medical device for human use under the stringent quality regulations for medical device manufacturers following EN ISO 13485.

This dermal probe concept, together with the concept of a versatile wearable pump, prompted the implementation of the quality management system for medical device manufactures at Joanneum Research and the subsequent development of probe and pump as regular medical devices with CE-certification for human use in Europe. These medical devices have been thoroughly evaluated for safety and performance and subsequently used in several trials which proved the utility of dOFM devices for PK-PD studies of lipophilic compounds, large antibodies and for highly demanding trials of topical bioequivalence. The research performed with current certified medical devices is summarized in a separate chapter, chapter 2.5.



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(12) **Patent Application Publication**

Bodenlenz et al.

(10) **Pub. No.: US 2011/0224628 A1**

(43) **Pub. Date: Sep. 15, 2011**

(54) **FILAMENT-BASED CATHETER**

(76) Inventors: **Manfred Bodenlenz**, Graz (AT); **Christian Hoefferer**, Graz (AT); **Thomas Birngruber**, Graz (AT); **Lukas Schaupp**, Graz (AT)

(21) Appl. No.: **13/119,527**

(22) PCT Filed: **Sep. 9, 2009**

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(51) **Int. Cl.**
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(52) **U.S. Cl.** **604/264**

(57) **ABSTRACT**

A membrane-free perfusion catheter comprising an exchange surface having a filament structure, a delivery unit for delivery of perfusion fluid to a lumen of the filament structure in a manner to allow for an exchange of substances between a medium surrounding the lumen and the perfusion fluid via the filament structure, and a drain unit for draining the medium surrounding the exchange surface and/or for draining the perfusion fluid delivered to the lumen of the filament structure after the exchange of substances between the medium surrounding the lumen and the perfusion fluid via the filament structure.

Figure 2-15: Title page of the US patent disclosing a “filament-based catheter” (patent was granted). Bottom: A picture of the first filament-based probe version under the microscope (outer diameter 0.36 mm).

2.4 From preclinical experiments to clinical research

This chapter summarizes the preclinical experiments which were the first to utilize dermal OFM probe prototypes for the purpose of intradermal drug sampling after topical application. It was probe type 2 “Helixcath” which enabled these first successful experiments on drugs in rats (for probe details see 2.3). These experiments mark the beginning of “dermal OFM” and its use for pharmaceutical testing. The success of these rat experiments and the probe’s robustness prompted the evaluation of the feasibility of using this dermal probe in human skin in vivo. These first human experiments did not yet involve any drug but used the vast knowledge from metabolic monitoring research to characterize the dermal probe and the dermis as novel tissue for OFM sampling. These human experiments are also summarized in this chapter as they paved the way for the first clinical study using dermal OFM for pharmaceutical testing. This first human drug study served as proof-of-concept for dermal OFM at the clinics and is summarized at the end of this chapter. The proof-of-concept prompted the design of regular medical devices for CE-certification. All studies performed thereafter with CE-certified OFM were larger clinical trials for pharmaceutical research and are therefore summarized in a separate chapter.

2.4.1 Experiments on topical ketoprofen in rats – Pickl et al. 2007

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ScienceDirect
Journal of Chromatography B, 850 (2007) 432–439

JOURNAL OF CHROMATOGRAPHY B
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Rapid online-SPE-MS/MS method for ketoprofen determination in dermal interstitial fluid samples from rats obtained by microdialysis or open-flow microperfusion

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Available online 30 December 2006

As outlined in chapter 2.3, the first experiments in rats were performed using a perforated steel cannula. As its usability was very restricted, the first experiments on drugs were performed with probe type 2 (Helixcath). The first drug investigated by dermal OFM was topical ketoprofen. The reason for this substance was that a project partner had developed a ketoprofen formulation including a carrier (Transfersomes®) for enhancement of the topical penetration.

The rat study on topical ketoprofen has been published in the Journal of Chromatography [12]. A copy of the paper is provided in the appendix, as it is in fact the first publication mentioning the use of OFM for sampling from the dermis of the skin. However, the paper does not disclose any details of the research results regarding the topical ketoprofen formulations nor does it disclose

details on dermal OFM methodology. Instead, the paper focused on the bioanalytical quantification of ketoprofen as the analyte in OFM and microdialysis samples. Therefore, the brief summary below provides some information on the methodology and the significance of these experiments for further research and device development. The synopsis table (Table 4) provides condensed information on study design and methodology.

This study was the first to use probe type 2 (Helixcath). Sampling from the dermis after application of the two different formulations clearly showed the differences between the solution including a carrier (Transfersomes®) and the solution without carrier. The ketoprofen profiles after the application of the carrier solution was significantly different 5 hours post-dose. The profile of the carrier solution showed a biphasic pattern, indicating the penetration of the fraction of ketoprofen transported by the carrier and the penetration of free ketoprofen.

The parallel use of several probes within the skin showed that the concentrations varied considerably between probes and between application areas within the test animals. This variation was not due to differences between the sampling probes or the relative recoveries. The stability of the relative recovery and the comparability between the two groups had been verified using sodium as the marker for the relative recovery (Figure 2-16). The variation of drug concentrations between probes was explained in the paper by variation in probe insertion depth, in skin/stratum corneum thicknesses, in dermal capillary perfusion, and in natural local shunts through the barrier by hair follicles. Years later the effects of probe depth variation and differences of the skin barrier between individuals have been closely investigated using clinical OFM material in humans. As will be shown in 2.5.2 the reproducibility of the further advanced clinical OFM (CE-certified materials) and procedures allowed to show that even small differences in probe depth affect the observed concentrations after topical administration. The rat model, however, has rarely been used thereafter for testing of topical penetration due to the fact that the dense fur (number of potential shunts by hair follicles) was not ideal to test penetration through intact skin. This drawback of the rat model was seen in a subsequent study on topical interferon-α (data not shown), when the drug penetrated the epidermal barrier towards the dermis much faster than expected. Therefore, thereafter pigs were used instead of rats for the testing of topical penetration. Finally, the use of human donor skin from plastic surgery was able to largely avoid the use of animals for the purpose of preclinical testing of topical formulations. Nevertheless, the rat model is in use in OFM research to this date, not for topical penetration testing but for a model of psoriasis-like skin inflammation (see “Current Research” in chapter 4).

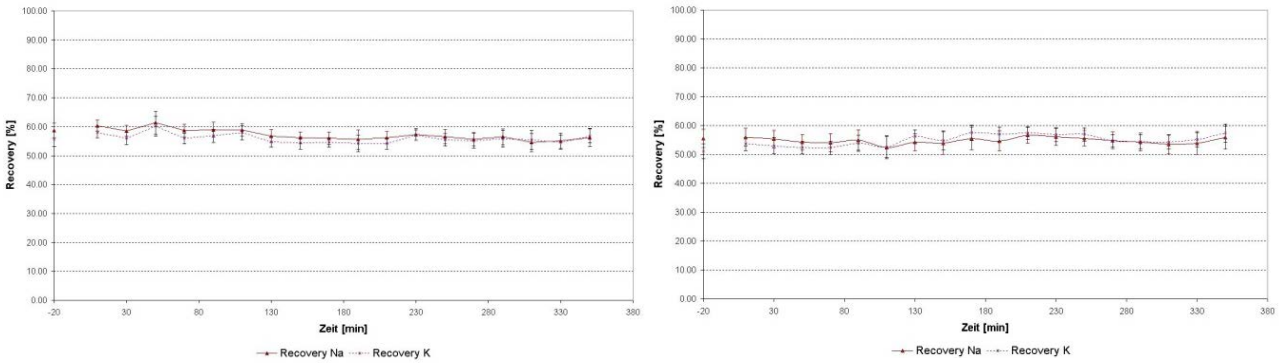

Figure 2-16: Sodium (Na) and potassium (K) relative recovery profiles in six control rats (left panel) and six carrier-treated rats (right panel; both mean ± SEM, N=6 rats, each with 4 probes). The profiles indicate that the sodium recovery was well comparable between control and carrier-treated rats (55.95 % vs. 54.86 %) and stable over time.

Author's contribution to research and publication: The author of this thesis conceived the design of the rat study, prepared the study, conducted the rat study with the preclinical research team, interpreted the data, and reviewed the manuscript.

Acknowledgements

These experiments have been performed in collaboration with IDEA AG in Munich, Bavaria, in the animal facilities of JSW Research Forschungslabor in Graz. Thanks to collaboration with the team at JSW Research (Gabriele Horwath, Dr. Birgit Reininger-Gutmann) and Anneliese Perrier from IDEA the author of this thesis became acquainted with animal research and was thereafter able to set up animal experiments in the centre of medical research (Zentrum für Medizinische Grundlagenforschung) of the Medical University Graz. This work was supported financially by the Austrian Federal Ministry of Transportation, Innovation and Technology (bmvit), Project 'ARTic' – Applied Research Technologies for the interstitial compartment – Grant No. Z1.805351.

Table 4: Synopsis of the topical ketoprofen study in rats

Description	
Type of study	Animal study comparing the transdermal penetration of ketoprofen from a solution including a carrier with the penetration of ketoprofen applied in a solution without the carrier.
Subjects	12 rats (rat strain: Wistar, weight 300-350g)
OFM materials	OFM Probe: Helixcath (permeable length 18 mm, OD 0.40 mm) Pump: Minipuls 3 by Gilson, France; Perfusate: Saline (Ringer) +0.1 % Bovine Serum Albumin
Design	Ketoprofen solution with carrier (Transfersomes®) and ketoprofen solution without carrier, each solution applied topically in 1 chamber per rat.

	2 x OFM per chamber, 1 µL/min sampling 6 hours of sampling in 60 min intervals (1 h baseline + 5 h post-dose)
Analyses	Ketoprofen
Outcome, Significance	The study showed that lead to an increased skin penetration of ketoprofen. This study was the first OFM study investigating the topical penetration of a drug. This study was the first using probe type 2 (Helixcath). The success prompted the use of dermal OFM (Helixcath) for the evaluation of further topical formulations (e.g. topical interferon-α-2b formulations, data not shown) and the probe's subsequent evaluation for human use.
Publication	K. E. Pickl, C. Magnes, M. Bodenlenz, T. R. Pieber, and F. M. Sinner, "**Rapid online-SPE-MS/MS method for ketoprofen determination in dermal interstitial fluid samples from rats obtained by microdialysis or open-flow microperfusion.**," J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., vol. 850, no. 1–2, pp. 432–9, May 2007. **[12]**

2.4.2 Experiments on topical fentanyl and benzoic acid – Holmgaard et al. 2012

Pharm Res
DOI 10.1007/s11095-012-0705-9

RESEARCH PAPER

Comparison of Open-Flow Microperfusion and Microdialysis Methodologies When Sampling Topically Applied Fentanyl and Benzoic Acid in Human Dermis *Ex Vivo*

R. Holmgaard • E. Benfeldt • J. B. Nielsen • C. Gatschelhofer • J. A. Sorensen • C. Höfferer • M. Bodenlenz • T. R. Pieber • F. Sinner

Received: 12 September 2011 / Accepted: 8 February 2012

Today, dermal OFM is frequently used in excised human donor skin (fresh or thawed, provided by the local Biobank Graz, Austria) in order to assess various properties of novel topical drug candidates and formulations. Thus, dermal OFM in the human skin model supports decisions on which compound/formulation is promising and can proceed into clinical development.

The use of OFM in donor skin goes back to a stimulating cooperation with the Danish researchers Prof. Eva Benfeldt[1], Prof. Jesper Bo Nielsen[2] and Dr. Rikke Holmgaard[3]. During her thesis Dr. Rikke Holmgaard performed a study in Graz, bringing donor skin with her from Denmark to Graz, thus introducing the author of this thesis to the use of human donor skin for the purpose of preclinical topical drug and formulation testing.

Figure 2-17: Prof. Eva Benfeldt (right) and Dr. Rikke Holmgaard (left) during the setup of the initial ex vivo experiments in Graz in December 2009

An international joint experiment was performed to compare dermal open-flow microperfusion (dOFM) and dermal microdialysis (dMD) in a single-laboratory setting, and in a joint publication to

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[2] Department of Environmental Medicine, University of Southern Denmark, Odense, Denmark
[3] Department of Environmental Medicine, University of Southern Denmark, Odense, Denmark

guide future researchers in the selection of the method. We compared dOFM and dMD techniques (one dOFM probe and two dMD probe types) for the capability to sample topically administered fentanyl and benzoic acid in human donor skin over 20 hours (Figure 2-19). Fentanyl is rather lipophilic with a log P (partition coefficient of the unionized species in octanol-water) of 4.05 and was selected as a representative compound for lipophilic compounds, while benzoic acid with its moderate log P of 1.87 was selected as a representative for more water-soluble compounds. Both drugs were applied in fluidic solutions to the skin. To ensure sufficient skin penetration of fentanyl and to ensure recovery by the membranes a 20 % ethanol solution was chosen for fentanyl, while benzoic acid was applied in aqueous solution. To prevent binding to the microdialysis membranes an aqueous perfusate containing 1 % albumin was used. Under these experimental conditions the dOFM and the two dMD techniques recovered the drugs reproducibly and to a well comparable degree.

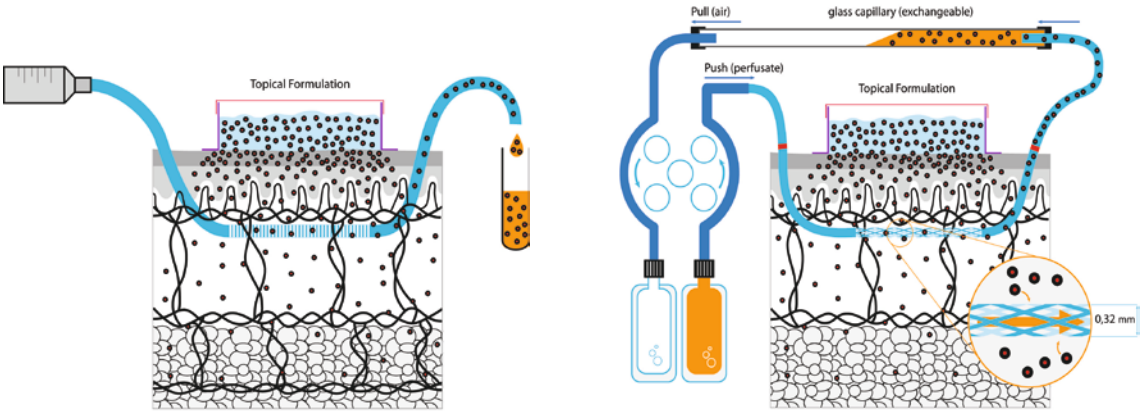

Figure 2-18: Dermal microdialysis (left) and dermal open-flow microperfusion (right) as used during the ex vivo experiments (pictures taken from Holmgaard et al. [11]).

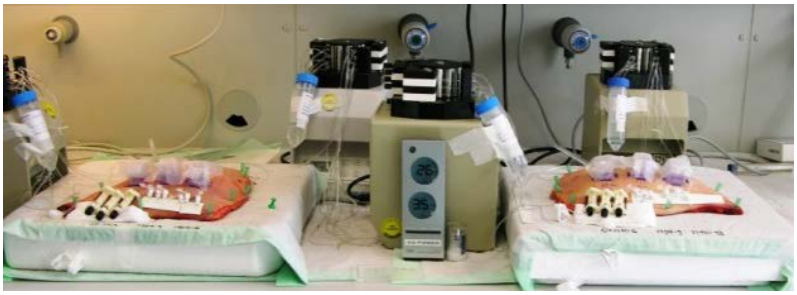

Figure 2-19: Ex vivo set-up comparing OFM and MD in two human skin explants. One explant was treated with fentanyl, the other with benzoic acid.

The subsequent joint publication provided the opportunity to discuss the advantages and limitations of the more or less established MD methodology and the novel OFM methodology with respect to their sampling capacity as well as their practical handling (see Table IV in Holmgaard et al. [11]). The discussion therein on the one hand pointed at the mechanical robustness of OFM probes, the strengths of OFM and its wide range of applicability compared to MD, but on the other hand pointed out the more demanding OFM equipment/handling due to use of push-pull fluidics. In the concluding sentences of that paper we stated that the study needs to be repeated with more lipophilic drugs as well as conducted under in vivo conditions in order to confirm the observations in the ex vivo experiment. To this date, such a direct in vivo comparison of OFM and

MD has not been performed. However, two independent clinical in vivo studies have been performed on the same (lipophilic) topical compound. Thus, these studies can provide clarification regarding the question which method to preferably choose when sampling lipophilic compounds (see section 2.5.2 on PK/PD of clobetasol-17-propionate).

Table 5 below provides the facts of the ex vivo study in human explanted skin and the subsequent publication. In the appendix the first page including the introductory section and the last page are reprinted, a full reprint is prevented by the copyright of the journal.

Table 5: Synopsis of topical fentanyl and benzoic acid studies in explanted skin

Description	
Type of study	Comparative study of topical penetration of two different compounds by dermal microdialysis and dermal OFM to compare the advantages and challenges of the two methods.
Subjects	Excised human skin from 9 healthy donors
Materials	OFM Probe: DEA15001 (permeable length 15 mm, OD 0.36 mm, ID 0.25 mm, CE-certified), MD Probe1: 100 kDa CMA66 (permeable length 10 mm, OD 0.5 mm, CE-certified) MD Probe2: 2 kDa Gambro (permeable throughout, OD 0.22 mm, non-CE) Pump: Minipuls 3 by Gilson, France; Perfusate: Saline + 1 % albumin OFM sampling in 50 µL glass capillaries, MD sampling in 0.2 ml PCR tubes
Design	Fentanyl (log P: 4.05) and benzoic acid (Log P: 1.87) test solutions, each applied in 3 chambers per donor; fentanyl solution contained 20 % ethanol. 3 x OFM, 3 x 100 kDa, 3 x 2 kDa probes for each compound, 1 µL/min sampling 20 hours of sampling in 2 h intervals
Analyses	Fentanyl/benzoic acid in OFM and MD samples; Measures of topical penetration such as AUC, Cmax, absorption rate and lag-time.
Outcome, Significance	This study showed that both techniques are suitable for ex vivo dermal sampling of topically applied benzoic acid in a pure aqueous solution and fentanyl in an ethanol-containing solution and a perfusate including 1 % albumin. Under these conditions the three probe types showed concordance in AUC and Cmax for both benzoic acid and fentanyl. The publication also outlined the advantages and challenges of both methods to guide future users in the choice of an adequate method for the study purpose and the physico-chemical properties of the target molecules.
Publication	R. Holmgaard, E. Benfeldt, J. B. Nielsen, C. Gatschelhofer, J. A. Sorensen, C. Höfferer, M. Bodenlenz, T. R. Pieber, and F. Sinner, "**Comparison of open-flow microperfusion and microdialysis methodologies when sampling topically applied fentanyl and benzoic acid in human dermis ex vivo.**," Pharm. Res., vol. 29, no. 7, pp. 1808–20, Jul. **2012. [11]**

Author's contribution to research and publication: The author of this thesis contributed to the study design, the preparation of the study, the conducting of the study, the interpretation of the data, and writing of the manuscript.

Acknowledgements
This collaboration was supported by **European COST-Action No. BM0903 "SKINBAD"**. The project supported scientific meetings, education and "Short Term Scientific Missions" (STSM) of young researchers in other labs and thus allowed the repeated trips made by Rikke Holmgaard from Copenhagen to Graz for the collaborative experiments. This STSM of Ms. Holmgaard in Graz contributed to the scientific aims of COST-SKINBAD – Work Package 4 to compare the methods potentially suitable for in vivo research into skin barrier penetration and function as required for SKINBAD.

2.4.3 Experiments towards human use

The superior safety properties of dermal probe no. 2 ("Helixcath") enabled its use in a few volunteers. These first experiments did not aim to investigate any drug yet, but aimed to obtain a basic understanding of the new probe's applicability, tolerability and performance as well as to get an understanding of the human dermis as a novel location for continuous interstitial fluid sampling using OFM.

The author utilized the research group's vast experience in the evaluation of subcutaneous sampling techniques (OFM and microdialysis) for the demanding purpose of continuous glucose monitoring and tight glycaemic control in diabetic patients and at intensive care units. This area of research had demanded intense characterization of sampling performance of different probes and therefore the methodology for probe characterization had already been established.

2.4.3.1 Feasibility of dermal use and glucose sampling

The main questions were:

- Is the new dermal probe applicable to human skin?
- Is the new dermal probe tolerable for the human skin?
- Is sampling in skin similar to what is known - subcutaneous sampling?
- Is the ionic reference technique (IRT) applicable also in dermal sampling?
- Is the interstitial/plasma relationship for glucose also true in the dermis?

A healthy subject received 3 probes into the dermis and 3 probes into subcutaneous adipose tissue. Moreover, the subject received an oral glucose load to induce a dynamic glucose excursion in order to investigate whether the intradermal and subcutaneous probes would be able to track the dynamic glucose concentration. Glucose, lactate and sodium were sampled from the skin and s.c. adipose tissue for 6 hours using an ion-free isotonic sterile perfusate (5 % mannitol in water). Glucose and lactate concentrations in OFM samples were corrected for the relative recovery of sodium according to the ionic reference technique [152] to obtain the dermal and subcutaneous interstitial fluid glucose and lactate concentrations. Finally, the interstitial glucose profile was calibrated to capillary glucose concentrations by a one-point calibration.

Figure 2-20 shows the results of this first human experiment using this probe for sampling from the dermis as well as from subcutaneous adipose tissue. The sodium recovery was stable for 6 hours and well comparable between dermal and s.c. probes. The degree of the recovery (~50 %) indicated that the probe was sufficiently effective to recover small analytes at a flow rate of 1 µL/min. Calibration of glucose by sodium yielded interstitial fluid glucose concentrations of approx. 70 % compared to blood levels. These results were well comparable to prior studies using concentric OFM probes [147]. When calibrated to the first capillary glucose concentration both the dermal and s.c. glucose profiles nicely followed the systemic glucose excursion. The lactate concentrations remained at an acceptable level and did not increase indicating satisfactory local tolerance of the probe.

The results of this basic experiment meant that (i) the new OFM probe effectively recovered small molecules in the human dermis and subcutaneous tissue and that (ii) all prior knowledge on relative recovery and calibration of probes did also apply to the human dermis. Thus this first human experiment on “standard analytes” was able to create confidence in the novel OFM probe type as well as the dermis as new tissue for OFM application.

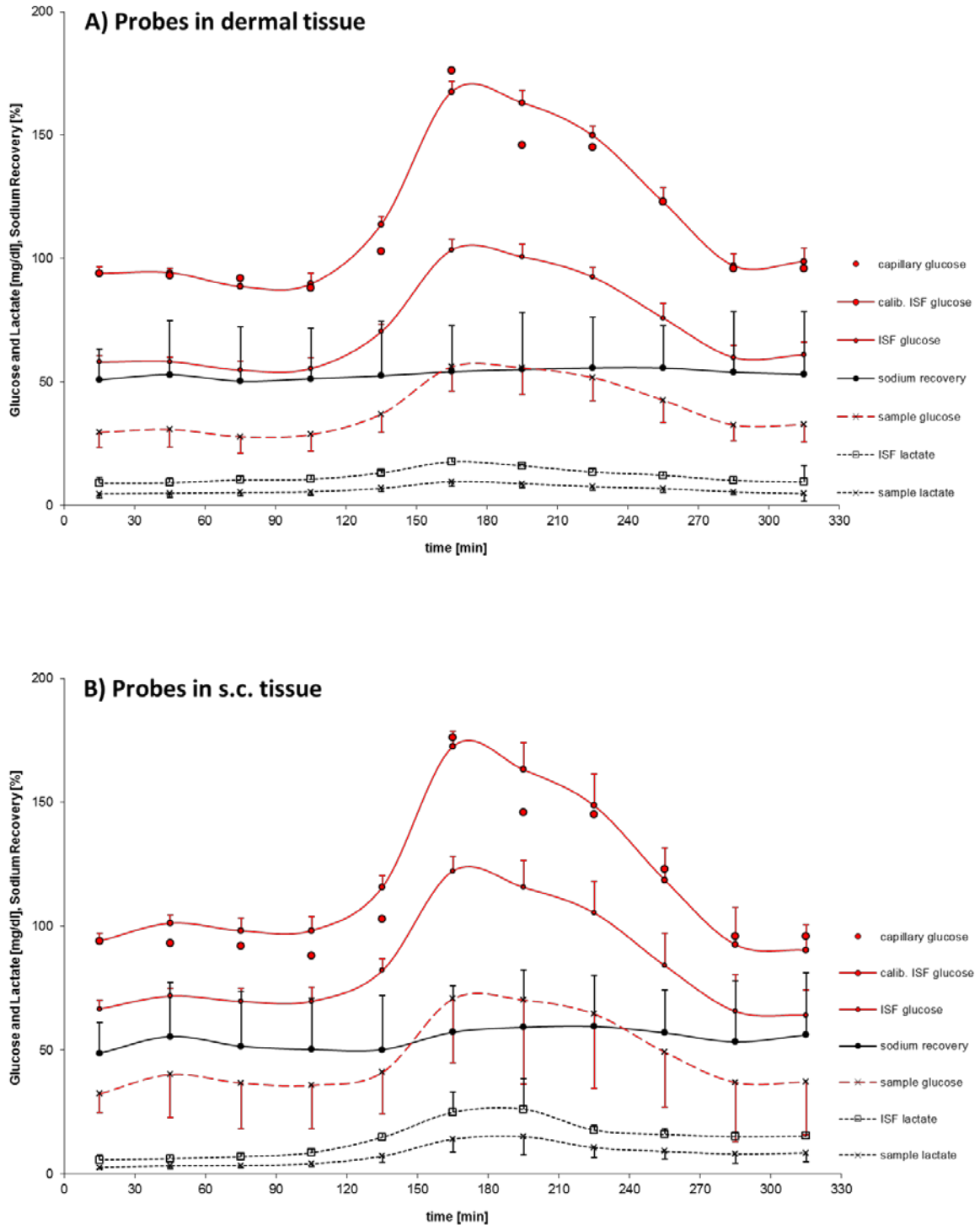

Figure 2-20: Results of the first human use of probe no. 2 “Helixcath” in the dermis (A) and subcutaneous (s.c.) adipose tissue (B). The use of this probe and sodium calibration enabled tracking of capillary glucose both in the dermis and in s.c. tissue. Data are mean ± SD for 3 probes.

As a result, patent protection was obtained for this first clinical dermal OFM probe [1]. Moreover, a sponsor was found to fund a first clinical dermal OFM drug study, which was the proof-of-

concept for dermal OFM for clinical pharmaceutical research. This study investigated a novel topical compound's pharmacokinetics and –dynamics (PK-PD) directly in inflamed and unaffected skin of patients (Summary in 2.4.3).

2.4.3.2 Feasibility of dermal lactate monitoring during exercise

After the encouraging results of the first human application, the new linear dermal probe was used for a few experiments on the feasibility of lactate monitoring. The dermal probe was of interest for this application because studies at the intensive care unit of the Medical University Graz had shown that lactate sampled from subcutaneous adipose tissue showed a low correlation to systemic lactate. The opportunity to test the feasibility of lactate monitoring using the dermal probe arose from a collaborative European project on continuous glucose monitoring and the presence of experts in sensor technology in Graz.

The ADICOL project and the collaboration with SensLab GmbH provided the opportunity to test the feasibility of continuous lactate monitoring using the wearable monitoring unit in combination with the first clinically applicable dermal OFM probe (Probe 2 - Helixcath, see details in 2.3.5). For this purpose the glucose sensor within the ADICOL monitor was exchanged for a SenLab lactate sensor.

The main questions prior to the lactate monitoring experiments were:

- Is the new dermal probe functional in human skin under conditions of heavy exercise and high skin capillary blood perfusion?
- Is the new dermal probe tolerable for the human skin in a way that local probe-induced lactate production does not prevent systemic lactate monitoring?
- Does the ionic reference technique (IRT) allow calibration of the OFM concentrations under such dynamic conditions?
- What is the relationship between lactate in dermal ISF and blood?

The feasibility of continuous lactate monitoring was successfully evaluated during periods with heavy treadmill exercise and dynamic lactate concentrations. Capillary blood from the earlobe served as the reference and was therefore sampled frequently during the exercise similarly as done during physical performance tests. Each sample was measured in duplicate using the device "Lactate Scout".

A novel dermal OFM probe was inserted into the dermis of the skin in each forearm, connected to portable monitoring devices which were fixed to the forearms (Figure 2-21). Probes were continuously perfused with ion-free, isotonic perfusate at a flow rate of 1 µl/min, and the lactate and ion concentration were measured online. The lactate signal was calibrated (online) using the conductivity signal (ionic reference technique). The actual dermal lactate status of the volunteer was measured with a delay of about 10 minutes.

The results of these two lactate measurement setups, the load of the treadmill, heart rate and reference lactate measurements of capillary blood are shown in Figure 2-22. Lactate signals of both portable devices correlated quite well with the reference lactate values determined with a lactate meter (Lactate Scout) in capillary blood. The results of this feasibility trial suggested that the novel dermal OFM probe can be used in combination with the portable ADICOL monitors and that the dermis may be more appropriate for continuous lactate measurements than the subcutaneous adipose tissue. Interestingly, the relative recovery changed considerably during the exercise exeriment as became evident from the conductivity sensor incorporated within the lacatate sensor. Thus, this experiment once again showed the effectiveness of the ionic reference technique to correct the sample concentrations.

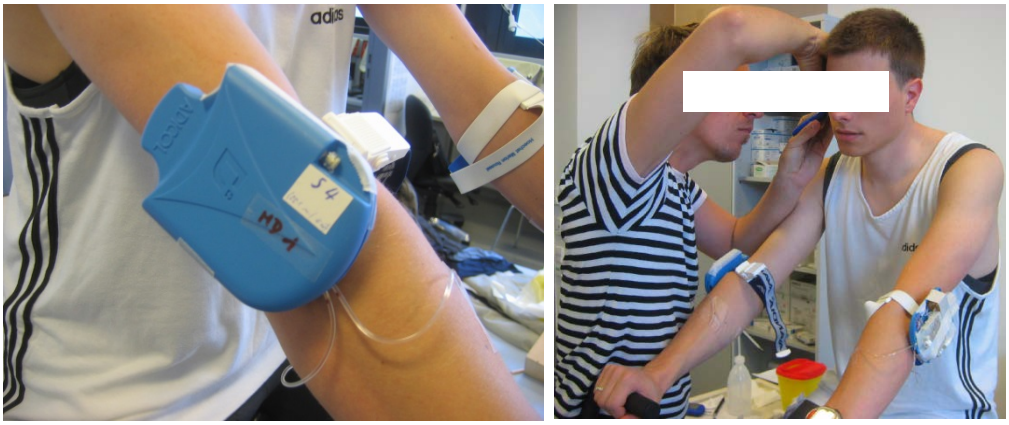

Figure 2-21: Lactate monitoring in forearm skin using a wearable pump and a dermal OFM probe

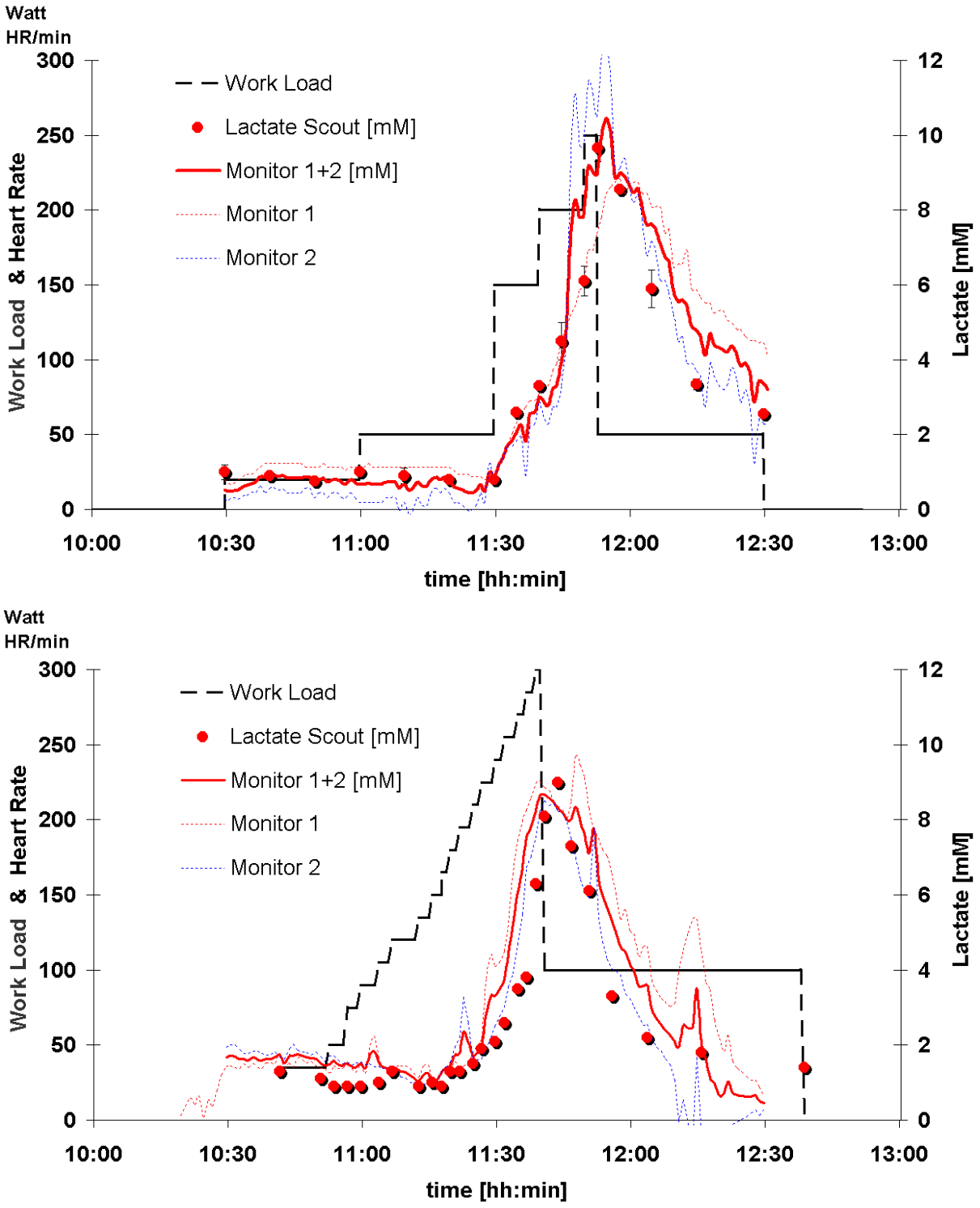

Figure 2-22: Lactate concentration profiles in the dermis during exercise in volunteer #1 (top) and volunteer #2 (bottom)

In summary, the second human experiment using this dermal probe was a success showing the technical feasibility of continuous lactate monitoring during exercise. This experiment indicated that the highly capillarized dermal layer of the skin would be appropriate for purposes of metabolic monitoring.

2.4.4 Clinical research on topical PK/PD of BCT194 – Bodenlenz et al. 2012

European Journal of Pharmaceutics and Biopharmaceutics 81 (2012) 635–641

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European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

Dermal PK/PD of a lipophilic topical drug in psoriatic patients by continuous intradermal membrane-free sampling

Manfred Bodenlenz [a], Christian Höfferer [a], Christoph Magnes [a], Roland Schaller-Ammann [a], Lukas Schaupp [a,b], Franz Feichtner [a], Maria Ratzer [a], Karin Pickl [a], Frank Sinner [a,b], Andrea Wutte [b], Stefan Korsatko [b], Gerd Köhler [b], Franz J. Legat [c], Eva M. Benfeldt [d], Andrew M. Wright [e], Daniel Neddermann [e], Thomas Jung [e], Thomas R. Pieber [a,b,*]

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[e] Novartis Pharma AG, Basel, Switzerland

Probe no. 2 (Helixcath) was the first dermal OFM probe used in humans. As described in the respective section on probe design 2.3.5, this probe enabled a number of preclinical dermal experiments and was thereafter successfully introduced into clinical research on the basis of small feasibility experiments to test the probe's applicability in humans.

Novartis Pharma AG, then having their global dermatological research headquarters in Vienna, could be convinced about the potential of the OFM methodology in dermatological drug research which resulted in funding for a first clinical dermal OFM drug study. This proof-of-concept for dermal OFM for clinical pharmaceutical research investigated the pharmacokinetics and –dynamics (PK-PD) of a novel topical compound directly in inflamed (lesional) and unaffected (non-lesional) skin of patients. The paper on the results of this research trial is provided in the appendix.

The novelty of the study and paper was given due to the fact that there was no method existing enabling the continuous sampling of lipophilic drugs and high-molecular solutes in the dermis. In the study we successfully utilized dOFM probes and the wearable multi-channel pump prototype to sample a lipophilic topical drug and the locally released biomarker (Tumor Necrosis Factor α - TNFα) in the dermis of non-lesional and lesional skin of psoriatic patients.

In brief, nine psoriatic patients received a topical inhibitor in a 0.5 % cream formulation on a lesional and a non-lesional application site once daily for 8 days. dOFM sampling with six probes was performed for 25 h from each site on day 1 and day 8. Patients were mobile and tolerated 25 h sampling well as dOFM probes were operated by the novel lightweight push–pull pump. We used ultrasound to verify correct intradermal probe placement.

In this first clinical drug study, dOFM was well tolerated and demonstrated significant drug concentrations in lesional as well as non-lesional skin after 8 days. The release of TNFα following probe insertion was significantly reduced after 8 days of treatment.

Thus, the novel membrane-free probes and wearable multi-channel pumps demonstrated to enable prolonged intradermal PK/PD profiling of a lipophilic topical drug and a high-molecular weight biomarker (TNFα, 51 kDa) in psoriatic patients. By this, this initial study demonstrated that dOFM overcomes the limitations of microdialysis sampling methodology, and it demonstrated the potential for PK/PD studies of topical products and formulations in a clinical setting.

A dependency of the sampled drug concentration on the probe depth could not be confirmed from the data in this first study on a topical drug. Most likely this was due to the fact that this first study in patients could not yet be standardized to the degree necessary to statistically show such an effect in a few subjects only. A major challenge was that the body location for sampling could not be selected, but 25 h sampling had to be performed where psoriatic skin lesions were situated and this was mostly near the joints (elbow, knee), which both could not be completely immobilized for more than 25 hours. This should have induced some more data variability. As will be shown in the summary of the clinical study on topical clobetasol in section 2.5.2, the proof of this concentration vs. depth relationship succeeded later using a further advanced setting and self-adhesive rings to stabilize the skin of the sampling sites for prolonged sampling periods.

Author's contribution to research and publication: The author of this thesis contributed to the study design, prepared the clinical protocol, performed the study with the clinical research team, analyzed the data, interpreted the data, and wrote the manuscript.

Table 6: Synopsis of the topical BCT194 study

Description	
Type of study	Kinetics and -dynamics of topical BCT194 cream 0.1 %
Subjects	8 patients with psoriasis
OFM material	OFM Probe: Helixcath, OFM Pump: 6-channel prototype
Design	8 days of topical treatment of a psoriatic lesion and a non-lesional control site Dermal OFM sampling day 1 and 8 6 probes, 3 in lesion, 3 in non-lesional skin, 1 µL/min sampling 25 hours of OFM sampling in 1 h intervals Skin biopsies to assess total concentration of BCT194
Analyses	OFM analytes: BCT194, TNFα; Other parameters: probe depth, PASI scoring
Outcomes, Significance	dOFM was well tolerated and demonstrated significant drug concentrations in lesional as well as non-lesional skin. TNFα release was significantly reduced following BCT194 treatment. This initial study shows that dOFM overcomes the limitations of microdialysis sampling methodology, and it demonstrates the potential for PK/PD studies of topical products and formulations in a clinical setting. This study served as the proof of concept for the use of dermal OFM in pharmaceutical studies. It prompted the effort for the design of medical devices and helped to acquire funding for prototyping.
Publication	M. Bodenlenz, C. Höfferer, C. Magnes, R. Schaller-Ammann, L. Schaupp, F. Feichtner, M. Ratzer, K. Pickl, F. Sinner, A. Wutte, S. Korsatko, G. Köhler, F. J. Legat, E. M. Benfeldt, A. M. Wright, D. Neddermann, T. Jung, and T. R. Pieber, "**Dermal PK/PD of a lipophilic topical drug in psoriatic patients by continuous intradermal membrane-free sampling.**," Eur. J. Pharm. Biopharm., vol. 81, no. 3, pp. 635–41, Aug. 2012. **[10]**

2.5 Clinical pharmaceutical research with CE-certified OFM

The successful first application of dermal OFM in clinical pharmaceutical research (read details on the proof-of-concept in chapter 2.4.4 and the according publication [10]) prompted the decision to invest in the development of regular CE-certified medical devices, i.e. devices fulfilling the European standards for regular use in humans. Following this proof a qualified decision for a “product development” was possible and at the same time without any option due to the increasingly stringent execution of the medical device regulations by the Austrian authority which precluded further clinical research using non-certified devices in human research.

The task required significant educational and organizational efforts as well as substantial financial resources to establish the standards of a regular medical device manufacturer at a research institute. The author identified a competitive Austrian funding program for the transformation of ideas into working prototypes (“Research Studios Austria”, funded by the Austrian Ministry for Technology, hosted by funding organisation FFG) and successfully applied for the funding of the implementation of “CASE - Clinically Applicable System for Evaluation of pharmaceutical products”. The research team was reorganized, transformed into a medical device development group and finally reinforced by personnel with industry and quality management experience. The product development including product testing in external laboratories and at the official Austrian institution for Quality Assurance (“Notified Body”) took more than two years and a CE-label for the novel OFM devices as medical devices for human use was obtained in 2010.

Already during the product design process the author of this thesis had changed his role to focus on the design and organization of the initial research trials for pharmaceutical industry utilizing the novel OFM devices. The initial clinical trial, however, did not investigate the pharmaceutical aspects of a drug but investigated the OFM devices itself for safety, tolerability and performance in a medical device study according to medical device law (Medizinproduktestudie lt. Medizinproduktegesetz summarized in 2.5.1 and a peer-reviewed publication [9]).

Since CE-certification in 2010, the clinical devices have been successfully applied by the author and colleagues in a number of clinical studies on dermatological and anti-diabetic drugs and most of them published in peer-reviewed pharmaceutical or dermatological journals (papers are provided in full-text in the appendix). The subsequent sections highlight (repeat) the most relevant pharmaceutical/medical findings of four clinical studies. Moreover, the challenges and further methodological advances are discussed as these aspects have not been explicitly discussed in the predominantly pharmaceutical or dermatological research oriented papers.

2.5.1 Clinical research on device tolerability – Bodenlenz et al. 2013

*Skin Research and Technology 2013; **0**: 1–10*
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doi: 10.1111/srt.12071

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Skin Research and Technology

Clinical applicability of dOFM devices for dermal sampling

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Prior to the first use of the medical devices for pharmaceutical research purpose, we performed a clinical study dedicated to the characterization of the most important properties of the medical devices ("Medical Device Study"): Tolerability of probe application and long-term use by the study volunteers, acceptance by the clinical user applying the devices, and sampling performance.

Physicians inserted 141 membrane-free dOFM probes into the dermis of 17 healthy and psoriatic volunteers and sampled dermal ISF for 25 h by using the novel wearable push-pull pumps. The tolerability, applicability, reproducibility, and reliability of multiple insertions and 25 h continuous sampling was assessed by pain scoring, physician feedback, ultrasound probe depth measurements, and 25 h drift and variability of the sodium relative recovery.

The volunteers reported the insertion pain as being moderate and the pain score decreased with each additional probe (first probe 3.3 ± 1.4 vs. ninth probe 1.9 ± 1.2; mean ± SD, $p < 0.001$, paired t-test). Probe insertion was precise, although deeper in lesional skin (0.75 ± 0.24 mm vs. 1.05 ± 0.38 mm, mean ± SD, $p<0.001$). The wearable push-pull pump enabled uninterrupted interstitial fluid sampling over 25 h resulting in a low variability of the relative recovery (CV of the relative recovery: 4.5 ± 2.12 %, mean ± SD). The relative recovery was virtually free of any drift over 25 hours of dermal sampling (<0.02 % change per hour).

The author of this thesis published these study results in a peer-reviewed journal in the field of dermatology and therein concluded that dOFM sampling devices were tolerable and reliable for prolonged continuous dermal sampling in a multi-probe clinical setting (Bodenlenz et. al 2013 [9], paper provided in the appendix). Table 7 at the end of this section provides a short tabular synopsis of this medical device study.

This device study and the subsequent publication might be considered as "outstanding" simply due to the fact that no similar device evaluation study existed at that time for microdialysis, and it still does not exist. In the publication a vision on future qualities of sampling devices was

presented. In Table 1 of the publication the author listed the “criteria for the ideal sampling technique”, to indicate the most important acceptance criteria for the participating study volunteer as well as for the clinical scientist as the user of the method. Thus, this list of criteria defined the ultimate design goal for the two sampling techniques, OFM and microdialysis, to serve as criteria for the assessment of the available sampling devices then and to serve as guidance for further development of both techniques. The publication outlined the essential differences to state-of-the-art microdialysis methodology and material which are still valid to this date.

Most important, the study volunteers accepted the procedures well (multiple probe insertion followed by 25 hours day-and-night sampling) with no scars visible at the end-of-study visit. This first study with the CE-certified devices created a sound basis for the subsequent extensive use of OFM probes in pharmaceutical studies as will be reported in the subsequent sections. As will be shown, the excellent tolerability of the OFM probe and the mobility provided by the wearable pump had thereafter enabled larger and larger study settings with 16 probes in parallel for up to 48 hours. Furthermore, the publication mentioned the protocols and sampling modes supported by the devices, as well as the wide range of accessible molecules from rather lipophilic topical drugs to extremely large therapeutic molecules like antibodies. At that time it was not known if and when sponsors would be found to sponsor such clinical studies, but as the subsequent summaries demonstrate sponsors have been found and all the capabilities mentioned in the paper have been successfully utilized in pharmaceutical studies.

Author’s contribution to research and publication: The author of this thesis conceived the study design, prepared the clinical protocol, performed the study with the clinical research team, analyzed the data and wrote the manuscript.

Table 7: Synopsis of the device evaluation study

Description	
Type of study	Medical device study (safety, tolerance, performance, etc.)
Subjects	17 heathy volunteers and patients with psoriasis
OFM-material	OFM Probe: DEA15001, OFM Pump: MPP101 Further: Skin stabilization
Design	OFM test on various sites on arms and legs, healthy and lesional skin 9 probes, 3 per skin site, 1 µL/min sampling with ion-free perfusate 25 hours of OFM sampling in 1 h intervals
Analyses	OFM analytes: sample volume, sodium recovery; Other parameters: probe depth, questionnaire and insertion pain evaluation using visual analogue score, healing process documentation
Outcome, Significance	This clinical study showed that dOFM sampling devices are tolerable and reliable for prolonged continuous dermal sampling in a multiprobe clinical setting. The reproducibility of the relative recovery and its stability for 25 hours was demonstrated. The subsequent publication is cited in all clinical study protocols (ethics applications) in order to indicate the devices' safety and tolerability for study participants.
Publication	M. Bodenlenz, B. Aigner, C. Dragatin, L. Liebenberger, S. Zahiragic, C. Höfferer, T. Birngruber, J. Priedl, F. Feichtner, L. Schaupp, S. Korsatko, M. Ratzer, C. Magnes, T. R. Pieber, and F. Sinner, "**Clinical applicability of dOFM devices for dermal sampling**.," Skin Res. Technol., vol. 19, no. 4, pp. 474–483, Apr. 2013. **[9]**

2.5.2 Clinical research on PK/PD of topical CP-17 – Bodenlenz et al. 2016

Pharm Res (2016) 33:2229–2238
DOI 10.1007/s11095-016-1960-y

RESEARCH PAPER

Kinetics of Clobetasol-17-Propionate in Psoriatic Lesional and Non-Lesional Skin Assessed by Dermal Open Flow Microperfusion with Time and Space Resolution

Manfred Bodenlenz[1] • Christian Dragatin[1] • Lisa Liebenberger[2] • Bernd Tschapeller[1] • Beate Boulgaropoulos[1,2] • Thomas Augustin[1] • Reingard Raml[1] • Christina Gatschelhofer[1] • Nathalie Wagner[3] • Khaled Benkali[3] • Francois Rony[3] • Thomas Pieber[1,2] • Frank Sinner[1,2]

Received: 1 February 2016 / Accepted: 27 May 2016 / Published online: 6 June 2016

The proof-of-concept study as well as the medical device evaluation study ([9,10]) had already included patients suffering from psoriasis and confirmed the applicability, tolerability and stable performance when sampling interstitial fluid from psoriatic skin lesions and non-lesional skin. This evidence could be well utilized when a sponsor was found for the first use of the CE-certified devices for a PK/PD study of a rather lipophilic topical drug (clobetasol-17-propionate, CP-17).

The clinical study evaluated the kinetics of topically applied CP-17 in lesional and non-lesional psoriatic skin when released from a commercially available low-strength cream, and the modulation of a set of biomarkers (cytokines). Twelve patients received a marketed low-strength CP-17 cream product (0.05 %) on small lesional and non-lesional skin test sites for 14 days, once daily. On day 1 and 14, dOFM samples were continuously taken in the dermis for 24 h post-dose and analyzed by LC-MS/MS for CP-17, and by ELISA assays for cytokines. Probe depths were assessed by 50 MHz ultrasound scanning. The clinical effect of treatment has been assessed by the so called “Plaque Total Severity (or Sign) Score” (TSS), which is calculated as the sum of 5-point rating scores for erythema, induration and scaling.

For the author of this thesis, the kinetic results had been of primary interest due to the fact that experts were not in agreement whether diseased skin would be more or less permeable for a lipophilic drug. Moreover, the author sought to prove by OFM what biophysics and diffusion theory suggests, i.e. that the concentration of the topically applied drug should decrease with distance from the skin surface. Pair-wise comparisons of adjacent probes for AUC and depths and clustering of probes according to probe depths confirmed the AUC vs. depth relationship, and prompted a more sophisticated statistical analysis. Mixed-effects modelling identified skin condition (lesional/non-lesional), treatment duration (day 1/day 15) and also probe depth as kinetics determining variables. The time- and depth-resolved intradermal data revealed (i) slower

penetration of CP-17 into lesional than into non-lesional skin, (ii) normalized (faster) skin penetration after repeated dosing, and (iii) no CP-17 accumulation within the dermis of lesional or non-lesional skin [6].

The effect of the CP-17 treatment was nicely visible after 14 days of treatment. The treated area appeared as a pale rectangular area within the psoriatic lesion, and the TSS score of the treated areas was reduced from 6.3 ± 0.5 on day 1 to 2.4 ±1.4 on day 15 ($p<0.001$). The cytokine data obtained by OFM (TNFα, IL-6, IL-8, IP-10) confirmed the clinical observation by showing the reduction of their intradermal release from day 1 to day 14 of topical CP-17 treatment. To enable a most reliable investigation and interpretation of cytokines results, a vehicle cream (placebo) was used to treat a 2^{nd} lesion and a 2^{nd} non-lesional site to obtain appropriate controls for the effects of CP-17 treatment on cytokines. For CP-17 and vehicle treatments the release of cytokines was compared between day 1 and day 15, and between lesional and non-lesional skin on both day 1 and day 15. For these comparisons the AUC was calculated for each cytokine profile. According to these AUCs daily topical CP-17 treatment decreased the intradermal release of these cytokines from day 1 to day 15 in both lesional and non-lesional skin by a factor between 2 and 7. In the vehicle treated lesional and non-lesional sites the release did not change from day 1 to day 15. Furthermore, the results indicated that the release of IP-10 might be specific for psoriatic lesions or for inflamed skin in general. Only IP-10 showed differences between lesional and non-lesional skin on day 1, with concentrations which were 2-fold compared to non-lesional skin. CP-17 treatment reduced the lesional IL-10 concentrations to non-lesional concentrations, while vehicle treatment was not able to normalize the IL-10 concentration. These cytokine results were not yet published in a peer-reviewed journal but instead reported on conferences [57,82,134]. It should be noted here, that many cytokines recovered by sampling probes (OFM or MD) are not present in the dermis at the level shown by the probes but released by the dermal tissue in an innate immune reaction to the sterile trauma induced by probe insertion [153,154]. This needs to be considered by users interpreting cytokine concentrations and changes following probe insertion, and ideally already considered by the insertion of extra probes as controls in untreated or placebo-treated sites. Cautionary notes have been published to make researchers aware of these probe-induced effects and the need of proper controls, for instance by Stenken et al. [155]. This means that a proper setting for the study of cytokines and other markers of disease does require an increased number of probes and thus a well-tolerable procedure of insertion as well as suitable pumps to support sampling from several probes in parallel.

This study again demonstrated the utility of dermal OFM for the investigation of lipophilic drugs, also enabling the detection and quantification of a drug within the dermis if released from a low-strength cream containing 0.05 % of drug only. A similar study on CP-17 had been undertaken by Au et al. [156] using microdialysis. Au et al., however, did not investigate CP17 penetration and

local drug action cytokines but concentrated on the feasibility of sampling of CP-17 for purposes of topical bioequivalence (for bioequivalence see 2.5.4). To succeed with microdialysis, the researchers dissolved CP-17 in an ethanolic solution, used a higher CP-17 concentration, and used a 20 % lipid solution as the perfusate to recover CP-17 from the dermis. The comparison of the experimental conditions and the results between the OFM and the MD study should be adequate to clarify the question raised in a prior study and publication (see section 2.4.2) whether OFM or MD should be preferred when sampling lipophilic drugs, in particular when a study aims to investigate the overall bioavailability of the lipophilic drug at tissue level.

The fact that probe depth could be confirmed as a relevant parameter for kinetic results meant that the overall methodological variability of OFM was low enough to reveal such small kinetic differences within the dermis. The finding of this stringent depth dependency was important, in particular as several previous microdialysis studies could not identify probe depth as a relevant or crucial factor for clinical kinetic studies though the depth-variation in those studies was not smaller but well comparable to our study (on average 0.80 ± 0.2 mm). In particular this finding was important for the use of OFM and microdialysis for the purpose of topical bioequivalence assessments in which two different products have to be reliably compared for the topical penetration of the active drug released by the different formulations.

Also from the perspective of the clinical scientist with a focus on the skin barrier properties in disease and the consequences for pharmacology this study was informative. The data of this study supported the assumption that the thickened psoriatic stratum corneum might act as a trap compartment which lowers the skin penetration rate for lipophilic topical drugs.

Author's contribution to research and publication: The author of this thesis contributed to the study design and the writing of the clinical protocol, performed the study with the clinical research team, largely performed the data analyses, interpreted the data, and wrote the manuscript.

Table 8: Synopsis of the topical CP-17 study

Description	
Type of study	Kinetics and -dynamics of topical clobetasol-17-proprionate cream 0.05 %
Subjects	8 patients with psoriasis
OFM-material	OFM Probe: DEA15001, OFM Pump: MPP101 Further: Skin stabilization
Design	Daily topical CP-17 treatment of 2 lesional + 2 non-lesional control sites Dermal OFM on day 1 and day 14 12 probes, 3 per site, 1 µL/min sampling 26 hours of OFM sampling in 2 h intervals
Analyses	OFM samples: CP-17, biomarkers; Other: probe depth, PASI scoring
Outcomes; Significance	OFM enabled the intradermal investigation of a highly lipophilic drug released from low-strength cream and timely and spatially, i.e., probe depth dependent, resolved kinetic data were delivered. These data support the assumption that the thickened psoriatic stratum corneum might act as a trap compartment which lowers the skin penetration rate for lipophilic topical drugs. This study is of great importance for future bioequivalence studies using OFM or microdialysis. It clearly indicates that the depth of each probe needs to be thoroughly assessed as depth differences may be critical for the validity of the outcomes of the bioequivalence study.
Publication	M. Bodenlenz, C. Dragatin, L. Liebenberger, B. Tschapeller, B. Boulgaropoulos, T. Augustin, R. Raml, C. Gatschelhofer, N. Wagner, K. Benkali, F. Rony, T. Pieber, and F. Sinner, "**Kinetics of Clobetasol-17-Propionate in Psoriatic Lesional and Non-Lesional Skin Assessed by Dermal Open Flow Microperfusion with Time and Space Resolution**" Pharm. Res., vol. 33, no. 9, pp.2229-2238, Sep. 2016. **[6]**

2.5.3 Clinical research on PK/PD of secukinumab – Dragatin et al. 2016, Kolbinger et al. 2017, Loesche et al. 2017

Secukinumab distributes into dermal interstitial fluid of psoriasis patients as demonstrated by open flow microperfusion

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**These authors contributed equally to this manuscript.*

β-Defensin 2 is a responsive biomarker of IL-17A–driven skin pathology in patients with psoriasis

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The clinical study on secukinumab was the most comprehensive PK/PD study using dermal OFM. A considerable effort was made to ensure the most reliable quantification of the antibody in dermal interstitial fluid, in order to verify that the single antibody dose of 300 mg results in a molar concentration exceeding the molar concentration of secukinumab's target molecule IL-17A in psoriatic plaques. The target molecule as well as other downstream markers has also been assessed in this study by OFM and other methods, and the study included OFM experiments which should be fundamental for future use of OFM in antibody research in psoriasis and other skin diseases.

The study's primary aim for quantification required following a step-wise approach. Consequently the study was split up into two studies: a preparatory study on healthy subjects and the study on psoriatic patients.

Eight healthy subjects and eight psoriasis subjects were investigated. The subjects came to the clinic for five study visits, including three visits with OFM investigation on day 1, day 8 and day 15. All subjects received a dose of 300 mg of the antibody subcutaneously at the end of day 1. In the healthy subjects the quantification of secukinumab with dOFM was established using sinistrin (inulin) as the interstitial reference molecule for calibration. To compare the relative recoveries of the antibody with recovery of the envisaged reference sinistrin, independent no-net-flux

procedures were performed for the antibody and for sinistrin, using 12 probes in parallel in the according study visits. A picture of a healthy volunteer including wearable OFM devices is shown in Figure 2-23.

The no-net-flux for secukinumab succeeded and indicated a dermal interstitial secukinumab concentration of ~23 % relative to plasma (Figure 2-23). The slope of the no-net-flux regression line revealed the Relative Recovery for Secukinumab, which due to its considerable size of 150 kDa, was below 10 %.

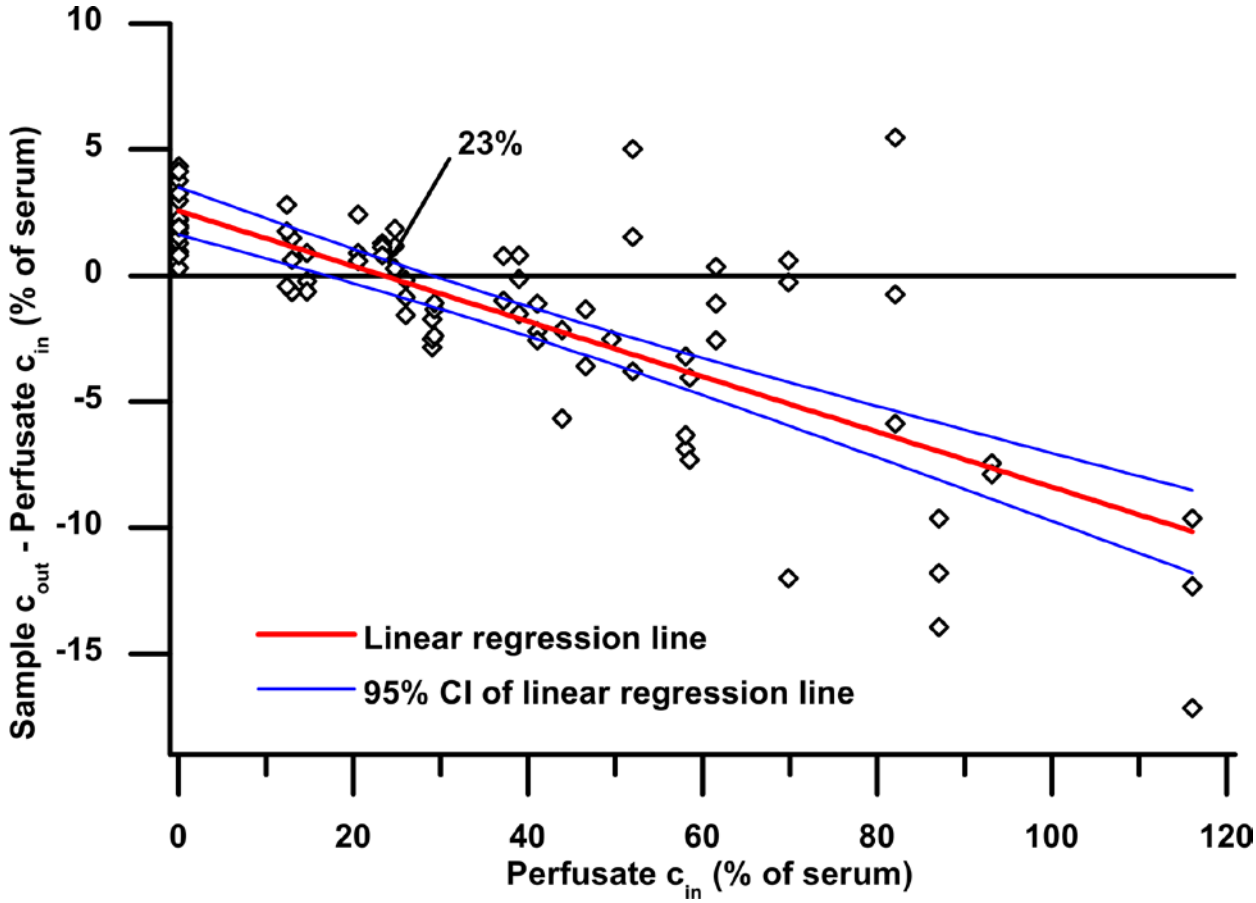

Figure 2-23: No-Net-Flux plot of secukinumab in dermal ISF on Day 15 with data points from six healthy subjects. Gain or loss in the sample concentration is plotted against the perfusate concentration. The intersection of the linear regression line (R^2=0.59) with the x-axis at a perfusate concentration of about 23 % represents the concentration of secukinumab in the dermal ISF relative to the serum concentration (Panels and text from Dragatin et al. [7]).

The no-net-flux for sinistrin (sinistrin had been given as primed-continuous intravenous infusion, i.e. by a constant infusion following a bolus) showed a reasonable linear relationship (determination coefficient r2 for linear regression was was 0.66) and an intercept close to 100 % of the serum concentration, thus confirming that at steady-state sinistrin was equally distributed between plasma/serum and the dermal interstitium (sinistrin geometric mean ratio dermal/serum: 1.003; 90 % CI between 0.91 and 1.10; [99]). The slope revealed a sinistrin recovery of ~17 %, which was perfectly in line with prior (unpublished) experiments on dermal sinistrin sampling, and which meant a ratio of 2.45 between sinistrin and secukinumab recovery. To assure that dOFM correctly mirrored the dermal secukinumab concentrations, also skin blisters were raised in the

healthy subjects by negative pressure applied to the skin (suction blister method) and punch biopsies were taken on day 15. The absolute secukinumab concentrations in suction blister fluid (6.9 ± 2.3 µg/ml) and punch biopsy (10.4 ± 4.0 µg/ml) corresponded to the dOFM results (8.0 ± 3.2 µg/ml; mean ± SD).

The established dOFM methodology with sinistrin as a reference was then used in psoriasis subjects to quantify secukinumab in lesional and non-lesional skin. Sinistrin recoveries were well comparable between the visits, between the skin sites and also comparable to the healthy subjects, indicating that dOFM recovery is well reproducible.

Sinistrin in the samples was also used to assess the absolute levels of the biomarker molecules based on their concentration in dOFM samples. The antibody concentration and its target molecule IL-17A was expressed as molar concentration which allowed the direct comparison. The mean secukinumab concentration (46 nM) measured on Day 8 in dermal ISF from lesional skin indicated a clear molar excess of secukinumab molecules compared to the mean level of IL-17A molecules (0.31 pM, with a maximum of 2.28 pM) in quantities that appeared sufficient to completely neutralize IL-17A in skin [7]. It has been concluded that dOFM can readily be used to quantify dermal interstitial fluid concentrations of therapeutic antibodies such as secukinumab in the skin of healthy subjects as well as psoriasis subjects.

All biomarker results including those in interstitial fluid sampled by dOFM have been published in journals with a stronger focus on immunology [4,157]. Those publications also report the results obtained by other methods, such as the tape stripping methodology, which was used to assess markers of keratinocyte differentiation in the epidermis.

The comprehensive work performed within this first study of a monoclonal antibody has created a sound basis for further use of dOFM in antibody studies. Sinistrin has been shown to be a valid interstitial reference, and the dOFM (sinistrin) recovery has been shown to be well comparable between different study visits, healthy and patients and lesional and non-lesional skin (healthy: 16 % ± 3 %, patients 15 ± 3 %; for all study visits and probes). Therefore, sinistrin can serve as a quality marker for OFM sampling procedure in future studies. Importantly, the relationship between the relative recoveries of sinistrin and the antibody (factor 2.45) should also apply to other monoclonal antibodies, which are currently in development for treatment of e.g. moderate to severe atopic dermatitis. This means, future antibody studies aiming at quantification should not need to repeat all steps of validation but may utilize the known relationship for calibration.

From the perspective of sampling and quantification methodology the most interesting result was delivered by an additional (i.e. optional) antibody no-net-flux in the patient group (Figure 2-23, a). This secukinumab no-net-flux in patients failed to show a linear relationship between gain/loss and the perfusate concentration (determination coefficient r^2 was 0.03), and thus failed to deliver the interstitial concentration or the point of no-net-flux, respectively. The loss of relationship, however, was predominantly observed with the most concentrated two of the five perfusates, where the antibody should have been lost (delivered) to the ambient interstitial space. That is, in

the gain region the relationship was still intact (r^2= 0.41) albeit – also due to the reduced data points - much lower compared to the relationship in the complete secukinumab no-net-flux in the healthy subjects (r^2 = 0.59). An extrapolation would have delivered an intercept (interstitial concentration) slightly above 40 % (Figure 2-23, b).

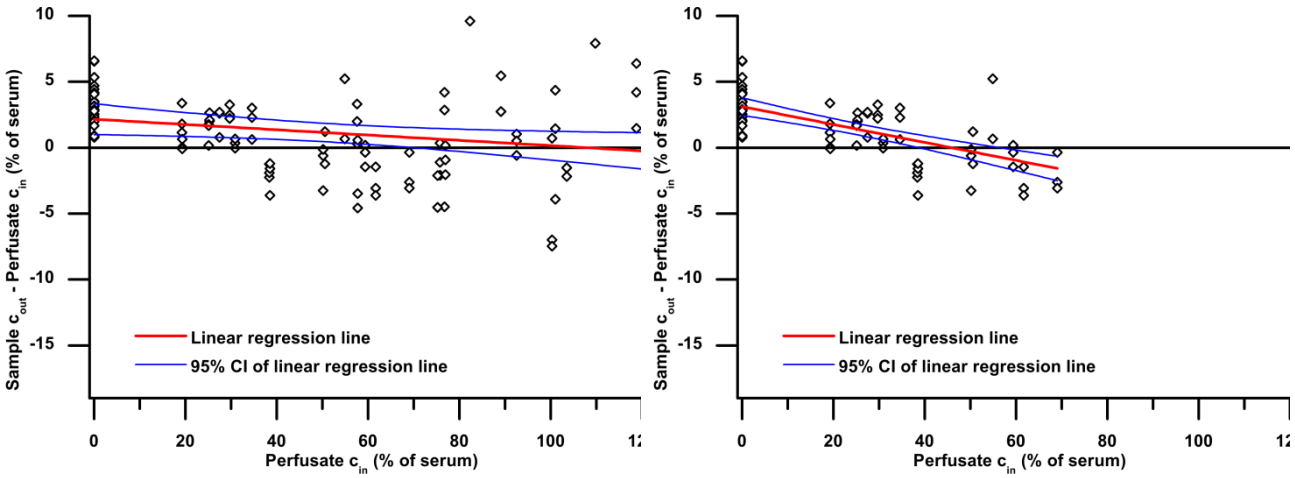

Figure 2-24: No-Net-Flux plot of secukinumab concentrations from eight psoriasis subjects at Day 15. **Left panel:** Data from all five perfusate concentrations are shown. Partly uninterpretable results were obtained because no stable losses were achieved when using concentrated secukinumab perfusates higher than the tissue concentration. Thus linear regression analysis (R^2=0.03) failed to show linear relationship. Consequently these data were not included into further data processing. **Right panel:** The removal of the perfusates with the two highest secukinumab concentrations would result in a reasonable linear regression line (R^2=0.41) and in an intersection point that is a little higher than that found in healthy volunteers. However, these data were not included into further data processing (Panels and text from Dragatin et al. [7]).

This observation may indicate that the conditions for a valid no-net-flux over a full range were not given any more for the large antibody in patients. The necessary condition may be that the rate for resupply of the sampled molecule should be equal to the rate of removal of the molecule when provided to the tissue. For small molecules, which are rapidly delivered over the capillary membrane and also cleared this way from the interstitial space, this condition may be valid for a wide range of concentrations. A huge antibody, however, may only be (slowly) removed from the spot of delivery via the lymphatic pathways, a hypothesis which would be in line with the very slow appearance of secukinumab in the circulation after subcutaneous injection.
In conclusion, future no-net-fluxes may need to avoid concentrated perfusates when investigating molecules of high molecular weight. Moreover, statistical analysis plans defining the processing of no-net-flux data may need to include the option to ignore the concentrated perfusates and to develop the linear relationship step-wise from the low-concentrated perfusates towards the intercept.

Author's contribution to research and publication: The author of this thesis contributed to the preparation of the study, the conducting of the study, acquisition of data, data analysis and data interpretation, and reviewed the manuscripts for two papers [4,7].

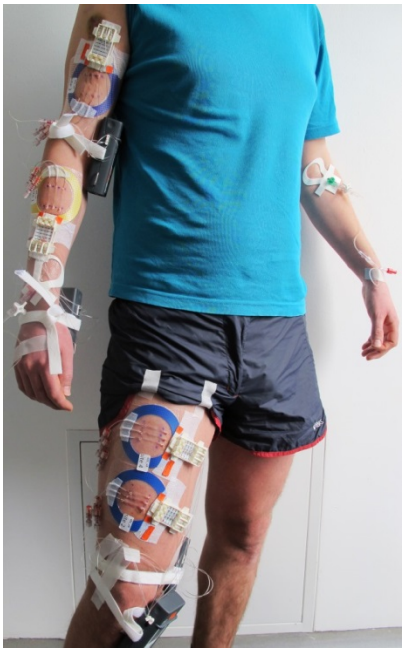

Figure 2-25: Picture of a healthy study volunteer in the antibody study wearing four pumps operating twelve dermal probes

Table 9: Synopsis of the secukinumab studies

Description	
Type of study	Kinetics and -dynamics of a monoclonal antibody after a single s.c. dose
Subjects	8 healthy subjects, 8 patients with psoriasis
OFM-material	OFM Probe: DEA15002, OFM Pump: MPP101 Further: Skin stabilization
Design	**Study 1** in healthy subjects to establish and validate methodology (methods: no-net-flux compared to sinistrin calibration, suction blisters and biopsies) **Study 2** in patients utilizing the validated OFM methodology 3 OFM study visits, on day 1, 8, 15 mAB injected subcutaneously at end of day 1 Up to 15 dOFM probes/subject, 1 µL/min sampling Up to 20 hours of OFM sampling in intervals ≥2 hours Suction blisters, tape stripping
Analyses	OFM analytes: secukinumab, sinistrin, several cytokines/biomarkers; Other: probe depth, PASI scoring; regression analyses on inulin and secukinumab perfusate concentrations
Outcomes, Significance	This study demonstrated the capability of OFM to reliably sample and quantify larger molecules such as therapeutic antibodies. Moreover, this study successfully validated inulin (sinistrin) as a marker for the relative recovery in dermal studies. Importantly, this study also identified specific limitations of the no-net-flux calibration approach. The results suggested that future no-net-fluxes on large molecules (with low

	recoveries) may be limited to perfusates with low concentrations to avoid the increased variation associated with the net-delivery of drug to the tissue from concentrated perfusates.
Publications	C. Dragatin, F. Polus, M. Bodenlenz, C. Calonder, B. Aigner, K. I. Tiffner, J. K. Mader, M. Ratzer, R. Woessner, T. R. Pieber, Y. Cheng, C. Loesche, F. Sinner, and G. Bruin, "**Secukinumab distributes into dermal interstitial fluid of psoriasis patients as demonstrated by open flow microperfusion**.," Exp. Dermatol., vol. 25, no. 2, pp. 157–9, Feb. **2016**. **[7]** F. Kolbinger, C. Loesche, M.-A. Valentin, X. Jiang, Y. Cheng, P. Jarvis, T. Peters, C. Calonder, G. Bruin, F. Polus, B. Aigner, D. M. Lee, M. Bodenlenz, F. Sinner, T. R. Pieber, and D. Patel, "**ß-defensin-2 is a responsive biomarker of IL-17A-driven skin pathology in psoriasis**," J Allergy Clin Immunol, vol. 139, no. 3., pp. 923-932.e8, Mar. **2017**. **[4]** C. Loesche, F. Kolbinger, M. Valentin, P. Jarvis, M. Ceci, G. Wieczorek, E. Khokhlovich, I. Koroleva, G. Bruin, F. Sinner, B. Aigner, and D. D. Patel, "**Interleukin-17A blockade with secukinumab results in decreased neutrophil infiltration in psoriasis: minimally-invasive measurement by tape stripping**," Adv. Precis. Med., vol. 1, no. 2, pp. 1–9, **2016**. [157]

2.5.4 Clinical research on topical bioequivalence – Bodenlenz et al. 2017

Clin Pharmacokinet
DOI 10.1007/s40262-016-0442-z

SHORT COMMUNICATION

Open Flow Microperfusion as a Dermal Pharmacokinetic Approach to Evaluate Topical Bioequivalence

Manfred Bodenlenz[1] · Katrin I. Tiffner[1] · Reingard Raml[1] · Thomas Augustin[1] · Christian Dragatin[1] · Thomas Birngruber[1] · Denise Schimek[1] · Gerd Schwagerle[2] · Thomas R. Pieber[1,2] · Sam G. Raney[3] · Isadore Kanfer[4,5] · Frank Sinner[1,2]

As has been stated in the introductory section of this thesis, the plan to develop und use dermal OFM methodology for topical bioequivalence assessments had been developed in 2007, when Benfeldt et al. published a study on the use of dermal microdialysis for this purpose in the internationally most renowned “Journal of Investigative Dermatology” [150].

The subject of “bioequivalence (BE)” is closely related with the subject of “bioavailability (BA)” and the definition of BE is based on the definition of BA. The close link between BA and BE is also reflected by the abbreviation “BABE” or “BA-BE” which is often used when writing about this subject. The term “bioequivalence” frequently causes confusion amongst scientists in research as well as the pharmaceutical industry and its meaning is a continuous matter of debate, presumably as the meaning for a certain drug product needs some interpretation or simply because the full definition of the term is not known. In the United States of America the terms bioavailability and bioequivalence are defined in the CFR-Code of Federal Regulation (CFR - Code of Federal Regulations, Title 21, PART 320 -- BIOAVAILABILITY AND BIOEQUIVALENCE REQUIREMENTS, citable as “21CFR320.1”] in the paragraphs a) and e) as follows:

- (a) **Bioavailability** means the **rate and extent** to which the active ingredient or active moiety is absorbed from a drug product and **becomes available at the site of action**. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action.
- (e) **Bioequivalence** means **the absence of a significant difference** in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered **at the same molar dose** under similar conditions **in an appropriately designed study**. Where there is an intentional difference in rate (e.g., in certain extended release dosage forms), certain pharmaceutical equivalents or alternatives may be considered bioequivalent if there is no significant difference in the extent to which the active ingredient or moiety from each product becomes available at the site of drug action. This applies only if the difference in the rate at which the active ingredient or moiety

becomes available at the site of drug action is intentional and is reflected in the proposed labeling, is not essential to the attainment of effective body drug concentrations on chronic use, and is considered medically insignificant for the drug.

The proof of the bioequivalence of a topical (test) product (usually a generic product) compared to the topical originator product (also termed the reference listed product – RLD) in a clinical study in volunteers may be more challenging than for oral dosage forms. On the one hand the challenge is due to the definition of bioequivalence, demanding the comparative assessment of the drug's BA "at the site of action", which for many topical drugs treating skin diseases is definitely within the layers of the skin. This poses a methodological challenge. On the other hand it is known that the penetration of topical drugs into and through the skin is highly variable between subjects, with a coefficient of variation (CV) typically in the range between 40 to 90 % [158,159]. This poses a challenge regarding the number of volunteers needed for the proof of topical bioequivalence. Many topical products are tested for bioequivalence in clinical endpoint studies, that is, the treatment effects of two products are compared. As both the topical penetration and the effects are highly variable, such endpoint studies do usually require hundreds of volunteers, meaning that these studies are time-consuming and costly but still may not be very sensitive for differences between topical drug products [160,161].

In 2014 the US-FDA searched for novel methods for topical bioequivalence due to the fact that availability of generic topical dermatological drug products in the United States is constrained by the limited methods established to assess topical bioequivalence. The institute (HEALTH, Joanneum Research) reacted to the subsequent US-FDA research call and submitted a research application proposing studies in human volunteers and in human donor skin using dermal OFM. The institute received the grant.

As a result, OFM materials were refined again, a number of standardizing tools developed in order to perform a comprehensive clinical study "to evaluate whether dOFM is an accurate, sensitive and reproducible in vivo method to characterize the intradermal bioavailability of acyclovir from 5 % acyclovir creams, comparing a reference (R) product either to itself or to a different test (T) product "[5].
In pilot studies the products were selected (acyclovir products with different formulations) and the essential study parameters for the main study such as required study duration and time resolution evaluated. In the pivotal study, T and R products were applied to six randomized treatment sites on the skin of 20 healthy human subjects. Two dOFM probes were inserted in each treatment site to monitor the intradermal aciclovir concentration for 36 h. Comparative bioavailability (of R versus R and T versus R) was evaluated based on conventional bioequivalence criteria for pharmacokinetic endpoints (AUC and Cmax) where the 90 % confidence interval of the geometric mean ratio between the T and R falls within 0.80 to 1.25 (for the log-transformed ratios within the range -0.223 to +0.223).

In the pivotal study involving 20 subjects, the positive control products (R versus R) were accurately and reproducibly confirmed to be bioequivalent, while the negative control products (T versus R) were sensitively discriminated not to be bioequivalent.. That is, though topical penetration is known to be highly variable [158,159,162], BE was successfully confirmed for

reference vs. reference for the kinetic endpoints AUC0–36h (0.86–1.18, point estimate 1.01) and Cmax (0.86–1.21, point estimate 1.02) in 20 subjects only, with point estimates close to 1 and a 90 % CI range which was small enough to not violate the conservative BE limits. Thus, dOFM accurately, sensitively and reproducibly characterized the dermal BA in a manner that can support BE evaluations for topical acyclovir 5 % creams in a study with 20 subjects.

This brief description summarizes the successful first OFM bioequivalence study similar as reported in conference abstracts and the paper published with the partner US-FDA (Bodenlenz et al. 2017, [5]). The full-text of this paper is provided in the appendix section. Table 9 at the end of this section provides a tabular synopsis of the bioequivalence study. Figure 2-24 provides an optical impression of the study setting and procedures.

The study demonstrated the feasibility to prove topical BE according to FDA criteria in 20 subjects only. This meant a significant advance with regard to the alternative of a clinical outcome BE study which requires hundreds to thousands of patients. This study also confirmed Benfeldt et al. [150] who, in 2007, based on a small microdialysis BE study, had estimated that the required power of a BE study could be achieved with such a low number of subjects.

Proper study design and standardization were the key for the success of this OFM BE study. Beyond the use of standardized OFM probes and their standardized application also all other materials and procedures were fully standardized, and potential influential factors were well-controlled. To achieve this degree of standardization the study team had also designed:

- Sterile templates for definition of test areas and probe positions
- Skin stabilization aids (self-adhesive) preventing any stretching of skin areas
- Plasters defining the topical dosing areas
- Non occlusive protective shields to protect the treated sites from unintended touching for 2 days

To provide stable and reproducible conditions for topical penetration we controlled room temperature as well as relative humidity in a narrow range (22 ± 1°C, 40 - 60 % RH). Other factors which could not be fully controlled were assessed at best in order to enable subsequent research regarding their impact on the primary outcome parameters:

- Probe depths were assessed by ultrasound over the full length of the probe.
- Skin temperature at t=0 was assessed by an infrared thermometer.
- Glucose was added to the perfusate to assess the relative recovery by loss over 37 hours day and night sampling.
- Skin impedance was used to characterize electrical properties of the skin (resistance, reactance)
- Trans-epidermal water loss (TEWL) was used to characterize the skin for transepidermal evaporation of water as a potential measure of (water) permeability.

Thus, the study has acquired a comprehensive data set which is currently under statistical evaluation. It is anticipated that the statistical evaluation of the data will contribute new knowledge

on the stratum corneum skin barrier and the variability of topical penetration in the population, on the mechanisms of topical drug penetration and key influential factors. Moreover, it is anticipated that the analysis of variability and errors will answer the most relevant question for this theses whether current OFM is a reproducible research tool (chapter 3).

Author's contribution to research and publication: The author of this thesis contributed to the design of the study, wrote the study protocol, prepared the study, conducted the study with the clinical research team, contributed to the acquisition of data, data analysis and data interpretation, and wrote the manuscript.

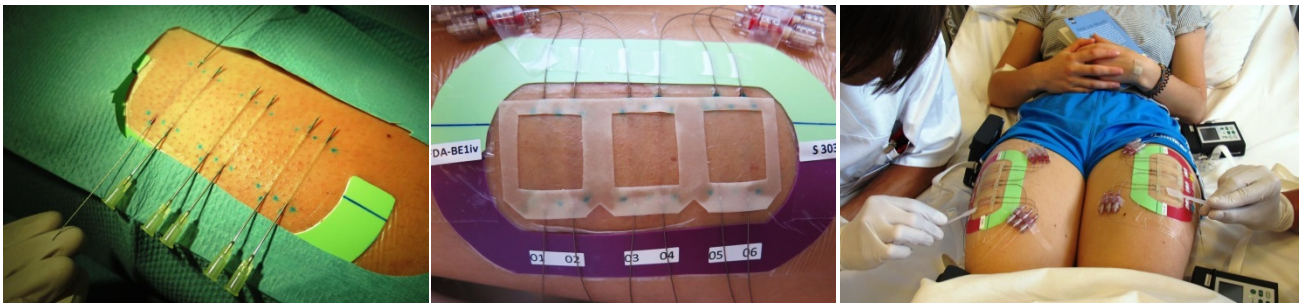

Figure 2-26: Left: Close-up view of a leg during dermal probe insertions. Mid: Close-up view of a leg with three topical test sites and 6 dermal probes prepared for topical dosing. Right: Picture of a volunteer with 6 topical test sites and 12 dermal probes recieving the topical doses.

Table 10: Synopsis of the topical aciclovir bioequivalence study

Description	
Type of study	Topical bioequivalence of two acyclovir products
Subjects	20 healthy subjects in the pivotal bioequivalence study
OFM-material	OFM Probe: DEA15003, OFM Pump: MPP101/MPP102 Further: Skin stabilization, non-occlusive protective shield, other novel tools
Design	6 topical application areas, 3 per thigh, product application randomized 12 dOFM probes/subject, 6 per thigh, 1 µL/min sampling 37 hours of sampling in 2 h / 4 h intervals
Analyses	OFM samples: acyclovir, glucose, deuterated water; Other: transepidermal water loss, skin temperature, skin impedance, probe depths
Outcome, Significance	dOFM accurately, sensitively and reproducibly characterized the dermal bioavailability in a manner that can support bioequivalence evaluations for topical acyclovir 5 % creams in a study with 20 subjects. This successful evaluation of dOFM for topical bioequivalence created confidence in continuous sampling methodology and led to a further research call of US-FDA explicitly inviting users of OFM and microdialysis to submit research proposals. HEALTH again applied successfully and currently evaluates dOFM also for BE of drug products. The publication created attention and interest in the industry for utilization of dOFM.
Publication	M. Bodenlenz, K. I. Tiffner, R. Raml, T. Augustin, C. Dragatin, T. Birngruber, D. Schimek, G. Schwagerle, T. R. Pieber, S. G. Raney, I. Kanfer, and F. Sinner, "**Open Flow Microperfusion as Dermal Pharmacokinetic Approach to Evaluate Topical Bioequivalence**" Clin. Pharmacokinet., vol. 56, no. 1, pp.91-98, Jan. **2017. [5]**

3 OFM today – a safe, tolerable and reproducible research tool?

Safety & tolerability

Since its CE-certification OFM has been utilized for clinical pharmaceutical research in more than 150 healthy volunteers and patients. In these studies each volunteer and patient received several probes (up to 16) for sampling from tissues for up to 48 hours. Usually a volunteer is investigated repeatedly in two or three OFM study visits to compare local drug concentrations (pharmacokinetics - PK) and local drug action (also “efficacy”, “pharmacodynamics – PD”) during a treatment period. Thus, more than 3000 probes have been used in vivo delivering far more than 30,000 hours of information from within the tissues. No cases of inflammation were observed in end-of-study visits and no scars remained. No serious adverse event occurred. In some cases probe insertion into the dermis caused visible bruising (hematoma) which was recorded as an adverse event, but also a transient effect. Dropouts of volunteers in OFM studies are rare and were not related to OFM. As the initial medical device evaluation study had shown, the OFM-related procedures (probe insertions, prolonged sampling using many probes) are well tolerated [9]. Since then, many of the participants asked whether they could participate in further studies. This should confirm the tolerability of OFM in clinical research. Taken together, current OFM devices can be considered as safe and tolerable in clinical research. Still, some scientists in academic research and industry who are not yet familiar with this or any other invasive or minimally-invasive clinical research method are reluctant when discussing the potential utilization for in vivo research. According to the experience of the author of this thesis, this psychologic hurdle of novel users is resolved once cautious scientists have the chance to watch the application of probes in study volunteers and to ask them about “How it was?”

Accuracy and Precision, Repeatability, Reproducibility

These terms are often used in a misleading way, in particular in everyday life but also in medical sciences and literature, though the terms are well described by ISO guidelines and also on Wikipedia. The discussion whether OFM is “accurate”, “precise” or “reproducible” requires providing a brief description of these terms here:

Accuracy is a description of *systematic errors*, a measure of *statistical bias*; as these cause a difference between a result and a "true" value, ISO calls this “trueness”. In an alternative less common definition by ISO high accuracy requires both high trueness and high precision.
Precision is a description of *random errors*, a measure *of statistical variability*. Often the terms repeatability and reproducibility are used to describe precision.

- **Repeatability** measures the variation in repeated measurements performed by a single instrument or person under the same conditions in one place in a short period of time.
- **Reproducibility** measures whether an entire study or experiment can be reproduced in its entirety. In other words it describes the ability to replicate the findings of others by

measuring the degree of agreement between the results of experiments conducted by different individuals, at different locations, with different instruments.

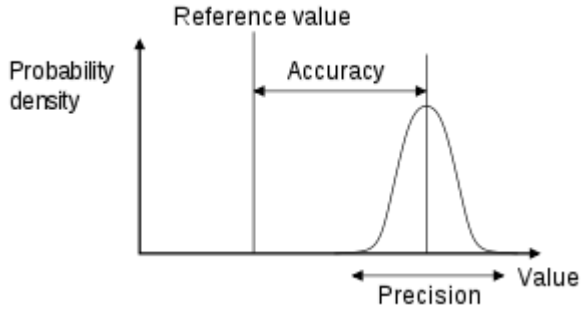

Figure 3-1: Accuracy is the proximity of measurement results to the true value; precision, the repeatability, or reproducibility of the measurement (Graphic illustration and description from Wikipedia).

Microdialysis literature repeatedly used the term “reproducibility” to describe the quality of the microdialysis study but the literature does not provide any informative data on the method’s accuracy, precision or repeatability in terms of their scientific definition. That is because the assessment of these parameters for in vivo research methods like MD and OFM should pose distinct challenges: First, a reasonable characterization of these in vivo methods requires a high number of repeated measurements of a well-known and stable ISF concentration of an analyte or the repeated measurement of an analyte which is well assessable by another recognized accurate as well as precise method serving as the “Golden Standard”. Second, the conductance of clinical studies for this purpose is laborious and requires considerable financial resources. Solely a producer of such research materials as well as authorities confronted with the resulting PK-PD drug data should have the motivation and resources to assess such characteristic data of instruments.

Studies including information on precision and accuracy

The studies on the monoclonal antibody secukinumab (see 2.5.3 and the according paper [7]) comprised a no-net-flux protocol which assessed the concentration of the metabolically inert polysaccharide inulin (sinistrin) in dermal ISF relative to the inulin concentration in serum serving as reference value during steady-state conditions following primed-continuous i.v. insulin infusion. The inulin concentration in dermal ISF was confirmed to be equal to the serum concentration (sinistrin geometric mean ratio dermal/serum: 1.003; 90 % CI between 0.91 and 1.10). This agreement with the reference value indicated (i) that inulin (sinistrin) can serve as interstitial reference in dermal OFM studies, and (ii) the accuracy of the OFM approach, in this case when combined with the no-net-flux procedure. Moreover, the secukinumab studies showed the precision (repeatability, reproducibility) of dOFM based on the relative recovery of inulin (sinistrin). Inulin (sinistrin) was intravenously administered to all healthy volunteers and all patients in all study visits using dOFM and was assessed in all dOFM probes/samples to calculate the relative recovery as a measure of precision (repeatability, reproducibility) and stability over time. The relative recovery was well comparable between different study visits, healthy and patients and lesional and non-lesional skin (healthy: 16 % ± 3 %, patients 15 ± 3 %;

for all study visits and probes) indicating repeatability and reproducibility of the sampling process (see 2.5.3.).

The most recent clinical study which evaluated dOFM for the purpose of topical bioequivalence assessments (see 2.5.4 and the paper [5]) was probably also the most appropriate to conclude on both the precision and the accuracy of dOFM. The primary aim was to show that (i) the reference product is bioequivalent to itself in 20 volunteers only, using the conventional (conservative) statistical BE-metrics and acceptance criteria (90 % CI of the geometric mean ratios for reference vs. reference within 0.8 – 1.25, or for the log-transformed variable within the range -0.223 to +0.223). The prerequisite to meet the acceptance in such a low number of volunteers was precision and accuracy. BE was successfully confirmed for reference vs. reference for the kinetic endpoints AUC0–36h (0.86–1.18, point estimate 1.01) and Cmax (0.86–1.21, point estimate 1.02). The point estimates were close to 1 indicating high accuracy of dOFM (including accuracy of application, bioanalytics etc.) in this BE study. The width of the 90 % CI interval for the main variable AUC0-36h (± 0.16 on the log-scale), which was symmetric did not violate the BE limits (± 0.223 on the log-scale), should indicate high precision of dOFM (here the "repeatability") which allowed to meet the conservative limits though topical drug penetration is known to be highly variable between as well as within individuals. The fact that the clinical study did not evaluate dOFM in one human volunteer on one single day (~repeatability) but evaluated dOFM in 20 subjects over a period of a month explains why also the term reproducibility was used: "*The results of this study suggest that an appropriately designed and well-controlled in vivo dOFM study could have the requisite accuracy, precision, reproducibility, and statistical power to compare the rate and extent to which a topically applied drug such as acyclovir becomes available in the dermis and that dOFM may provide a viable dermal PK approach for the BE assessment of topical drug products"* [5]). Nevertheless, the "reproducibility" of dOFM results has not yet been proven fully yet shown as the definition of the term requires that an entire study or experiment is reproduced in its entirety by other researchers in another location.

In the study on topical clobetasol-17-propionate (see 2.5.2 and paper [6]) dOFM enabled the intradermal investigation of this lipophilic drug when released from a low-strength cream and it delivered timely and spatially, i.e. probe depth dependent, resolved kinetic data. dOFM showed that the concentration of the topically applied drug depends on probe depth and that the concentration is significantly different if the insertion depth is varied by more than 0.15 mm. This should mean that all methodological random errors were sufficiently low and the accuracy of the method sufficient in order to be able to reveal this concentration-depth relationship in vivo in humans.

Precision, random and systematic errors

The result of this collaborative BE study with the US-FDA was very encouraging for dermal OFM and stimulated efforts to evaluate OFM for further topical drugs. However, the overall variability of the Area-Under-the-Curve (AUC) data among all subjects was high (CV ~ 40 %), albeit not higher than in other studies of topical penetration reporting CVs higher than 40 % [158,159,162]. The

variability should therefore not be irritating. Nevertheless, this variability was not understood and the causes also not described in literature, a situation which was unsatisfactory for the author and the OFM research team striving for reproducibility and searching for ways to minimize methodological random and systematic errors.

As a consequence the large data set of the acyclovir BE study has been systematically investigated by statistical means for the sources of variability to get insights into the potential causes of AUC differences between the individual subjects, but also between the test sites and between the probes within each subject. An analysis of variance (ANOVA) revealed that 82 % to 91 % of the overall AUC variability was inter-subject-variability (also “between-subject-variability”), while the remaining intra-subject variability was only 9 % to 18 %, consisting of site-to-site variability (4 % to 11 %) and probe-to-probe variability (5 % to 7 %). This meant that most of the variability was clearly attributable to the differences between the skin permeabilities of the individuals (anatomical/biological variability), while the remaining biological-methodological variability (variability of topical dosing + insertion depths + OFM fluidics + bioanalytics) was surprisingly low. In 2007 Benfeldt et al. [150] performed an excellent microdialysis BE study setting the benchmark regarding quality and study design. The team of Benfeldt et al. thoroughly characterized the sources of variability for a well-penetrating drug (lidocaine) and found an inter-subject-variability of 61 % and a remaining intra-subject variability of 39 % (sites 20 %, probes 19 %). The fact that the intra-subject variability in the OFM BE-study for a poorly penetrating drug as acyclovir was less than half of its microdialysis benchmark indicates that the reproducibility of OFM should have reached a high level, in fact a level which had not been expected.

Further analysis identified the main physical factors responsible for each type of variability, for the large inter-subject variability as well as the (low) within-subject variability (also site-to-site or “methodological” variability) which should be the decisive variability for the statistical power of BE studies using OFM or similar methods.

(i) Inter-subject variability

According to a step-wise regression model, most of the inter-subject variability of AUCs can be explained by differences in skin impedance (device developed by Joanneum Research), while the well-established parameter “Trans-Epidermal Water Loss (TEWL)” does explain a minor portion of the variability, i.e. is less predictive for penetration than the novel impedance method. This result (yet unpublished) should be of interest and relevance for the dermatological pharmaceutical community which is searching for reliable parameters to characterize the skin barrier of individuals as well as human donor skin to understand the test results (including variability) in skin penetration testing. However, these anatomical differences from subject to subject are in fact – irrespective of the magnitude of the differences - not at all relevant for BE studies utilizing OFM, as in OFM studies each subject serves as its own control by investigating drug penetration from both test and reference product simultaneously on multiple test sites on “the same skin”. For the advance of OFM those factors had to be identified which contributed to

site-to-site variability of the AUCs derived by dermal OFM probes from the parallel and equally dosed test sites.

(ii) site-to-site, methodological and OFM variability

Similar to the approach applied for the CP-17 study (Bodenlenz et al. 2016, [6]), pairs of probes within the same test sites were compared for differences in AUCs but also for differences in probe depths, for sample volume as the surrogate for flow rate and glucose loss from perfusate as the surrogate for the exchange rate or relative recovery. This paired analysis was not affected by the inter-subject variability and thus was sensitive enough to confirm that these parameters do impact the observed AUC as had to be anticipated considering the physics of continuous analyte recovery. However, solely probe-depth differences between sites ("the variability of the user") were found to have the potential to impact BE test results negatively also in an otherwise fully standardized study. As a consequence future BE studies using dermal sampling probes (microdialysis or OFM) need to assess probe depth accurately to verify that no significant differences between sites existed ("systematical error", "bias") that could impact the validity of the BE assessment between two products.

These results from research in topical bioequivalence (unpublished, analysis is ongoing) demonstrate that the variability caused by current dermal OFM devices is acceptably low, and that PK results assessed by dermal OFM are repeatable. Newest hints from data analysis suggest that the methodological component of overall variation of topical data might be even lower (and precision as well as accuracy higher) than suggested from the initial analysis of variance and the bioequivalence tests. More information on this ongoing research for a fully understanding of data variability in topical penetration studies is provided in the subsequent chapter "Current Research".

Precision of dOFM in other studies

A high level of precision (repeatability, reproducibility) has also been seen in other studies which included monitoring of the relative recovery. The ionic reference technique using sodium has repeatedly shown that the recovery is well comparable between different probes within the same subjects and between probes in different subjects. In a number of studies the interstitial marker inulin (sinistrin) was used to compare the recovery between probes, between test sites, and between healthy subjects and patients. Also these inulin results indicated that the recovery was reproducible. Moreover, for both the ions as well as inulin it has been shown that the recovery was stable for prolonged durations with no or a negligible drift over time. Reproducibility and stability of OFM can now be considered as proven. Moreover, the reproducibility of the sampling probe dimensions and the exclusive use of a standard flow rate (1 µL/min) helped to conclude on reproducibility of the relative recovery of typical analytes. In a number of experiments on topical drug candidates (data not shown) we found that the recovery of topical drugs, typically having a molecular weight between 300 to 500 Dalton, are recovered with a reproducible relative recovery of ~50 % (40 % to 60 %). Reproducibility, standardization and the knowledge about typical

recoveries should facilitate future drug studies: In future studies the monitoring of the recovery could be simply omitted. The dermal interstitial fluid concentration of topical drugs can be assessed by simply correcting the sample concentrations by a factor of 2. For mediators of inflammation, whose molecular weight are typically in the range between 10 to 20 kDa a correction factor of 5 can be used to estimate their molar concentration in dermal interstitial fluid.

It is noteworthy that the reproducibility seen in OFM studies to date does not mean that any OFM study result can be reproduced: The reproducibility of each clinical study, also of OFM studies, is still limited or defined by the variability of pharmacological and biological factors and not the least by the “variability of the clinical user”.

In conclusion, dermal OFM is a safe and tolerable, accurate and reproducible and thus powerful research tool in the hands of a disciplined research team in a well-designed and thoroughly standardized clinical study. The proven precision will keep future protocols simple and thus facilitate future drug research using (dermal) OFM.

4 Current research

Open-flow microperfusion methodology has reached a degree of maturity enabling its routine use in pharmaceutical research in humans and animals. The primary objective of most ongoing trials is the investigation of the pharmacokinetics (PK) of the active drug directly at the designated site of action. In the ideal case the kinetic readout is combined with the investigation of the drug's local pharmacodynamic action on biomarkers (PD), therefore those most data-rich trials are also termed 'PK-PD' trials.

Clinical research

A current clinical research trial uses OFM to investigate the distribution of different anti-infective drugs to the skin after single and repeated oral intake. The trial uses dermal OFM for a combined pharmacokinetic-pharmacodynamic investigation to understand both the drugs' differences in dermal bioavailability and their intradermal anti-inflammatory actions (PK-PD). This comparative study benefits from the fact that OFM reliably recovers both anti-infective drugs with a well-comparable sampling efficiency ("relative recovery") though their somewhat different physico-chemical properties. The preliminary results seem to confirm that the drug with a reduced degree of plasma protein binding achieves significantly higher levels at the dermal target, such that half the dose might be bioequivalent to the standard drug.

Dermal OFM has been successfully used in patients with psoriasis to assess the PK-PD of a monoclonal antibody. In the near future patients suffering from moderate to severe atopic dermatitis may also benefit from antibody therapies. Currently a trial is in conception which aims to investigate the PK-PD of a novel antibody against atopic dermatitis. Such a trial would be able to fully benefit from the strengths of OFM in the sampling of large molecules and biomarkers.

The most recent application of OFM is in trials using multi-probe settings in order to compare novel topical drug formulations head-to-head for dermal bioavailability. These head-to-head settings with OFM have proven particularly useful for the evaluation of topical generic products which need to demonstrate bioequivalence (BE) to the reference listed drug (RLD) product of the innovator in order to obtain market approval. The initial BE trials provided an enormous amount of data on topical penetration (8640 h of profiles in the pivotal study) and anatomical, physiological and methodological factors which potentially may influence the drug profile observed in the dermis by OFM. This data set is currently the subject of comprehensive statistical analysis and provides insights which are invaluable. As mentioned in the chapter 3 before, the preliminary results (unpublished) demonstrate that only a small fraction of about 5 % of the overall variance in such a topical drug study is probe-to-probe variability and in part attributable to OFM. Though this variability is low, it would be of value to fully understand probe-to-probe variability, as this small variability component together with site-to-site variability determines the statistical power of an OFM BE study, and thus the required number of subjects. Therefore, current research involves further statistical analysis to better identify the causes of variability and to generate hypotheses which can be investigated in subsequent studies. One such hypothesis of the author

is that larger differences between the AUCs of two adjacent probes, which have been observed repeatedly, are primarily caused by natural shunts or local defects in the stratum corneum above the probe. OFM sampling and the relative recovery should not contribute significantly to variability and cannot explain such large differences. However, a microscopic defect in the stratum corneum above the probe could easily multiply the local skin penetration of an analyte which otherwise hardly penetrates the intact barrier (like acyclovir, its gradient from skin surface to the dermal probe is 1 to 50 Mio). Local defects should lead to a distribution of AUCs - when observed with lots of OFM probes - which is very skewed towards higher AUCs. Consequently, if only two probes are used per test site, the lower AUC in each site should be more characteristic for the penetration into intact skin than the higher AUC. Preliminary data seem to confirm this: Test-wise exclusions the higher AUC from each pair of AUCs (i.e. 50 % of the probes excluded) led to a reduction of the overall coefficient of variation for the AUCs. More elegant and also effective to suppress “outliers” was the use of the geometric mean instead of the arithmetic mean for averaging the profiles of the probes or the AUC and Cmax data within subjects. The use of the geometric mean at the raw data level decreased the variation in the BE outcome parameters and decreased the width of the 90 % CI interval for the BE ratios. This seems to confirm the assumption on the highly skewed distribution within subjects and supports the author’s hypothesis that the main source of variability is the stratum corneum, and that a dermal probe with its high spatial resolution is just sensitive enough to reflect locally increased concentrations. Preliminary plots of normalized intra-subject AUC data of all probes do also show a highly skewed distribution. If further analysis can statistically prove the absence of a normal distribution of within-subject AUC data, this would mean that future studies of topical BE studies using OFM (or MD) should not compare two products between test sites with 2 or 3 probes only because even the (geometric) mean of 2 or 3 probes should not be best to inform about the topical formulation’s ability to deliver the drug through intact skin (AUC, Cmax). Instead, each product might just be randomly applied above a sufficient number of dermal probes per subject (e.g. 6 - 8 probes for each product) and the obtained 6 - 8 AUCs (Cmax values) per product used to calculate the geometric mean or median as the best parameter describing the distribution and thus the decisive property of the formulation. Thus, a very informative T/R ratio might be obtained from each single subject with a low variability of this T/R ratio among the study subjects - the best prerequisite for a sensitive and discriminative topical BE study.

Statistical analysis elaborating this and other hypotheses is ongoing. Certainly, knowledge on the causes of probe-to-probe variability will allow designing topical bioequivalence/bioavailability studies with an even lower variability. This will further reduce the (already low) number of subjects needed for clinical studies using dermal OFM. Further clinical trials are currently in preparation which aim to evaluate the utility of OFM for BE of other drugs. Those studies will assess more potential influential parameters during BE assessment and should thus provide further insights into the sources of variability and the absolute minimum number of subjects required in future BE studies. In these upcoming studies the setting will include 16 probes. The excellent tolerability of the OFM probe and the mobility provided by the wearable pump enable such large study settings with a multitude of probes in parallel for up to 48 hours. Such a test setting is shown in Figure 4-1.

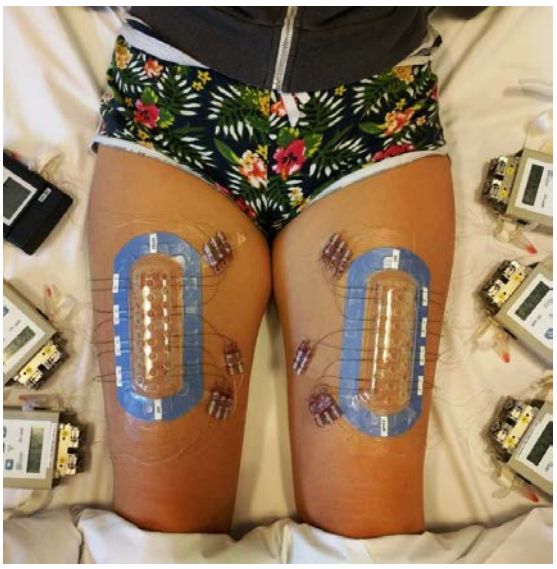

Figure 4-1: Topical bioequivalence test setting with 8 topical test sites and 16 dermal probes. Skin is stabilized with self-adhesive rings (blue) and the test sites are covered with non-occlusive transparent protection shields

Preclinical research

While designed for human use, OFM is increasingly used in preclinical research. This allows investigation of novel drugs at an earlier stage of development and carrying forward OFM from preclinical development to clinical development. As material and methodology is identical for animal and human use the results can be compared thus providing continuity during preclinical and clinical development of the drug product. In other words, if the animal model is appropriate for the purpose, the kinetics results obtained by OFM in animals should be predictive for the kinetics in humans.

As has been mentioned earlier, dermal OFM is also in use to test topical penetration in freshly explanted human skin which is obtained via the Biobank from plastic surgery. Also freshly explanted animal skin (e.g. pig skin) has been used to investigate topical penetration. The usability of dermal OFM in animal skin in vivo and ex vivo and in explanted human skin ex vivo might be of particular value for the development of topical formulations, as respective OFM experiments should allow the estimation of drug concentrations which can be expected in human skin in vivo. The knowledge of the in vivo/ex vivo relationship of the dermal concentrations in animals, and the ex vivo/ex vivo relationship between animals and humans should enable such a prediction of the situation in humans in vivo before a clinical formulation is developed.

An interesting new application is the use of OFM for the continuous sampling of immune cells from inflamed tissue. Already in 2009 a microscopic evaluation of interstitial fluid samples showed that cells are present in dermal OFM samples confirming that macroscopically fenestrated OFM probes are permeable for cells. Today's FACS methodology enables differentiation between immune cell types and a "true count" of the cell numbers for each subtype providing the "concentration of cells" (cells per µL). Current basic research characterizes the immune cell population over time and is already able to differentiate between inflamed skin and unaffected skin solely based on immune cell counts. A feasibility study in a rat model for psoriasis showed that the specific action of novel drugs can be seen in the immune cell population in OFM samples

[61] and in changes in T-cell subtypes such as Th17, CD8+, IL17+, IL4+, IFNγ+ cells [72]. In a next step the immune cell count and cell differentiation will be combined with large biomarker assays, thus to obtain a complete picture of local tissue inflammation in psoriasis and other inflammatory diseases. This will provide the basis for the investigation of the specific immunomodulatory effects of known but poorly understood therapies as well as new therapies.

Table 11 provides an overview of current OFM applications in clinical and preclinical research.

Table 11: Overview of current OFM applications

Type of studies	Populations, models	Types of drugs and studies
Clinical studies	healthy volunteers, patients with psoriasis, patients with atopic dermatitis, patients with diabetes	Topicals, oral drugs, antiviral drugs, anti-infective drugs(antibiotics), anti-inflammatory drugs, monoclonal antibodies, PK-PD investigation, bioequivalence (BE), immune-cell sampling, insulin analogues
Animal studies	domestic pigs, rats, rats with psoriasis-like inflamed skin	Topical and oral drugs, anti-inflammatory drugs, PK-PD investigation, formulation testing, immune cell-sampling, preparation of clinical trials
In vitro studies	Human skin explants, skin from domestic pigs	Topical anti-inflammatory drugs, drug metabolism, preparation of clinical trials, prediction of concentrations in human dermis in vivo

5 Future challenges

The design of novel sampling devices has been driven by the general need for a universal sampling method to enable access to a broad range of analytes at target tissue level for clinical pharmaceutical research. The specific design of current OFM sampling devices, however, has been clearly inspired and guided by the concepts and statistical considerations for topical bioequivalence trials as published by Eva Benfeldt et al. [150,151]. The recent dOFM bioequivalence trial on acyclovir products [59] demonstrated the utility of the current devices for topical bioequivalence studies. OFM was sufficiently reliable and reproducible to prove topical bioequivalence based on conventional criteria in not more than 20 volunteers. Thus the aims of this thesis regarding methodological progress were achieved. Notwithstanding, this successful bioequivalence trial did not mean the end of a long process but it actually marked the beginning of comprehensive research aiming at a deeper understanding of topical drug penetration and comparative bioavailability (bioequivalence) as basis for the future use of OFM and microdialysis in this field of pharmaceutical research. The new research activities will investigate further drugs and the potential factors that may influence bioequivalence test results in such clinical settings. The new challenges in this field of OFM research may be summarized best by the following research questions:

i. Which factors of biological and methodological variation exist and may influence bioequivalence results in a multi-probe head-to-head test setting?
ii. Do the skin properties of an individual subject (e.g. high or low impedance, high or low transepidermal water loss) have an impact on the discrimination of different formulations?
iii. If e.g. probe depth is confirmed as a relevant factor: Can we find a reliable model that can account for the potential impact of that factor on the pharmacokinetic endpoints AUC_{0-t} and C_{max} thus allowing the reduction of the required sample size (n volunteers) to less than twenty volunteers for a topical BE study?
iv. Is it conceivable that ingredients of topical formations can alter the local conditions within the skin such that OFM or microdialysis sampling can lead to erroneous BE results?

The literature on microdialysis studies provides a treasure chest of ideas which should be helpful for the further advancement of OFM. The challenge lies in finding the time for the study of the vast literature. Without doubt the literature study will be worth the effort. Christopher Anderson, the author of the stimulating commentary “Cutaneous Microdialysis: Is it worth the sweat?” [163] would probably agree. Summarizing past research in this thesis created an opportunity to read “old literature”: In the first publication of Anderson et al. on the use of “a new bioanalytical sampling technique” in 1991 [164] they reported considerable inter-subject variability in the amount of ethanol they found in dermal microdialysis samples after topical ethanol application. The use of topical ethanol in a clinical study might be a simple way to further characterize the variability in topical OFM/microdialysis studies, and to find out to which degree such variability is indeed “inter-subject” and to which degree it is caused by normal local skin defects which are accurately registered by intradermal probes providing very high spatial resolution[6].

Dermal open-flow microperfusion has repeatedly provided simultaneous access to inflamed skin and unaffected skin in trials with patients and thus provided insights into the pharmacokinetics and –dynamics (PK-PD) of potent drugs in psoriasis. OFM has addressed very specific questions therein and thus investigated a few biomarkers only, although the unfiltered OFM sample includes a lot more information on the local biomilieu (metabolites, cytokines, chemokines, and other markers of inflammation) that could be analyzed by novel sensitive assays with low volume needs. It seems also reasonable to characterize the dermal biomilieu of a healthy population by OFM to obtain a reference data set for the healthy condition and to use this information to identify individuals at risk, similarly as done in microdialysis by Sjögren and Anderson et al. [154]. For this kind of work the challenge lies in (i) the amount of data generated, (ii) their meaningful interpretation, and last but not least (iii) in finding funding for such basic medical and biomarker research.

Open-flow microperfusion and microdialysis are similar in methodology but very different in their sample composition. A very nice future challenge that will combine and fully utilize the complementary strengths of both sampling techniques will be the quantification of the total, the free and the protein-bound concentration of a drug at tissue level in vivo. The methodology for such quantification has already been established and successfully applied in feasibility studies. The challenge of this quantification lies in the reliable calibration of each probe type to obtain the recovery for different fractions. More drugs of different protein binding will have to be investigated in order to conclude on the reliability of the combined approach.

Dermal open-flow microperfusion methodology has undergone several loops of optimization during the first decade since its invention. Yet the OFM method – the same holds true for microdialysis - will benefit from further methodological improvements and promotion in medical pharmaceutical sciences in order to achieve general acceptance and usability in clinical and preclinical research.

Further methodological improvements should be achievable with respect to:

i. insertion methodology and precision
ii. minimal invasiveness and tissue reaction
iii. stability of relative recovery for durations >48 h
iv. device design for easy use and wide user acceptance (“Plug&Play”)

Promotion should mean to continue to create awareness amongst scientists and authorities that most drugs have their target in a certain organ or (peripheral) tissue and should therefore be characterized at the target level for their PK and PD rather than in blood as is still often done.

6 Summary and conclusions

Within this thesis devices for the continuous sampling of interstitial fluid from peripheral tissues in clinical research were successfully created, evaluated for their applicability in healthy volunteers as well as patients and the devices' utility for clinical and pharmaceutical research in a number of trials was proven.

The novel devices comprise a sterile sampling probe and a versatile wearable pump for clinical use. The novel probe provides minimally invasive access to the interstitial fluid biomilieu of the human dermis as well as subcutaneous adipose tissue in humans in vivo. In contrast to state-of-the-art microdialysis probes, the novel OFM probes are free of a filtering membrane and thus provide access to virtually all analytes of interest in the interstitial compartment irrespective the analytes' molecular size or lipophilicity. The probes enable research into a wide range of drugs and biomarkers directly in human target tissues in vivo; in particular these probes facilitate research on the mostly lipophilic topical drugs that are used to treat inflammatory skin diseases. The wearable multi-channel pump serves several OFM or microdialysis probes in parallel and thus facilitates prolonged and highly informative multi-probe study protocols. Its push-pull capacity fosters the utilization of probes requiring active volume control (OFM probes and large pore/high molecular weight cut-off microdialysis probes).

The author of this thesis successfully utilized the devices in a number of clinical studies and published the results as peer-reviewed papers in renowned international journals in the field of diabetology, dermatology and pharmacology. Clinical research involved more than a hundred healthy volunteers and patients and focused on the distribution of drugs to their target tissues (bioavailability, pharmacokinetics) and their effect on mediators of inflammation (biomarkers, pharmacodynamics). The research provided significant contributions to the basic knowledge on the role of the skin barrier in psoriasis, provided in vivo and ex vivo data on drug candidates to the pharmaceutical industry and supported the release of a novel highly efficacious antibody therapy against psoriasis. Moreover, the devices recently proved accuracy, sensitivity, and reproducibility in the characterization of the dermal bioavailability of a reference drug vs. a test drug in a manner that can support bioequivalence evaluation of topical generic drug products. Therefore, the devices currently are under closer evaluation for this most challenging application of continuous sampling devices, performing further clinical studies in collaboration with a health authority controlling the market release of medicines and generics.

The goals of the thesis have been achieved. Current OFM methodology overcomes the limitations of state-of-the-art continuous sampling methodology and enables the study of virtually all drugs and biomolecules directly at tissue level irrespective of the drugs' or biomolecules' molecular size or lipophilicity. The novel OFM devices have been shown to be safe, tolerable, reproducible and thus powerful research tools in a number of clinical pharmaceutical research studies. OFM has reached a degree of maturity enabling its routine use in pharmaceutical research in humans and animals, and has lately also been proven utility for topical bioavailability and bioequivalence assessments.

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8 Appendix - research papers in copy

The appendix provides the main papers in full-text if the copyright allowed a reprint of the entire contents. Otherwise the title page and last page (references) is provided only. To get the complete papers or to get papers in color (herein they provided in black/white), please download the papers from the websites of the journals or request a personal copy from the author of this thesis. The table below provides an overview on the papers in the appendix.

Table 9-1: Papers in the appendix

Publication	**What is provided?**	**Page**
Bodenlenz al. 2017 – Clin Pharmacokinet (open access paper)	entire paper	104
Kolbinger al. 2017 – J Allergy Clin Immunol (open access paper)	entire paper	111
Bodenlenz al. 2016 – Pharm Res (open access paper)	entire paper	129
Dragatin et al. 2016 – Exp Dermatol (open access paper)	entire paper	139
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8.1 Bodenlenz al. 2017 – Clin Pharmacokinet (open access)

Clin Pharmacokinet
DOI 10.1007/s40262-016-0442-z

SHORT COMMUNICATION

Open Flow Microperfusion as a Dermal Pharmacokinetic Approach to Evaluate Topical Bioequivalence

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Abstract

Background The availability of generic topical dermatological drug products is constrained by the limited methods established to assess topical bioequivalence (BE). A novel cutaneous pharmacokinetic approach, dermal open-flow microperfusion (dOFM), can continuously assess the rate and extent to which a topical drug becomes available in the dermis, to compare in vivo dermal bioavailability (BA) and support BE evaluations for topical products.

Objective To evaluate whether dOFM is an accurate, sensitive, and reproducible in vivo method to characterize the intradermal BA of acyclovir from 5 % acyclovir creams, comparing a reference (*R*) product either to itself or to a different test (*T*) product.

Methods In a single-center clinical study, *R* or *T* products were applied to six randomized treatment sites on the skin of 20 healthy human subjects. Two dOFM probes were inserted in each treatment site to monitor the intradermal acyclovir concentration for 36 h. Comparative BA (of *R* vs. *R* and *T* vs. *R*) was evaluated based on conventional BE criteria for pharmacokinetic endpoints (area under the curve and maximum ~~plasma~~ concentration) where the 90 % confidence interval of the geometric mean ratio between the *T* and *R* falls within 0.80–1.25.

Results The positive control products (*R* vs. *R*) were accurately and reproducibly confirmed to be bioequivalent, while the negative control products (*T* vs. *R*) were sensitively discriminated not to be bioequivalent.

Conclusions dOFM accurately, sensitively, and reproducibly characterized the dermal BA in a manner that can support BE evaluations for topical acyclovir 5 % creams in a study with $n = 40$ (20 subjects in this study).

Key Points

This is the first study showing the utility of clinical dermal open-flow microperfusion (dOFM) as a dermal pharmacokinetic approach to compare dermal bioavailability (BA) and support bioequivalence (BE) evaluations for a topical (locally acting) drug product.

dOFM is capable of directly measuring the penetration of topically applied acyclovir in human subjects in vivo with low variability for prolonged durations.

dOFM has the necessary accuracy and reproducibility to confirm BE for a reference acyclovir cream 5 % compared with itself, and is sufficiently sensitive to discriminate inequivalent BA between two different topical acyclovir cream 5 % products, in both cases based upon conventional BE criteria and pharmacokinetic endpoints.

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2.2 Topical Study Drugs

Acyclovir cream 5 % (Zovirax®; Valeant, Bridgewater, NJ, USA) was used as the *R* product. Acyclovir cream 5 % (Aciclovir 1A Pharma—Creme; 1A Pharma GmbH, Vienna, Austria), which has a different formulation composition and from which the topical BA of acyclovir was observed to be different relative to the *R* product (unpublished results from exploratory, pilot, in vivo dOFM studies), was used as the *T* product.

2.3 dOFM

dOFM probes were inserted intradermally (two probes at each of the three treatment sites on each limb) and dermal interstitial fluid samples were continuously collected (1 µL/min) from a pre-dose baseline time period (−1 h to 0 h) to 36 h post-dose. The dOFM devices (sterile probes, wearable pumps, accessories; CE certified for human use) were developed by HEALTH—Joanneum Research GmbH (Graz, Austria). The devices and their clinical use have been described in detail previously [23, 24]. In this study, the newest version of a CE-certified dOFM probe (DEA15003) was used. Like its precursors, this new dOFM probe is a highly flexible linear probe with a demarcated 15-mm fully permeable section, and it also has imprinted position markers to facilitate the precise positioning of the permeable section of the probe below the treatment area. The probe's outer diameter is 0.5 mm and it is inserted over a length of 30 mm using a standard 0.9-mm (outer diameter) hollow insertion needle.

2.4 Study Design

After enrollment and qualification of study subjects based upon the protocol inclusion and exclusion criteria, a set of three treatment sites (referred to as the 'test triad') was demarcated on each thigh as depicted in Fig. 2 (providing a total of six treatment sites per subject, each 5.5 cm^2). Twelve dOFM probes were inserted intradermally (two replicate probes per treatment site) and dermal interstitial fluid was continuously sampled at 1 µL/min using sterile perfusate that included 1 % albumin and 600 mg/dL glucose. The skin at a treatment site was cooled using a sterile ice bag prior to probe insertion as well as during the initial hour following probe insertion.

At baseline, the transepidermal water loss was measured on the skin of each leg (Aquaflux AF200; Biox Ltd, London, UK) and the baseline serum and dOFM samples were collected.

At $t = 0$, 15 mg cream/cm^2 of each of the *R* and *T* products were applied to the respective treatment sites on the skin using a tared spatula. The spreading procedure was of standardized duration (1 min) for all treatment sites and care was taken to consistently dose the cream as a homogenous layer. The *R* product was applied at two sites on each thigh (R_1 central, R_2 non-central) and *T* non-central (Fig. 2a) in a randomized order, using treatment randomization sequences of either 'R_2–R_1–T' or 'T–R_1–R_2' according to a randomization scheme that had been predefined in the study protocol to rule out the impact of anatomical location or procedural factors. Treatment sites were protected by a transparent, non-occlusive, dome-shaped, perforated plastic shield (Fig. 2b) and samples of the continuous perfusion from the dOFM probes in the dermis were collected at 4-h intervals, up to 36 h post-dose. Glucose in dOFM samples was measured at the bedside (Super GL; Dr. Müller Gerätebau GmbH, Freital, Germany) and the relative glucose loss from the perfusate (relative to 600 mg/dL in %) was calculated to monitor the exchange rate ('relative recovery') across the probe.

Study subjects rested in bed most of the time and slept in a supine position during the night. The sampling procedure during the night was identical to that used during the day. After termination of the study at 36 h, the intradermal position of each probe was assessed by a longitudinal ultrasound scan (GE LOGIQ e R6 device with linear 22 MHz probe; GE Healthcare, Vienna, Austria).

To reduce perturbations to the probes and to minimize variability in the kinetic data, any torsional strains or skin stretching from anatomical flexion at the test triads across 36 h were minimized through the use of self-adhesive stabilization rings (HEALTH-Joanneum Research GmbH). As additional controls to reduce experimental variability and optimize the precision and reproducibility of the study, room temperature and relative humidity were tightly controlled throughout the study (22 ± 1 °C, 40–60 % relative humidity).

2.5 Sample Analyses

Acyclovir determination: Samples (20 µL of perfusate plus D4-deuterated acyclovir internal standard) were processed by solid-phase extraction (Oasis MCX µElution plate; Waters, Milford, MA, USA). Samples were washed with formic acid/water (5 %/95 %, v/v) followed by methanol; eluted with NH_4OH/methanol (5 %/95 %, v/v); evaporated to dryness; and finally reconstituted in 20 µL methanol/water (5 %/95 %, v/v). High-performance liquid chromatography (HPLC) analysis of acyclovir in dOFM samples was performed with an Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA); ACQUITY-UPLC-HSS-T3 column (50 × 1.0 mm; Waters) using an isocratic method and a methanol/water (95 %/5 %, v/v) mobile phase with a flow rate of 300 µL/min and an injection volume of 3 µL. Acyclovir detection and quantification

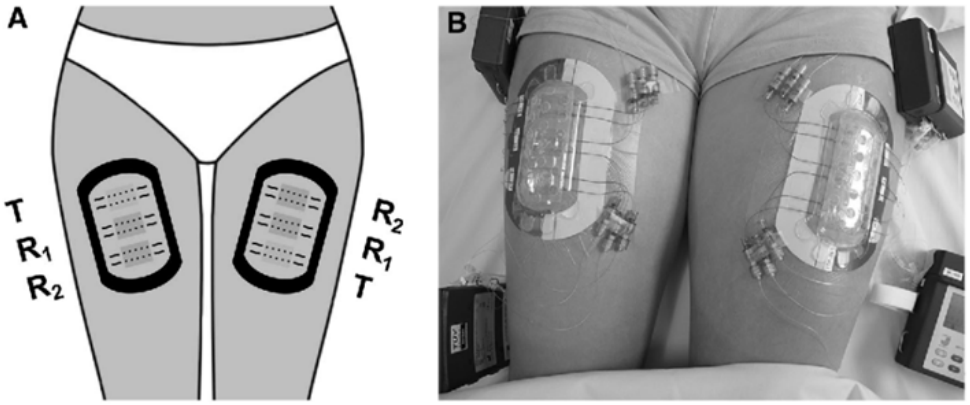

Fig. 2 Scheme (**a**) and photograph (**b**) of the duplicate test triad for comparative bioavailability assessment. Two treatment sites per test triad were dosed with the reference product (R_1: central, R_2: non-central) and one treatment site was dosed with the test product (T)

was performed by mass spectrometry (MS) using a Q-Exactive (Thermo) MS/MS in positive heated-electrospray mode (*m/z* 226.0935–152.05635 for acyclovir and 230.1196–152.05635 for the acyclovir-D4 internal standard).

2.6 Pharmacokinetic Data Evaluation

All clinical data were collected with an electronic case report form (eCRF) within OpenClinica Enterprise Edition. The eCRF system was hosted by Joanneum Research GmbH and is 21 CFR Part 11 compliant. All data management activities were performed and documented according to international standards and the data management plan.

The statistical analysis plan predefined data analysis, including criteria for the identification and treatment of possible outliers from intradermal 36-h concentration profiles. Possible outliers were identified by a statistical approach comparing each value with the moving median. Values that were <50 or >200 % of the moving median were reanalyzed and the reanalyzed values were used. Values that were still regarded as outliers according to this criterion were imputed by the arithmetic mean value of the preceding and subsequent time point samples, or by using the carry-last-value-forward approach in situations where there was no subsequent timepoint, to obtain complete profiles for PK endpoint calculation. The dermal PK endpoint AUC_{0-36h} was calculated as sum of the dOFM sample concentrations over 36 h of continuous sampling. The dermal PK endpoint C_{max} was identified as the maximum concentration during the same duration.

An exploratory pilot study in six subjects where an even higher topical acyclovir dose was applied over 36 h showed no detectable acyclovir in the central test sites when the central test sites were left untreated, and it also did not show any detectable acyclovir in serum. Therefore, there was no evidence of any potential for cross-talk between test sites and all 40 test triads were considered independent for BE analysis in this proof-of-principle study.

BE criteria were used to evaluate the dermal PK endpoints for the positive control for BE (R_2 vs. R_1) and the negative control for BE (T vs. R_1) based on the typical acceptance criteria for BE. The dermal PK endpoints AUC_{0-36h} and C_{max} were log transformed prior to analysis. The results were evaluated to determine whether the 90 % confidence interval of the mean ratios of *T*/*R* fell within the conventional BE limits of 0.80–1.25.

3 Results

3.1 dOFM Data Acquisition

All subjects tolerated the 36-h continuous dOFM sampling and no dropouts or serious adverse events occurred. No adverse event (or serious adverse event) related to the dOFM technique occurred during the study. Dermal sampling and the probe perfusion equilibrium were stable for 36 h, verified by stable glucose exchange rates (of approximately 60 %) across the probes. Applying the criterion for possible outliers within acyclovir profiles yielded a sample reanalysis rate of 3 %. After reanalysis, the rate of possible outliers was reduced to 1 % of all samples. All the acyclovir profiles (36 h each) of the 240 dOFM probes in the pivotal study (six probes per thigh on each of two thighs on each of 20 subjects) were included in the statistical evaluation. A characteristic steady decline in the later phase of the PK profile after C_{max}, which is usually seen in systemic blood concentration PK studies, when the rate of drug clearance from the systemic circulation dominates over the rate of drug input into the systemic circulation (e.g., from an oral dose), was not clearly observed for acyclovir in the dermis after topical administration in this study, even across 36 h of sampling (Fig. 3).

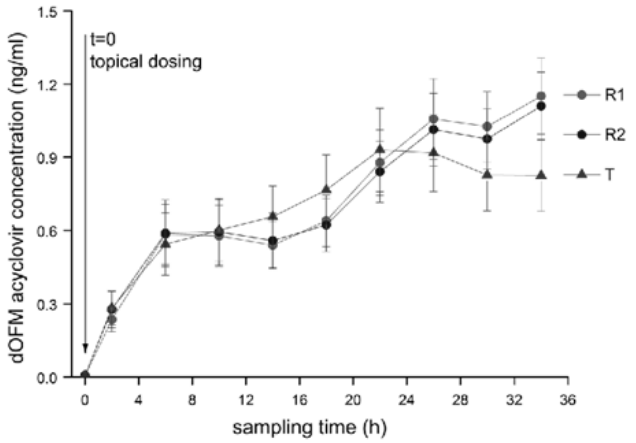

Fig. 3 Dermal open flow microperfusion (dOFM) acyclovir concentration profiles for the test product (*T*) site and the two reference (R_1 and R_2) sites (mean ± standard error of the mean, $n = 40$ test triads in 20 subjects). Acyclovir was analyzed from one pre-dose sample (spanning −1 to 0 h) and nine pooled post-dose samples (spanning 0–4, 4–8 … 32–36 h). The post-dose concentrations are plotted at the mid-point of the time intervals (2, 6 … 34 h)

3.2 Comparative BA

The statistical comparison of PK endpoints using BE criteria are summarized in Table 1. The mean (and geometric mean) results across treatment groups for each PK endpoint are provided in Table 2.

BE was confirmed for the positive control products (R_2 vs. R_1) for AUC_{0-36h} (0.86–1.18) and C_{max} (0.86–1.21). An exploratory statistical re-sampling procedure showed that $n = 36$ (18 subjects in this study design) would have been sufficient to demonstrate BE for R_2 vs. R_1 based on AUC_{0-36h} and $n = 38$ (19 subjects in this study design) would have been sufficient based on C_{max}.

The negative control products (*T* vs. R_1) failed to demonstrate BE for both parameters, AUC_{0-36h} (0.69–1.05) and C_{max} (0.61–1.02).

Consistent probe depths were confirmed for the *R* treatment sites (R_1: 0.83 ± 0.20 mm, R_2: 0.81 ± 0.22 mm, $p = 0.5329$). The mean probe depth (*T*: 0.73 ± 0.18 mm, $p = 0.0007$) was lower in the *T* treatment sites than it was in either *R* site, but a regression analysis indicated that there was no significant impact of probe depth on the AUC for acyclovir ($p = 0.1001$).

4 Discussion

This in vivo study illustrates the feasibility of dOFM to compare dermal PK and to assess BA of acyclovir from *T* and *R* topical creams in situ in the dermis. The results demonstrate that dOFM accurately and reproducibly determined the positive control products to be BE (R_2 vs. R_1) and that it had the sensitivity to differentiate the negative control products (*T* vs. R_1) as not being BE, in both cases based upon PK endpoints and the usual BE acceptance criteria.

The dOFM probes facilitated a stable intradermal sampling for 36 h from 4 *R* and 2 *T* treatment sites simultaneously in each subject, and thereby enabled characterization of the dermal PK profile for a relatively slow and low level of permeation of topical acyclovir. All subjects tolerated 36-h post-dose sampling well and no subject withdrew from the study prematurely. The positive subject compliance may be, in part, attributed to the compact wearable open-flow microperfusion devices, allowing enhanced mobility of the subjects. Bedside glucose analysis in dermal samples showed that exchange across the dOFM probe (the 'relative recovery') was stable over 36 h and thus indicated that skin cooling and the maintenance of an equilibrium period of 60 min post insertion was appropriate for this study.

dOFM delivered stable acyclovir profiles (<1 % outliers) and thereby demonstrated good reliability. Importantly, the high precision and reproducibility of the study results are attributable to several specific controls implemented to standardize and optimize study procedures, such as stabilization of the treatment sites to reduce strain and stretching, pain management during probe insertion to improve consistency and control of probe depth insertion, cooling after implementation to reduce trauma formation, and control of ambient temperature and humidity. Furthermore, several factors that were not necessarily amenable to better control, but which had a potential to influence the variability, were monitored so that their influence on variability could be evaluated and potentially corrected for: transepidermal water loss and impedance were monitored to evaluate potential subgroups in the population with skin permeability that may be more or less discriminating to differences in topical BA; glucose and deuterated water were monitored in the perfusate to identify any perturbation in the sampling environment; probe depth of the entire perfusate exchange area and flow rates of each time interval of each probe were monitored. The PK endpoint data in this study were not corrected by any of those factors.

Acyclovir products were chosen for this proof-of-principle dOFM study of comparative BA owing to the availability of 5 % cream products with different compositions and potentially different BA, and also because a dermal PK approach may have particular value to assess BE of topical products intended for local action rather than a clinical endpoint study in patients. The hydrophilicity of this drug, however, is not representative of most topical drugs and further research with hydrophobic drugs is warranted. The C_{max} was not seen for the reference product within 36 h,

Table 1 Statistical evaluation comparing PK endpoints using typical BE criteria ($n = 40$ test triads in 20 subjects)

Comparison	PK endpoint	90 % confidence interval	*T/R* (point estimate)	Outcome
R_2 vs. R_1	$AUC_{0-36\ h}$	0.86–1.18	1.01	Positive BE result Confirmed
	C_{max}	0.86–1.21	1.02	R_2 is considered BE to R_1
T vs. R_1	$AUC_{0-36\ h}$	0.69–1.05	0.85	Negative BE result Confirmed
	C_{max}	0.61–1.02	0.79	T is not considered BE to R_1

AUC area under the curve, *BE* bioequivalence, C_{max} maximum plasma concentration, *PK* pharmacokinetic, *R* reference, *T* test product

Table 2 Pharmacokinetic endpoints $AUC_{0-36\ h}$ and C_{max}

Product	$AUC_{0-36\ h}$ (ng h/mL)		C_{max} (ng/mL)	
	Mean ± SEM[a]	Geometric mean ± SEM[b]	Mean ± SEM	Geometric mean ± SEM
R_1	26.75 ± 3.85	16.23 ± 1.19	1.32 ± 0.18	0.85 ± 1.18
R_2	26.32 ± 4.07	16.34 ± 1.18	1.29 ± 0.17	0.86 ± 1.17
T	25.38 ± 4.52	13.84 ± 1.20	1.12 ± 0.19	0.67 ± 1.19

AUC area under the curve, C_{max} maximum plasma concentration, *R* reference *SEM* standard error of the mean, *T* test product

[a] Mean refers to the arithmetic mean and corresponds to the arithmetic mean profiles in Fig. 2

[b] Geometric mean refers to the geometric mean that was used in the statistical evaluation reported in Table 1

and the slow and ongoing permeation of acyclovir from both 5 % cream products produced a PK profile from which it was not possible to calculate the $AUC_{0-\infty}$. Hydrophilic drugs such as acyclovir may be poorly absorbed owing to a low partitioning through the skin's hydrophobic permeability barrier, the stratum corneum [26]. Low levels of percutaneous absorption and slow kinetics are among the reasons why prior studies using conventional dermal microdialysis, which were limited to a 5-h post-dose sampling duration, only succeeded to sample acyclovir when simultaneously inhibiting local skin blood flow [26, 27].

This study was intended to evaluate how accurately, precisely, and reproducibly dOFM could monitor the rate and extent to which a topically applied drug (acyclovir) becomes available in the dermis, and to compare the dermal BA of acyclovir between *T* and *R* creams administered using the same clinically relevant dose amount under essentially identical study conditions, using a single-dose study design. The labeled use of these acyclovir cream products involves a repeated dosing regimen throughout each day of use. Repeated dosing (e.g., every 4–5 h) is an effective strategy to maintain therapeutic drug concentrations at the site of action. However, differences in BA that might not be evident in a study where the products were re-dosed every 4 h may become evident in clinical use, where the product may not be re-dosed precisely every 4 h, particularly if one product dries more rapidly. In addition, a multiple dosing study design could obscure or convolute PK endpoints such as C_{max}, which were of interest to compare. Therefore, a single-dose study design was considered to be the most appropriate for the purposes of comparing acyclovir BA between the *T* and *R* products precisely because it may be more sensitive and discriminating than a multiple dosing study design. Indeed, it is possible that the differences in BA observed between the *T* and *R* products in this study may not be distinguishable in the clinical use of these products.

The application of dOFM accurately and reproducibly confirmed BE when acyclovir cream 5 % (Zovirax®) was compared with itself (R_2 vs. R_1). The statistical power estimation revealed that the sample size of $n = 40$ (20 subjects in this study) was well chosen, as $n = 38$ (19 subjects) would have been sufficient to demonstrate BE with a confidence level above 80 %. Both PK endpoints for the *T* vs. *R* product comparison failed to satisfy the conventional criteria for BE, demonstrating that dOFM was sufficiently sensitive to discriminate between the *T* and *R* products.

5 Conclusion

In this study, dOFM showed low variability and high robustness, successfully characterizing the dermal BA of acyclovir from *T* and *R* products in a manner that could support evaluations of BE for topical acyclovir cream 5 %

products in vivo with $n = 40$ (20 subjects in the independent replicate leg design used for this study). Factors contributing to variability of in vivo dermal PK data were well controlled in this clinical study, which is the first to evaluate clinical dOFM for comparative dermal BA/BE assessment.

The results of this study suggest that an appropriately designed and well-controlled in vivo dOFM study could have the requisite accuracy, precision, reproducibility, and statistical power to compare the rate and extent to which a topically applied drug such as acyclovir becomes available in the dermis and that dOFM may provide a viable dermal PK approach for the BE assessment of topical drug products.

In the future, the sensitivity of dOFM may be even further improved for compounds permeating the skin more rapidly and to a greater extent than acyclovir, by refinements in subject inclusion/exclusion criteria, by correcting for perturbations to the dermal sampling caused by changes in blood flow or variations in probe depth, or by choosing shorter dose durations and monitoring the dermal PK for the T and R products during the period when the drug is being cleared from the skin. Further research exploring dOFM and other cutaneous PK methodologies to evaluate topical BA/BE is warranted based upon the results of this study.

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Compliance with Ethical Standards

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Conflict of interest MB, KIT, RR, BT, TA, CD, TB, SK, DS, TRP, and FS are employees of Joanneum Research holding patents on OFM devices. SGR is an employee of US FDA. GS and IK declare that there are no conflicts of interest.

Ethics, informed consent, research involving human participants The study was conducted with the full informed consent of all participating subjects, under the authority of the Ethical Committee of the Medical University of Graz, the Austrian health authority AGES, and the FDA's Research Involving Human Subjects Committee (RIHSC) and was performed in accordance with Good Clinical Practice and the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The study has been registered in the European Clinical Trials Register (EudraCT No. 2013-005062-19) and at ClinicalTrials.gov (NCT02711267).

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β-Defensin 2 is a responsive biomarker of IL-17A–driven skin pathology in patients with psoriasis

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Background: IL-17A is a key driver of human autoimmune diseases, particularly psoriasis.
Objective: We sought to determine the role of IL-17A in psoriasis pathogenesis and to identify a robust and measurable biomarker of IL-17A–driven pathology.
Methods: We studied 8 healthy subjects and 8 patients with psoriasis before and after administration of secukinumab, a fully human anti–IL-17A mAb, and used a combination of classical techniques and a novel skin microperfusion assay to evaluate the expression of 170 proteins in blood, nonlesional skin, and lesional skin. For validation, we also tested stored sera from 601 patients with a variety of autoimmune diseases.
Results: IL-17A was specifically expressed in lesional compared with nonlesional psoriatic skin (9.8 vs 0.8 pg/mL, $P < .001$). Proteomic and gene transcription analyses revealed dysregulated antimicrobial peptides, proinflammatory cytokines, and neutrophil chemoattractants, levels of which returned to normal after treatment with secukinumab. β-Defensin 2 (BD-2) was identified as a biomarker of IL-17A–driven pathology by comparing protein expression in patients with psoriasis versus that in healthy subjects (5746 vs 82 pg/mL in serum, $P < .0001$; 2747 vs <218 pg/mL in dermis, $P < .001$), responsiveness to secukinumab therapy, and synergistic induction by IL-17A and TNF-α in epidermal keratinocytes. In a validation set of sera from 601 patients with autoimmune diseases thought to be IL-17A driven, we found that BD-2 levels are most highly increased in patients with psoriatic skin lesions, and in patients with psoriasis, BD-2 levels correlated well with IL-17A levels ($r = 0.70$, $n = 199$, $P < .001$) and Psoriasis Area and Severity Index scores ($r = 0.53$, $n = 281$, $P < .001$).
Conclusion: IL-17A is a primary driver of skin pathology in patients with psoriasis, and serum BD-2 is an easily measurable biomarker of IL-17A–driven skin pathology. (J Allergy Clin Immunol 2017;139:923-32.)

Key words: *IL-17, psoriasis, secukinumab, β-defensin 2, biomarker, dermal interstitial fluid, microperfusion, psoriatic arthritis, ankylosing spondylitis, rheumatoid arthritis, multiple sclerosis, autoimmunity*

T_H17 cells and the IL-17 family of cytokines are key drivers of autoimmune diseases, such as psoriasis, psoriatic arthritis (PsA), ankylosing spondylitis (AS), rheumatoid arthritis (RA), and multiple sclerosis (MS).[1] Psoriasis is the best studied of these diseases, with multiple interventions targeted at T_H17 cells and their cytokines showing efficacy in its treatment.

The IL-17 family of cytokines includes 6 members (IL-17A to IL-17F) that function as homodimers or heterodimers to signal through a family of cytokine receptors with multiple chains (IL-17RA to IL-17RE), with IL-17A and IL-17F signaling through a receptor complex composed of IL-17RA and IL-17RC.[2,3] Circulating levels of IL-17A protein have been shown to be higher in patients with psoriasis than in healthy control subjects and correlate with disease severity,[4] whereas mRNA expression of the *IL17A*, *IL17C*, and *IL17F* genes is higher in psoriatic lesional tissue than nonlesional tissue.[5-7] Indeed, therapies that target either IL-17A or IL-17RA are similarly highly effective in reducing disease in patients with psoriasis.[1,8-11] Although these numerous lines of evidence implicate an important role for IL-17A, a nonredundant role for IL-17F has not been precluded.

Much of our current understanding of psoriasis pathophysiology and the effects of IL-17A blockade has largely been based on studies evaluating gene expression from skin biopsy specimens.[6,12-14] Although these studies have provided significant insights into the pathways that might be involved in IL-17A–mediated pathogenesis, they are limited by the frequent incongruence between changes in mRNA and protein expression. Furthermore, they do not quantify the early microanatomically specific changes in protein expression, in particular large proteins,

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Abbreviations used
AS: Ankylosing spondylitis
BD-2: β-Defensin 2
CD: Crohn disease
dISF: Dermal interstitial fluid
GM: Geometric mean
IHC: Immunohistochemistry
LLOQ: Lower limit of quantification
MMP: Matrix metalloproteinase
MS: Multiple sclerosis
qRT-PCR: Quantitative RT-PCR
PASI: Psoriasis Area and Severity Index
PsA: Psoriatic arthritis
RA: Rheumatoid arthritis
RQ: Relative quantity

such as cytokines and chemokines. To date, protein expression response to therapy in psoriatic skin has been mostly demonstrated on a qualitative rather than quantitative level through the use of histology and immunohistochemistry (IHC).[12,14-16]

To increase our understanding of the inflammatory pathophysiology in psoriatic skin at a protein expression level and to identify a protein biomarker of IL-17A–mediated pathology, we conducted an exploratory study in 8 healthy subjects and 8 patients with psoriasis to quantify and compare the soluble proteomic profiles of lesional versus nonlesional skin before and after single-dose systemic treatment (300 mg administered subcutaneously) with secukinumab (a fully human anti–IL-17A mAb with a half-life of approximately 27 days) and/or healthy skin. Dermal open flow microperfusion, a novel technique that provides minimally invasive access to dermal interstitial fluid (dISF), was used to sample the dermis.[17-19] Dermal (dISF) samples were then analyzed for 170 proteins encompassing cytokines, chemokines, growth factors, cell adhesion molecules, and soluble receptors. Early proteomic changes that occur within the dermis after secukinumab treatment were complemented with gene expression changes extracted from full skin biopsy specimens. Our aim was to identify which pathways might be central to psoriasis pathology in skin and whether the antimicrobial peptide β-defensin 2 (BD-2) might be a protein biomarker of IL-17A–mediated pathology.

METHODS

Study design

This single-center clinical study was conducted according to ethical principles and good clinical practice at the Medizinische Universität in Graz, Austria, after being approved by the ethics committee and Austrian health authority. Enrolled patients needed to sign informed consent forms and comply with the protocol requirements (ClinicalTrials.gov Identifier NCT01539213).

We enrolled 8 healthy subjects (mean age, 26.1 years) and 8 patients with psoriasis (mean age, 38.8 years). All 16 subjects were white, and all patients with psoriasis were male, as were 6 healthy subjects. All subjects received a single 300-mg subcutaneous injection of secukinumab on study day 1. Skin biopsy specimens (4-mm skin punch), serum samples, and dermal samples were obtained on study days 1 (before secukinumab treatment), 8 and 15 (7 and 14 days after secukinumab treatment). Details of the dermal sampling methodology were described previously.[19]

In addition, baseline serum from patients with various inflammatory diseases sampled in multicenter trials conducted after ethics committee and health authority approval have been analyzed. Sera analyzed were from 289 patients with psoriasis (NCT01539213 and NCT00941031), 37 patients with PsA (NCT00809614), 56 patients with AS (NCT00809159), 68 patients with MS (NCT01051817), 94 patients with RA (NCT01426789), and 57 patients with Crohn disease (CD; NCT00584740).

Protein and RNA measurements

Free IL-17A and IL-17F levels in serum and dISF were quantified by using microparticle-based fluorescent sandwich immunoassays based on Erenna technology validated in human serum (Singulex IL-17A Human Immunoassay Kit, catalog no. 03-0017-05; Singulex IL-17F Human Immunoassay Kit, catalog no. 03-0018-03; Singulex, Alameda, Calif). The lower limit of quantification (LLOQ) for IL-17A was 0.64 pg/mL in dISF and 0.096 pg/mL in serum, and that for IL-17F was 96.6 pg/mL in dISF and 14.4 pg/mL in serum. BD-2 was quantified by using ELISA (Alpha Diagnostic, catalog no. 100-250-BD-2). The LLOQ for BD-2 was 218 pg/mL in dISF and 32.5 pg/mL in serum. Sinistrin served as a reference substance and was used to estimate the absolute concentrations of biomarkers in dISF.[19]

The chemiluminescent multiplex enzyme immunoassay platform from Aushon BioSystems (Billerica, Mass) was used to profile and quantify the levels of 170 proteins distributed over 43 panels. The panel of 170 proteins encompasses cytokines, cytokines receptor, chemokines, cell adhesion molecules, angiogenesis factors, matrix metalloproteinases (MMPs), growth factors, and neurotrophic factors (see Table E1 in this article's Online Repository at www.jacionline.org).

Total RNA was isolated from skin biopsy specimens by using the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA isolated from commercial skin biopsy specimens (Asterand UK, Royston, United Kingdom) from healthy subjects (n = 10) served as controls for gene expression data. Gene expression analysis was done by using quantitative RT-PCR (qRT-PCR) or NanoString technology (NanoString Technologies, Seattle, Wash). See the Methods section and Table E2 in this article's Online Repository at www.jacionline.org for further details.

Cell culture

Human primary skin cells were obtained from PromoCell (Heidelberg, Germany). Epidermal keratinocytes (adult, pooled donors, passage 5, 30,300 cells/cm^2), dermal fibroblasts (adult, single donor, passage 6, 30,300 cells/cm^2), and dermal microvascular endothelial cells (juvenile, pooled donors, passage 5, 22,700 cells/cm^2) were seeded onto 96-well plates and incubated overnight. Thereafter, cells were incubated for another 20 hours in medium alone or 0.03 to 960 ng/mL IL-17A in the absence or presence of 1 ng/mL TNF-α, 0.03 to 960 ng/mL IL-17F in the absence or presence of 1 ng/mL TNF-α, or 0.03 to 960 ng/mL TNF-α. Supernatants were examined for the release of BD-2.

Statistical analysis

For the skin microperfusion study, log-transformed baseline (day 1) IL-17A and IL-17F protein levels were analyzed by using a mixed effects model with site (healthy volunteer or patient for serum levels and lesional or nonlesional psoriatic skin for skin levels) as a fixed effect and subject as a random effect, respectively. The adjusted geometric means (GMs) at each site were provided on the original scale together with the *P* values for comparisons between sites. Pearson correlation coefficients (denoted by *r*) between baseline protein levels (IL-17A and IL-17F) and efficacy scores (Psoriasis Area and Severity Index [PASI] score) were calculated together with the corresponding *P* values by site. Log-transformed baseline BD-2 levels in serum and skin were also analyzed by using a mixed effects model with site (healthy volunteer or patient for serum levels and healthy and lesional or nonlesional psoriatic skin for skin levels) and subject as a random effect, respectively. The adjusted GMs at each site were provided on the original scale together with the *P* values for comparisons between sites. IL-17F levels in serum from patients with psoriasis and BD-2 levels over time in serum and skin were log-transformed and analyzed by site by using a mixed effects model with visit day (baseline, day 8, and day 15) as a fixed effect and subject as a random effect, respectively. The adjusted GMs and 95% CIs at each visit

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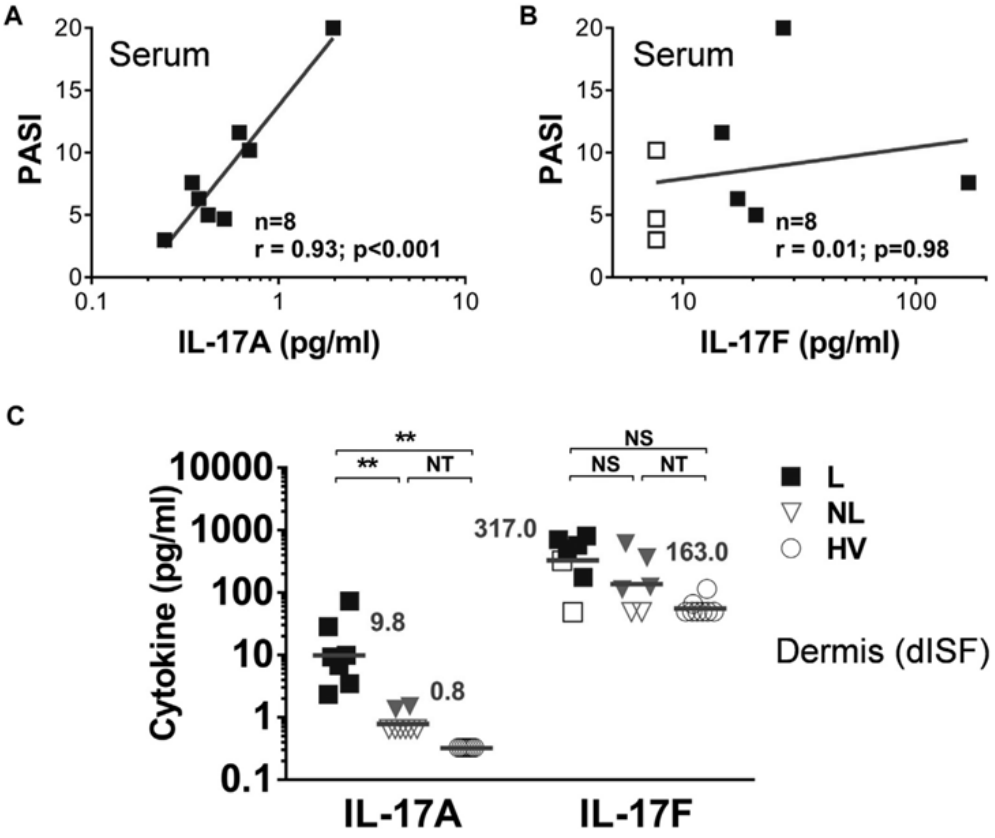

FIG 1. Baseline dermal protein levels of IL-17A are significantly increased in lesional versus nonlesional psoriatic skin and correlate with disease activity (PASI). **A** and **B,** Correlation plots of PASI scores versus IL-17A levels (Fig 1, *A*) and PASI scores versus IL-17F levels (Fig 1, *B*). A linear regression fit is depicted by a *red line,* the strength of the linear relationship is indicated by using the Pearson correlation coefficient *r, n* indicates the number of values on each plot, and the *P* value shows the probability that the slope of the true relationship is zero. **C,** Baseline IL-17A and IL-17F levels in the dermis (dISF) of healthy volunteers (*HV, circles*) and lesional (*L, squares*) and nonlesional (*NL, triangles*) skin from patients with psoriasis. *Red lines* and values represent the adjusted GMs and are adjusted for relative recovery of the reference substance sinistrin (Dragatin et al[19]). Data less than the LLOQ were imputed as half LLOQ and are shown as *open symbols.* ***P* < .01. *NS,* Not significant (*P* > .05); *NT,* not testable because of the number of samples less than the LLOQ in both groups.

were provided on the original scale together with *P* values for comparisons between visits. Pearson correlation coefficients between BD-2 levels and efficacy (PASI) scores were calculated together with the corresponding *P* values by site and visit. Any values less than the LLOQ (note: values less than the LLOQ differ for some samples because of a different sample dilution factor applied) were imputed by half the LLOQ.

In the secukinumab dose regimen study in patients with moderate-to-severe psoriasis (NCT00941031), serum samples were taken and analyzed for BD-2, IL-17A, and IL-17F. Correlations between PASI scores and BD-2, IL-17A, and IL-17F levels were investigated by using a Pearson correlation coefficient. BD-2, IL-17A, and IL-17F values were log-transformed before analysis. Values of less than the LLOQ were not included in the calculations.

Analyses were performed with PROC MIXED and PROC CORR in SAS software, version 9.4 (SAS Institute, Cary, NC).

RESULTS

Baseline IL-17A protein levels are increased in lesional versus nonlesional skin of patients with psoriasis

To understand the relative roles of IL-17A and IL-17F in psoriasis, we evaluated protein levels in the circulation (serum) and skin (dISF) of the 16 subjects in our study (NCT01539213). Consistent with previous studies, baseline serum IL-17A protein levels were higher in patients with psoriasis than in healthy subjects (GM: 0.5 vs 0.2 pg/mL, respectively; $P < .05$). Posttreatment serum levels of free IL-17A could not be assessed because the IL-17A assay used measures not only free IL-17A but also IL-17A bound to secukinumab. No validated assay exists to measure free IL-17A in the presence of secukinumab. Serum IL-17F levels in patients with psoriasis (17.5 pg/mL) did not change significantly at day 15 from baseline after secukinumab (14.9 pg/mL, P = not significant). At baseline, serum IL-17A protein levels correlated well with PASI scores ($r = 0.93$, $P < .001$; Fig 1, *A*), whereas baseline IL-17F levels did not ($r = 0.01$, $P = .98$; Fig 1, *B*).

Dermal (dISF) levels of IL-17A protein at baseline were significantly higher in lesional than nonlesional psoriatic skin (GM: 9.8 vs 0.8 pg/mL, respectively; $P < .01$; Fig 1, *C*). IL-17A was not detectable in the normal skin of healthy subjects (less than the LLOQ: <0.64 pg/mL). In contrast, baseline IL-17F levels in dISF were not significantly different between lesional and nonlesional skin, although IL-17F levels tended to be higher in lesional than nonlesional skin (GM: 317 vs 163 pg/mL, respectively; $P = .105$; Fig 1, *C*). IL-17F protein levels were

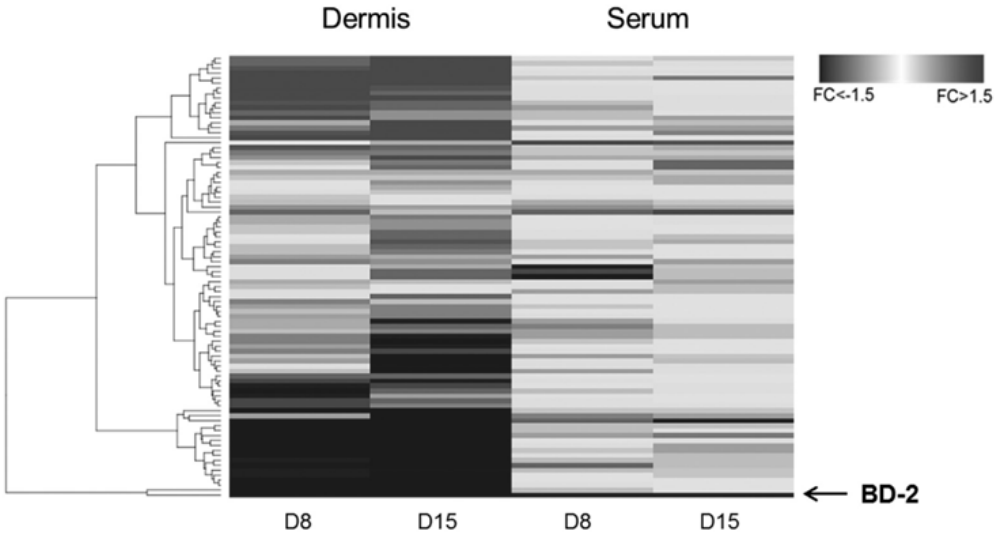

FIG 2. Proteomic analysis of dermis (dISF) and serum from patients with psoriasis after IL-17A blockade reveals early molecular changes and identifies BD-2 as a potential IL-17A response biomarker. Heat map of 89 proteins with at least a 1.5-fold change *(FC)* at either day 8 or day 15 compared with baseline in the dermis (dISF) or circulation (serum) of patients with psoriasis treated at day 1 with secukinumab. Dermal samples were taken from lesional skin. Hierarchical clustering was performed on the protein concentration data to show patterns of expression of related proteins in different compartments at 2 time points. *Blue* represents downregulation from baseline, and *red* indicates upregulation.

not measurable in the normal skin of healthy subjects (less than the LLOQ of 96.6 pg/mL; Fig 1, *C*). Transcriptional analysis of IL-17 family members (IL-17A to IL-17F) in skin biopsy specimens confirmed these findings by demonstrating upregulation of mRNAs for IL-17A, IL-17F, IL-17C, and potentially IL-17E (IL-25) in psoriatic lesional skin compared with healthy skin, whereas expression of IL-17B was unchanged and that of IL-17D was reduced (see Fig E1 in this article's Online Repository at www.jacionline.org). Consistent with changes in protein levels, *IL17A* and *IL17F* mRNA levels were reduced after secukinumab treatment (see Fig E1).

Proteomics screening of serum and dISF for IL-17A–responsive markers

To identify potential markers of IL-17A response proteins, we used a hypothesis-free proteomics screening approach measuring 170 proteins in dISF and serum before and after secukinumab treatment. By using a 1.5-fold change from baseline as a threshold, 89 proteins were shown to be dysregulated in dISF, serum, or both after treatment with secukinumab (Fig 2). The 10 proteins showing the greatest fold decrease were antimicrobial peptides (BD-2 and lipocalin-2 [NGAL or LCN2]), MMPs (MMP-1 and MMP-8), IL-1 pathway members (IL-1β and IL-1ra), neutrophil proteins (myeloperoxidase), and neutrophil-attracting (growth-regulated alpha protein [GRO-α] or CXCL1 and epithelial cell–derived neutrophil-activating peptide 78 [ENA-78] or CXCL5) or T_H17 cell–attracting (macrophage inflammatory protein 3α or CCL20) chemokines (Table I). Levels of other chemokines, including GRO-γ/CXCL3 and I-309/CCL1, were also decreased after secukinumab. We could not detect a change in TNF-α levels. The 5 proteins showing the greatest fold increase were T_H2 cell–associated proteins (IgE and eotaxin-2/CCL24), adiponectin (Acrp-30), leptin, and endoglin. An early downregulation of the antimicrobial peptides BD-2 (encoded by the gene *DEFB4A*), BD-3 *(DEFB103B)*, lipocalin-2 *(LCN2)*, LL-37 *(CAMP)*, *S100A8*, and *S100A9*, as well as neutrophil- and T_H17-attracting chemokines (*CXCL1*, IL-8/*CXCL8*, and *CCL20*, respectively) and the IL-1 family members IL-36α *(IL36A)*, IL-36β *(IL36B)*, IL-36γ *(IL36G)*, and IL-36RN *(IL36RN)*, was also observed on the mRNA level (see Figs E2-E4 in this article's Online Repository at www.jacionline.org), thereby confirming and extending observations made on the protein level. BD-2 (also known as skin antimicrobial peptide 1) was the protein showing the highest fold change in both dermis and serum and was selected for further study.

BD-2 levels correlate with IL-17A and psoriasis clinical severity and decrease after IL-17A blockade

BD-2 levels were further and more specifically quantified by using ELISA in skin (dISF) and serum before and after secukinumab treatment (Fig 3). At baseline, serum levels of BD-2 protein were significantly higher in patients with psoriasis than in healthy subjects (GM: 5746 vs 82 pg/mL, respectively; $P < .001$; Fig 3). Serum BD-2 levels decreased rapidly in patients with psoriasis after a single 300-mg subcutaneous dose of secukinumab (GM: 971 pg/mL at day 8 and 649 pg/mL at day 15; $P < .0001$ vs baseline for both time points). Dermal (dISF) BD-2 levels were also significantly higher in lesional skin than in nonlesional psoriatic skin at baseline (GM: 2747 vs 417 pg/mL, respectively, $P < .001$; Fig 3). In contrast, dermal BD-2 levels in healthy subjects were all less than the LLOQ ($P < .001$ vs lesional skin in patients with psoriasis, Fig 3). After secukinumab treatment, mean BD-2 levels in lesional skin decreased by 80% at day 8 and approached levels close to the LLOQ at day 15 after treatment with secukinumab (GM: 550 pg/mL at day 8 [$P < .05$] and 196 pg/mL at day 15 [$P < .01$], Fig 3). Thus BD-2 in both serum and dISF is responsive to IL-17A inhibition.

To confirm that IL-17A mediates BD-2 expression in the skin, we tested cytokine-stimulated BD-2 expression in epidermal

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TABLE I. Proteins with the greatest fold change and selected proteins of interest in skin (dermis) or circulation (serum) of patients with psoriasis after treatment with a single dose of the IL-17A inhibitor secukinumab

	Fold change relative to baseline			
	Dermis (dISF)		Serum	
Protein	Day 8	Day 15	Day 8	Day 15
Top 10 downregulated				
BD-2	−18.73	−32.20	−3.95	−3.66
MMP-1	−6.20	−15.19	−1.11	1.04
IL-1β	−2.71	−5.47	1.14	1.14
IL-1 receptor antagonist (IL-1ra)	−2.19	−4.37	−1.47	−2.32
MMP-8	−1.91	−3.42	−1.16	−1.07
Myeloperoxidase	−1.18	−3.20	−1.27	−1.18
CXCL1 (GRO-α, *CXCL1*)	−2.63	−3.13	−1.08	−1.17
Lipocalin-2 (NGAL, *LCN2*)	−2.14	−2.98	−1.11	−1.12
CCL20 (Macrophage inflammatory protein 3α, *CCL20*)	−2.62	−2.64	−1.24	1.45
CXCL5 (ENA-78, *CXCL5*)	−3.00	−2.50	1.05	−1.02
Other proteins of interest				
CXCL3 (GRO-γ, *CXCL3*)	−1.61	−2.20	−1.16	−1.08
CCL1 (I-309, *CCL1*)	−1.34	−1.88	1.09	1.03
TNF-α	1.00	1.18	1.04	1.03
Top 5 upregulated				
Endoglin	2.51	2.52	1.04	1.08
Leptin	2.59	2.62	1.09	1.39
Adiponectin (Acrp-30)	1.50	2.72	1.13	−1.04
Eotaxin-2 (*CCL24*)	1.56	2.77	1.06	1.14
IgE	1.92	3.19	−1.00	−1.06

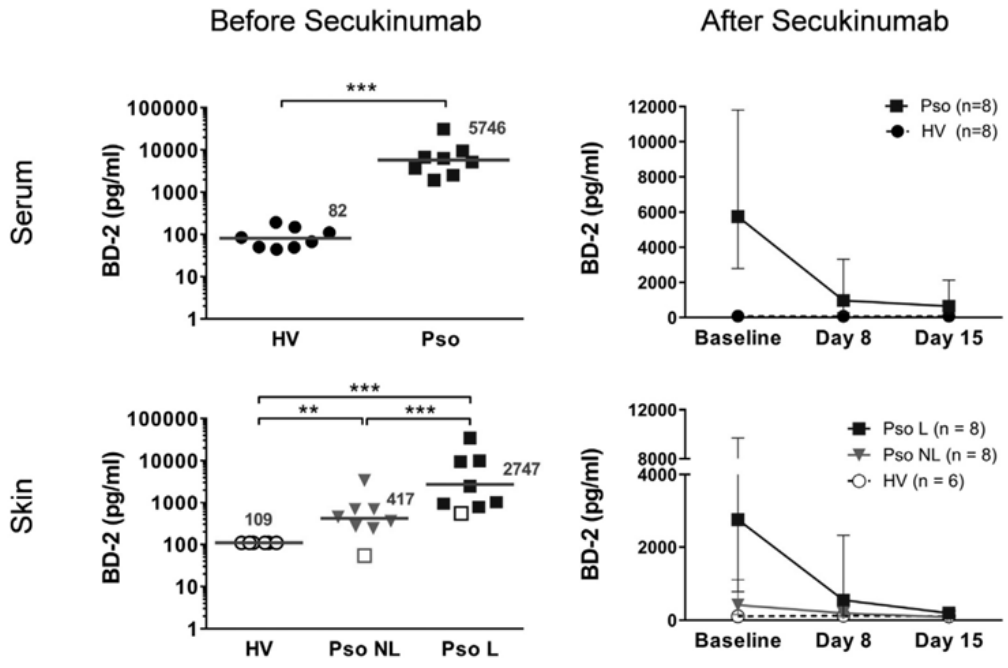

FIG 3. BD-2 protein levels are increased in patients with psoriasis and rapidly decrease in dermis and serum after secukinumab treatment. *Upper panel, left,* Baseline human BD-2 levels in the serum of healthy volunteers *(HV)* and patients with psoriasis *(Pso). Upper panel, right,* Serum BD-2 levels after a single dose of 300 mg of secukinumab administered subcutaneously (GM ± 95% CI). *Lower panel, left,* Baseline BD-2 levels in the dermis (dISF) of lesional *(Pso L)* and nonlesional *(Pso NL)* skin from patients with psoriasis and skin from healthy subjects *(HV). Lower panel, right,* Dermal BD-2 levels after a single dose of 300 mg of secukinumab administered subcutaneously (GM ± 95% CI). *Red horizontal bars* depicts the GM concentration. All data less than the LLOQ (218 pg/mL for healthy volunteers and 109 pg/mL for patients with psoriasis) were imputed as half LLOQ and are shown as *open symbols.* $^{**}P < .01$ and $^{***}P < .001$.

keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells. Of the 3 cell types, only epidermal keratinocytes produced BD-2 (Fig 4). Although IL-17A, IL-17F, and TNF-α all induced low levels of BD-2 at high concentrations, the combination of IL-17A and TNF-α was synergistic and induced high levels of BD-2 expression. Thus epidermal

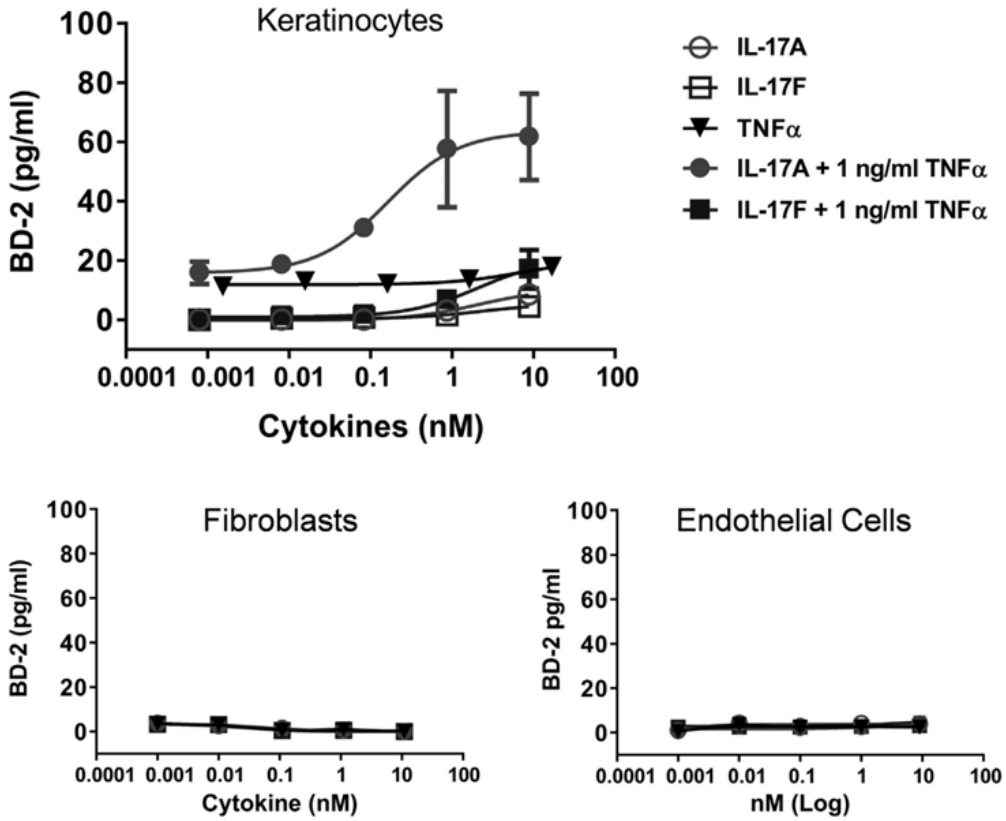

FIG 4. BD-2 expression by different primary human skin cell types. Levels of BD-2 (means ± SDs, n = 3) in supernatants of either epidermal keratinocytes, dermal fibroblasts, or dermal microvascular endothelial cells stimulated with different concentrations of human IL-17A, IL-17F, or TNF-α alone or IL-17A and IL-17F in combination with a low concentration of TNF-α.

keratinocytes are the IL-17A–responsive skin cell type for BD-2 production.

To determine whether BD-2 could be a marker of psoriasis disease activity, we compared BD-2 serum levels with PASI scores. The clinical efficacy of secukinumab has been previously documented, and similarly in this study, the mean PASI score decreased by 62.5% at day 22 after a single dose of 300 mg of secukinumab administered subcutaneously ($P < .0001$ vs baseline). BD-2 levels also decreased in correlation with reductions in clinical disease severity (PASI scores at baseline, day 8, and day 15; $r = 0.87$-0.89; $P < .01$). These data were confirmed by analyzing stored baseline serum samples from a second, larger phase 2b study in patients with psoriasis (NCT00941031).[20] In this validation cohort there was a significant correlation between serum BD-2 and IL-17A levels (n = 199; $r = 0.70$, $P < .001$, Fig 5), as well as serum BD-2 levels and PASI disease activity (n = 281, $r = 0.53$, $P < .001$; Fig 5). Thus serum BD-2 levels correlate with both serum IL-17A levels and psoriasis disease activity. Furthermore and in line with the close correlation of serum levels of BD-2 with IL-17A, serum levels of IL-17A show a significant correlation with psoriasis disease activity (n = 206, $r = 0.44$, $P < .001$; Fig 5). Little correlation was observed for IL-17F with either psoriasis disease activity (n = 177, $r = 0.15$, $P < .05$; Fig 5) or serum BD-2 levels (n = 173, $r = 0.15$, $P < .05$; Fig 5).

Because IL-17A levels are increased and IL-17A is a pathogenic driver in multiple autoimmune diseases, we sought to determine whether BD-2 could be a marker of IL-17A activity in patients with diseases other than psoriasis. We analyzed BD-2 levels in stored baseline serum (n = 601) from previous clinical studies in several autoimmune diseases. BD-2 levels were highly increased in the sera of patients with diseases having skin involvement, such as psoriasis and PsA, and only at moderate levels in those with diseases with minimal to no skin involvement, such as AS, RA, MS, and CD (Fig 6). Although previous or current psoriatic inflammation of the skin is required for the diagnosis of PsA, many patients with PsA do not have detectable skin lesions at the time of evaluation. Thus we evaluated whether BD-2 levels were different in subjects with or without ongoing skin involvement and found that BD-2 levels were higher in those patients with active skin involvement compared with those who did not (GM: 1493 vs 454 pg/mL, $P < .01$). We also assessed IL-17A and IL-17F levels in patients with psoriasis, PsA, and AS and found that they were expressed at similar levels in PsA patients with or without skin involvement (Fig 6). Thus BD-2 appears to be a biomarker of IL-17A pathogenesis in the skin.

DISCUSSION

In this study our aims were to (1) better understand the role of IL-17A in psoriasis pathogenesis by defining the early effects of IL-17A blockade on inflammatory proteins in both serum and the dermis and (2) identify soluble biomarkers of IL-17A pathway activation.

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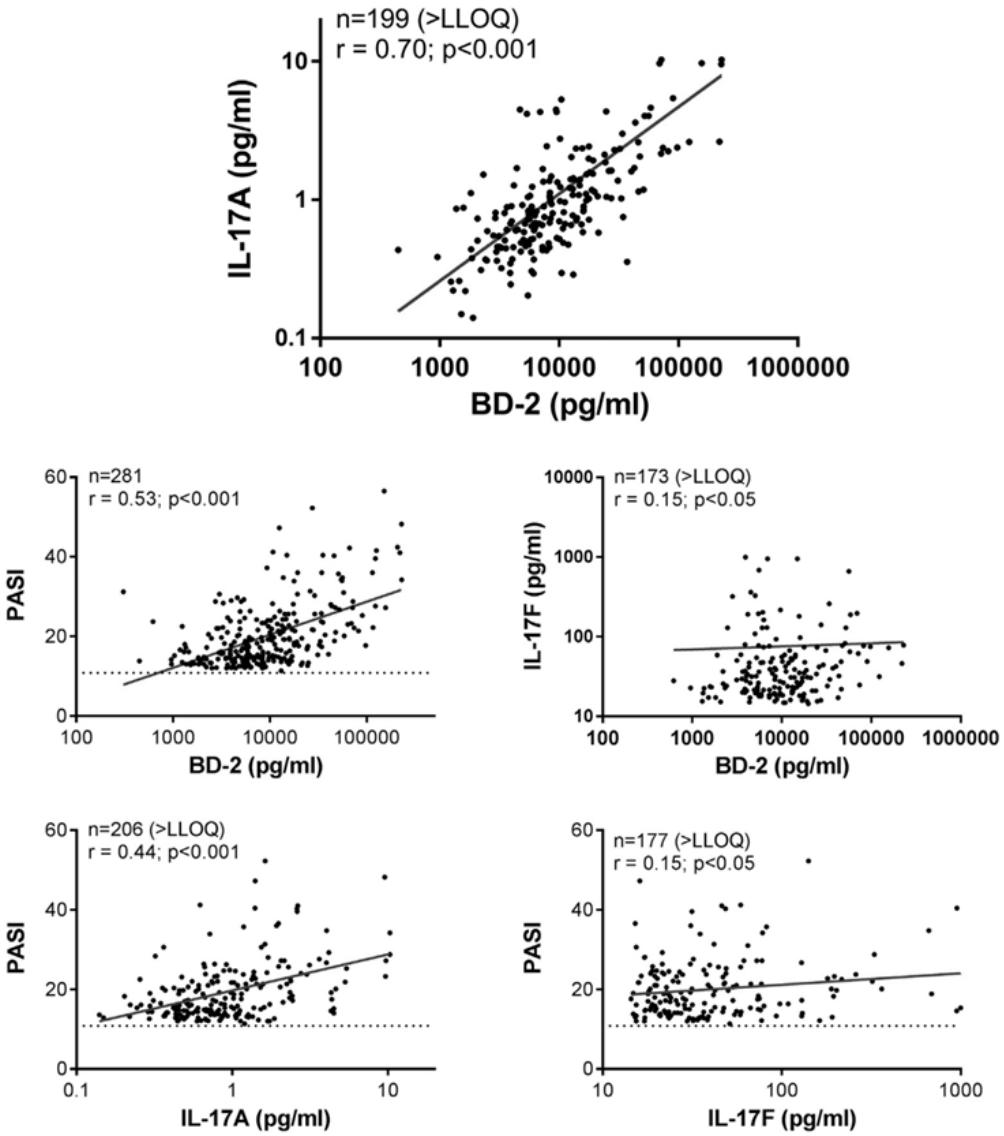

FIG 5. Baseline serum BD-2 levels correlate with IL-17A levels and disease activity (PASI scores). Correlation plots of PASI scores and protein levels from stored samples from the AIN457 Regimen Finding Study in Patients with Moderate to Severe Psoriasis (ClinicalTrials.gov identifier: NCT00941031). *Upper panel,* IL-17A level versus BD-2 level. *Middle panel, left,* PASI score versus BD-2 level. *Middle panel, right,* IL-17F level versus BD-2 level. *Lower panel, left,* PASI score versus IL-17A level. *Lower panel, right,* PASI score versus IL-17F level. In each plot the *red line* is from a linear regression fit, the strength of the linear relationship is indicated by using the Pearson correlation coefficient *r*, and *n* indicates the number of values on each plot. The *P* value is from a test that the slope of each linear regression line equal zero; the smaller the *P* value, the less likely it is that the slope of the true relationship equals zero.

Previous studies quantifying skin gene expression by using transcriptomics or qualitatively assessing protein expression by means of IHC before and after anti–IL-17A therapy have provided important information,[12,13] but the conclusions are limited by the often discrepant timing and levels of gene and protein expression. This might be of particular relevance because proteins are the final effector molecules in biological systems and significant differences to their corresponding mRNA levels can exist.[21] To quantify soluble skin proteins involved in psoriasis pathogenesis, we used dermal open flow microperfusion.[18]

As predicted based on IHC and mRNA transcription data, we found high IL-17A protein levels in lesional skin from patients with psoriasis. Similarly and consistent with previous[12,13] and our own mRNA transcription data, we could not detect either IL-17A or IL-17F protein in skin of healthy volunteers. Interestingly, we found detectable but lower IL-17A protein levels in nonlesional skin of patients with psoriasis (2/8 tested), suggesting that nonlesional skin is not normal and has a low level of ongoing IL-17A pathway activation. In the serum we confirmed previous data, now using a highly sensitive and validated assay, that IL-17A levels in our small study of 8 subjects are increased in patients with psoriasis and correlate with disease activity. Although IL-17F protein levels in patients with psoriasis were much higher than IL-17A levels, they were not consistently upregulated in lesional versus nonlesional skin in all psoriasis plaques and did not

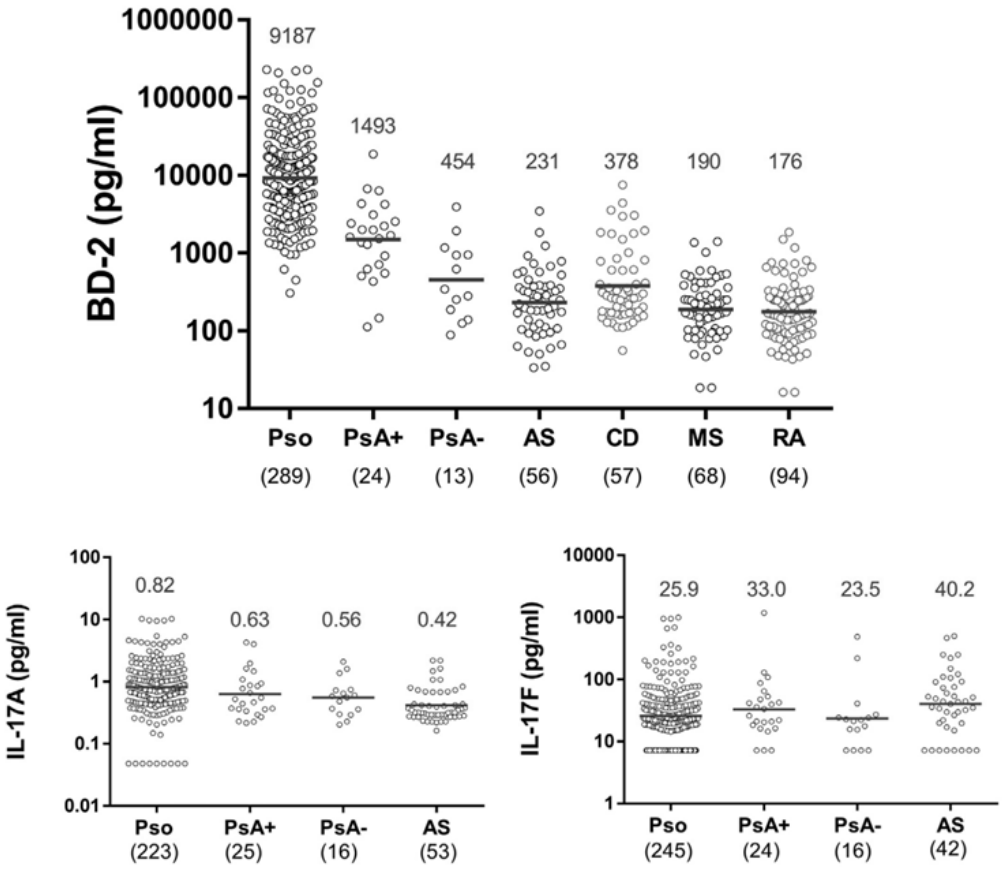

FIG 6. Baseline BD-2, IL-17A, and IL-17F serum levels in patients with autoimmune diseases (log scale). Distribution of BD-2, IL-17A, and IL-17F baseline levels across indications from 6 different clinical studies. PsA+ indicates data from patients with skin psoriasis and PsA- indicates data from patients without any skin involvement. The *number in parentheses* denotes the number of samples tested. The *red line* indicates the location of each GM value, and its value is indicated by *red* text.

correlate with psoriasis disease activity as measured by using PASI scores. One possible explanation for this discrepancy is that IL-17A is about 100-fold more potent than IL-17F for signaling through IL-17R,[22] a feature also seen for BD-2 induction in keratinocytes in Fig 4. We sought to validate this finding using a larger independent cohort of patients with psoriasis. In the validation set serum IL-17A levels continued to have a good correlation, whereas IL-17F levels had a weaker if not poor correlation with PASI scores. Combined with the excellent and similar efficacy of compounds that target IL-17RA (which blocks IL-17A, IL-17C, IL-17E, and IL-17F) and IL-17A only (reviewed in Patel and Kuchroo[1]), we conclude that IL-17A has a more dominant role in driving pathologic changes in psoriatic skin than IL-17F.

To identify *in vivo* IL-17A–responsive proteins, we treated patients with psoriasis with a single dose of secukinumab, a selective anti–IL-17A antibody with previously demonstrated[11,12,23] pharmacology and clinical efficacy in patients with psoriasis. Secukinumab has been shown in the patients evaluated in this study to reach pharmacologically active skin concentrations at the time of sampling.[19] Proteomics analysis after selective blockade with secukinumab showed that most changes occurred in the dermis. Many of the most strongly downmodulated proteins mapped to the canonical IL-17A pathway (MetaCore from Thomson Reuters) and were linked to keratinocyte activation, inflammation, and neutrophil and leukocyte/dendritic cell recruitment.[24] Three of the 10 top downregulated proteins (myeloperoxidase, CXCL1, and CXCL5) were neutrophil associated, verifying previously described effects of anti–IL-17A therapy on this hallmark feature of psoriasis[25] and the established role of IL-17A in inducing neutrophil recruitment. As previously seen with transcriptional analysis, the protein expression of IL-1β, MMP-1, and MMP-8 in skin decreased after IL-17A blockade. However, the changes were limited to the skin and not seen in serum, likely because of the restricted local production and action of IL-1β and MMPs. Similarly, the chemokines CXCL1, CXCL3, CXCL5, CCL1, and CCL20 were also decreased in the skin but not serum after secukinumab, destroying the chemokine gradient from serum into skin. Although TNF-α mRNA expression is decreased after secukinumab,[12] we did not detect changes in protein levels in this study. The 2 proteins with levels that increased most in dISF were IgE and CCL24, suggesting a skewing of the skin immune response from T_H17 to T_H2. Interestingly, mRNA for IL-17E (IL-25), which promotes T_H2, was also slightly increased in skin after secukinumab therapy. Thus IL-17A blockade leads to decreases in skin proinflammatory cytokines, decreased destruction of the connective tissues, skewing of the immune response away from T_H17, and decreased recruitment of leukocytes, particularly neutrophils, into the skin.

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BD-2 was the protein with by far the biggest change in the dermis and serum after anti–IL-17A treatment (Fig 2 and Table I). This confirms on a protein level the finding that levels of *DEFB4A* mRNA encoding BD-2 protein are decreased after IL-17A blockade.[12,13] *DEFB4A* mRNA is highly induced by IL-17A in epidermal keratinocytes and reconstituted human epidermis,[13,26-28] and BD-2 protein is primarily located in the epidermal layer of the skin,[29,30] where it is a potent antimicrobial peptide.[31] In addition, BD-2 is chemotactic for cells that express CCR2,[32] CCR6,[33] or both, such as neutrophils,[32,34] monocytes/macrophages,[32] immature dendritic cells, and T cells,[35] and is involved in arrest of T_H17 cells on inflamed endothelium.[27] Our study confirms BD-2 is expressed in skin by epidermal keratinocytes, and recent results show that its expression is highly induced by IL-17A in a synergistic manner with TNF-α.[36] Thus BD-2 is an IL-17A–responsive innate inflammatory protein produced in the skin by epidermal keratinocytes.

With the above information, we sought to define whether BD-2 could be validated as a biomarker of IL-17A–mediated skin pathology in patients with psoriasis. We also recognized that a serum biomarker would be more useful than one that would require a skin biopsy or microperfusion. Previously, Jansen et al[29] showed that serum BD-2 levels correlated with psoriasis disease severity, as defined by PASI score. We have confirmed this finding with a larger cohort of patients with psoriasis and have extended it by showing that serum BD-2 levels correlate even more strongly with IL-17A. Importantly, neutralizing IL-17A with secukinumab decreased BD-2 levels not only in skin but also in serum, and this reduction occurred within 1 week, the earliest time point that we measured. Given the dramatic reduction seen at 1 week after secukinumab treatment, it is likely that BD-2 responds even earlier. Lastly, we tested BD-2 levels in baseline serum samples from patients with diseases thought to be IL-17A driven and showed that BD-2 levels were most highly increased in patients with psoriatic skin inflammation.

However, there are caveats and limitations of this study. First, many of the mechanistic findings in the dermis were made in a relatively small number of subjects. However, we could confirm a link between the dermis and serum in our cohort and then reproduce many findings in serum from a larger cohort. Larger studies with skin microdialysis would be needed to confirm the dermal rather than serum data. By using IHC, other candidates for soluble biomarkers of IL-17A activity in patients with psoriasis have emerged, including S100A7 and S100A8,[13] but these proteins were not evaluated here. BD-2 might also respond to mediators other than IL-17A, and our study shows that IL-17A acts in synergy with TNF-α to induce BD-2 by skin epithelium. There are conflicting data regarding the effect of TNF-α blockade or other effective psoriasis treatments on BD-2 levels. In one study *DEFB4A* mRNA levels in skin biopsy specimens did not change after etanercept, whereas they did decrease dramatically with IL-17A blockade.[13] In another study BD-2 protein staining was qualitatively decreased by IHC after etanercept.[15] There was no decrease, and possibly an increase, in serum BD-2 levels after treatment with fumaric acid esters.[37] Increased serum BD-2 levels have been linked to atopic dermatitis and systemic lupus erythematosus,[38-40] and BD-2 can be expressed by other epithelia than skin, including lung and gastrointestinal epithelia.[41,42] Although we did not detect high BD-2 levels in patients with gut inflammation (CD), we have not tested lung diseases. BD-2 is not an exclusive biomarker for IL-17A–mediated skin pathology in psoriasis. However, all studies (including the current one) testing the association of serum or skin BD-2 protein levels and/or skin *DEFB4A* mRNA expression with psoriasis disease activity, serum IL-17A levels, or response to IL-17A blockade are consistent and robust.

In summary, our study shows that IL-17A is a dominant driver of skin pathology in patients with psoriasis, and serum BD-2 is an easily measurable biomarker of psoriasis disease activity that responds rapidly and robustly to IL-17A inhibition by secukinumab. Serum BD-2 levels might be a surrogate for IL-17A activity and could be used to monitor responses to IL-17A–targeted therapies.

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Clinical implications: Serum BD-2 might be a valuable biomarker to predict and/or monitor response to IL-17A–based therapies.

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METHODS

Gene expression analysis

Total RNA extraction. Four-millimeter punch skin biopsy tissue samples were collected and immersed in 1.5 mL of RNAlater RNA Stabilization Reagent. Total RNA was isolated from tissue samples by using the Qiagen RNeasy Micro Kit and eluted in 12 μL of RNase-free water. RNA isolated from commercial skin biopsy specimens (Asterand UK) from healthy subjects (n = 10) served as controls for gene expression data. Twenty to 50 ng of RNA was preamplified by using the Affymetrix SensationPlus FFPE Amplification kit (Affymetrix, Santa Clara, Calif).

NanoString nCounter gene expression analysis. RNA samples were processed with the nCounter Prep Station and Digital Analyzer of NanoString Technologies. The custom-designed nCounter Gene Expression CodeSets C1933_Amadeus and C2018_Everest overall contained probe sets for 314 target transcripts, 7 candidate reference genes for normalization, and 2 sex control genes. Probe sequences for genes reported in this study are shown in Table E2. Three hundred nanograms (C1933_Amadeus) or 25 ng (C2018_Everest) of the preamplified sense RNA was hybridized with the respective CodeSet at 65°C for 16 hours.

Posthybridization processing procedures were carried out, as recommended by NanoString Technologies. Cartridges were scanned at a resolution of 600 fields of view. Gene expression barcode counts were analyzed with nSolver Analysis software v1.1 (NanoString Technologies). Raw NanoString barcode counts for each gene were subjected to an mRNA content-related normalization by using the GM of the 3 reference genes (*RPLI3A*, *RPLI9*, and *UBC*).

qRT-PCR gene expression analysis. qRT-PCR was applied for expression analysis of IL-17 family member genes. However, at the time of qRT-PCR analysis, 2 individual RNA samples from 2 different subjects (one from baseline and the other from day 8, which had the lowest total RNA yield after extraction) had already been exhausted by preceding microarray and NanoString gene expression analyses. cDNA synthesis from mRNA was performed with the iScript advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif) with 2000 ng of input of preamplified sense RNA generated, as described above. Samples were processed with a cDNA (total RNA equivalent) input of 10 ng per reaction (*RPL13A*, *RPL19*, and *UBC*) or 40 ng per reaction (*IL17A*, *IL17B*, *IL17C*, *IL17D*, *IL17E*, and *IL17F*) by using predesigned probe-based qPCR assays from Integrated DNA Technologies (Coralville, Iowa) or Life Technologies (Grand Island, NY; *IL17A*: Hs.PT.56a.19213466; *IL17B*: Hs.PT.58.2931881; *IL17C*: Hs.PT.56a.1093657; *IL17D*: Hs00370528_m1; *IL25 [IL17E]*: Hs03044841_m1; *IL17F*: Hs.PT.56a.20717359; *RPL13A*: Hs.PT.56a.39648480.g; *RPL19*: Hs.PT.56a.39989087; *UBC*: Hs.PT.39a.22214853). All samples were measured in triplicate by using a QuantStudio 12K Flex instrument (Applied Biosystems, Foster City, Calif). The real-time program was 50°C for 2 minutes, 95°C for 10 minutes (1 cycle), 95°C for 15 seconds, and 60°C for 60 seconds (40 cycles). Automatic threshold settings were used. qRT-PCR results were evaluated by using the QuantStudio 12k Flex software v1.1.1 (Applied Biosystems). For qRT-PCR, normalized relative quantities (RQs) were calculated according to the method described by Hellemans et al.[E1] The GM of the quantification cycle values of all Asterand healthy skin reference samples was used as a reference quantification cycle value for the calculation of RQs. RQ values were then normalized by using the GM of the RQs of the 3 reference genes *RPL13A*, *RPL19*, and *UBC*. According to the applied calculations, a normalized RQ value of 1 reflects the GM of the respective gene expression in Asterand healthy skin reference samples.

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E1. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007;8:R19.

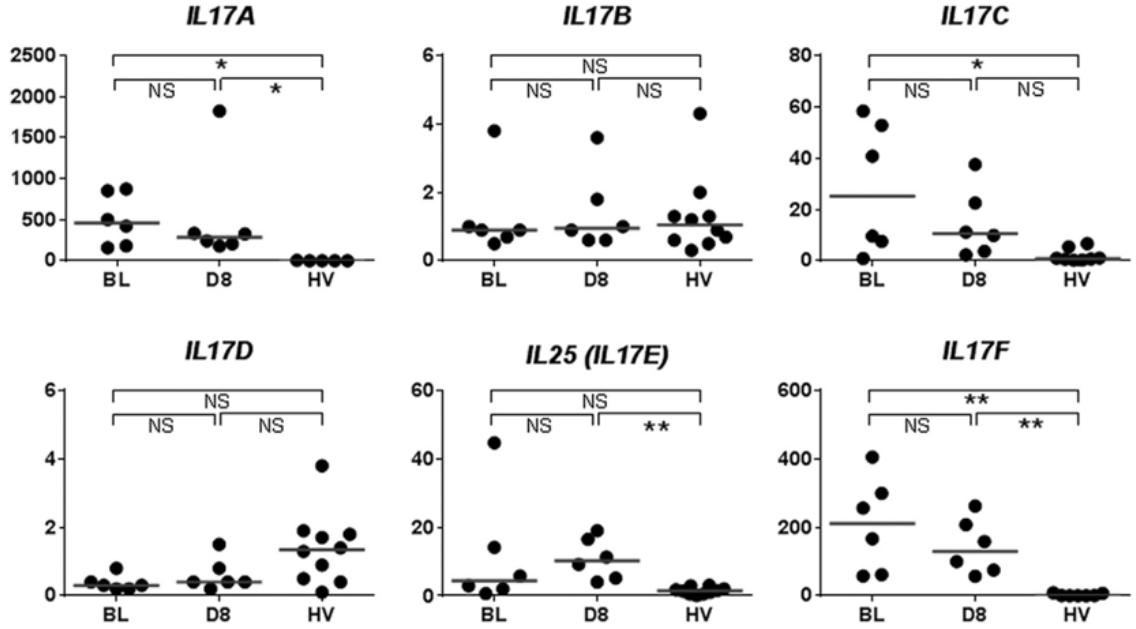

FIG E1. Expression of IL-17 family member genes in skin biopsy specimens from patients with psoriasis and healthy subjects before and after treatment with secukinumab. qRT-PCR was performed on skin biopsy specimens from lesional skin of patients with psoriasis enrolled in the study (n = 6; data from 2 subjects are not shown because of missing data at either baseline or day 8). Healthy control skin biopsy specimens (n = 10) were obtained from a commercial source (Asterand). *Y-axes* show normalized RQs. *Red lines* depict median values. $*P < .05$, $**P < .01$. *NS,* Not significant ($P > .05$). *BL,* Baseline; *HV,* healthy volunteers.

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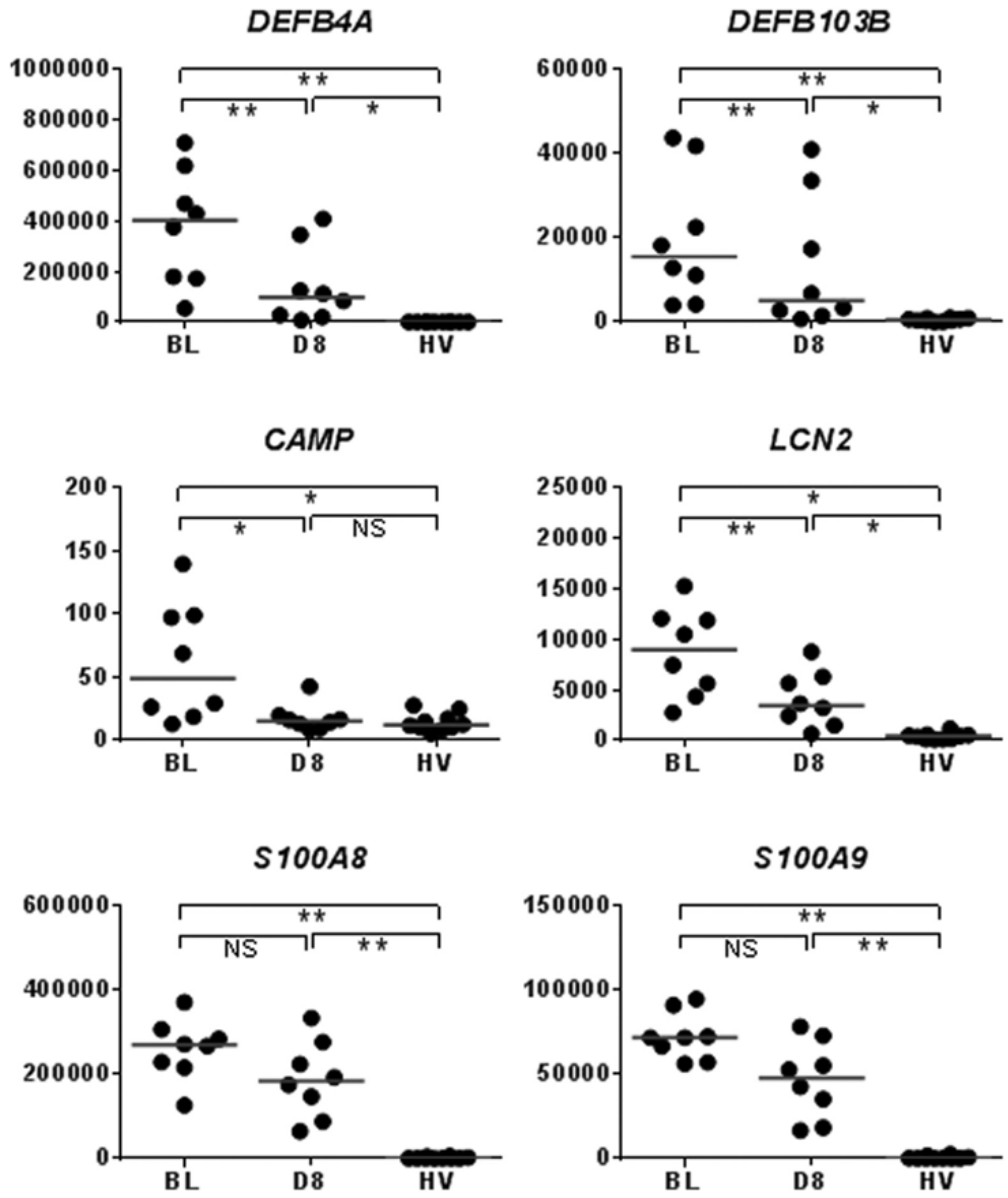

FIG E2. Early transcriptional downregulation of β-defensins and antimicrobial peptides. mRNA gene expression from lesional skin biopsy specimens and healthy subjects were all determined by using NanoString technology. Healthy control skin biopsy specimens (n = 10) were obtained from a commercial source (Asterand). *Y-axes* show normalized RQs. *Red lines* depict median values. **P* < .05, ***P* < .01. *NS,* Not significant (*P* > .05). *BL,* Baseline; *HV,* healthy volunteers.

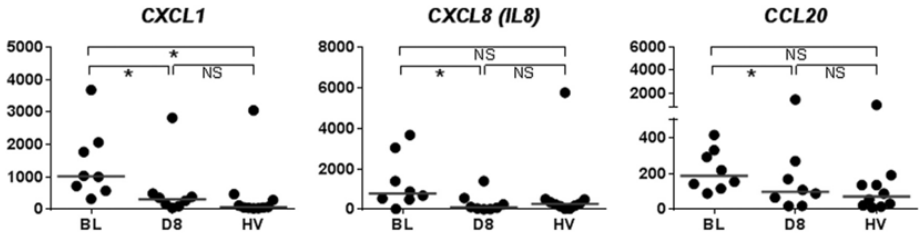

FIG E3. mRNA expression analysis indicates early effects on T_H17- and neutrophil-attracting chemokines. mRNA gene expression from lesional skin biopsy specimens and healthy subjects were all determined by using NanoString technology. Healthy control skin biopsy specimens (n = 10) were obtained from a commercial source (Asterand). *Y-axes* show normalized RQs. *Red lines* depict median values. *$P < .05$. *NS,* Not significant ($P > .05$). *BL,* Baseline; *HV,* healthy volunteers.

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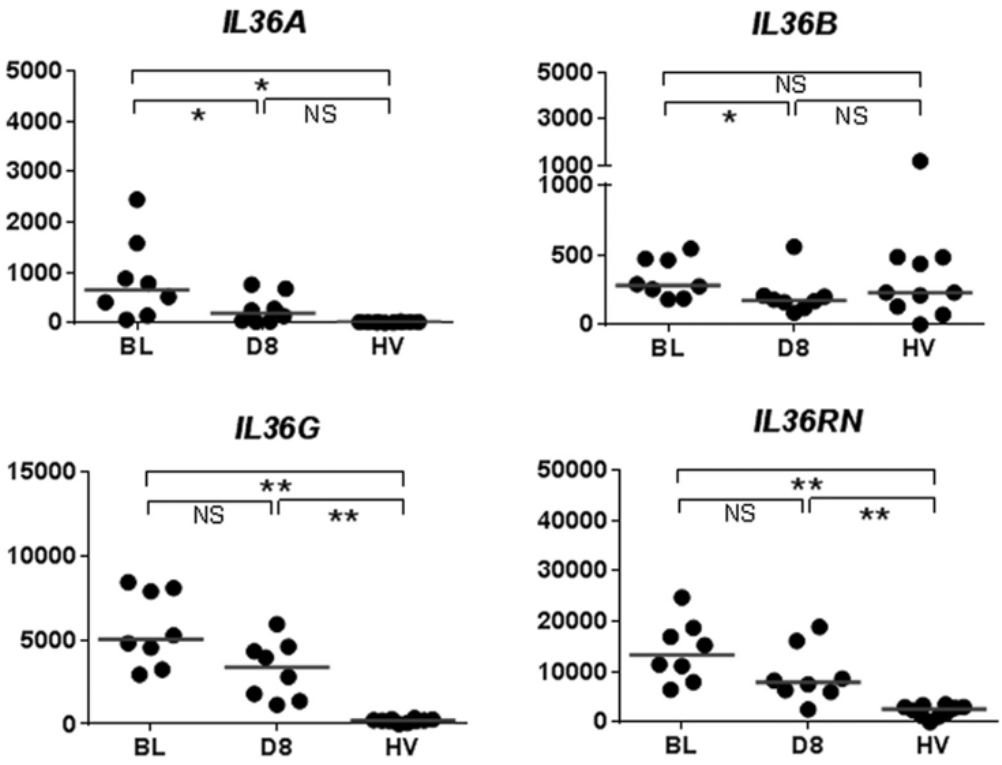

FIG E4. mRNA expression analysis indicates early effects of IL-17A blockade on the IL-1 family cytokines IL-36α, IL-36β, and IL-36γ. mRNA gene expression from lesional skin biopsy specimens and healthy subjects was determined by using NanoString technology. Healthy control skin biopsy specimens (n = 10) were obtained from a commercial source (Asterand). *Y-axes* show normalized RQs. *Red lines* depict median values. **P* < .05, ***P* < .01. *NS*, Not significant (*P* > .05). *BL*, Baseline; *HV*, healthy volunteers.

TABLE E1. Aushon protein analysis panel

Protein	Entrez gene ID
α_2-Macroglobulin	2
Acrp-30 (adiponectin)	9370
Ang-2 (angiopoietin 2)	285
Apo A-1 (apolipoprotein A-1)	335
Apo B-100 (apolipoprotein B-100)	338
AR (amphiregulin)	374
β-NGF (β nerve growth factor)	4803
sAPPβ (soluble amyloid precursor protein β)	351
BCA-1/CXCL13 (B cell–attracting chemokine)	10563
BD-2 (β-defensin 2)	1673
BDNF (brain-derived neurotrophic factor)	627
BMP-9 (bone morphogenetic protein 9)	2658
Cathepsin D	1509
CC16 (Clara cell protein)	7356
CD14 (cluster of differentiation 14)	929
CD30/TNFRSF8 (cluster of differentiation 30)	943
CD40L (cluster of differentiation 40 ligand)	959
CGα (chorionic gonadotropin α)	1081
Clusterin	1191
CNTF (ciliary neurotrophic factor)	1270
COX-2 (cyclooxygenase 2)	5743
C-peptide	3630
CRP (C-reactive protein)	1401
E-cadherin	999
EGF (epidermal growth factor)	1950
EGFR (epidermal growth factor receptor)	1956
ENA-78 (epithelial cell–derived neutrophil-activating peptide 78)	6374
Endoglin	2022
Eotaxin	6356
Eotaxin-2	6369
Eotaxin-3	10344
ER (epiregulin)	2069
ErbB2/Her2 (human epidermal growth factor receptor 2)	2064
E-selectin	6401
Exodus-2 (6Ckine)	6366
FasL (Fas ligand)	356
FGF basic (fibroblast growth factor basic)	2247
Fibrinogen	2243
Fibronectin	2335
G-CSF (granulocyte colony-stimulating factor)	1440
GDNF (glial cell–derived neurotrophic factor)	2668
GM-CSF (granulocyte macrophage colony-stimulating factor)	1437
gp130 (glycoprotein 130)	3572
GROα (growth-regulated protein α)	2919
GROγ (growth-regulated protein γ)	2921
HCC-4/CCL-16 (hemofiltrate CC chemokine 4)	6360
HGF (hepatocyte growth factor)	3082
HGH (human growth hormone)	4261
I-309	6346
ICAM-1 (intercellular adhesion molecule 1)	3383
ICAM-3 (intercellular adhesion molecule 3)	3385
IFN-α (interferon α)	3440
IFN-γ (interferon γ)	3458
IgE (immunoglobulin E)	
IGFBP-1 (insulin-like growth factor binding protein 1)	3484
IGFBP-2 (insulin-like growth factor binding protein 2)	3485
IGFBP-3 (insulin-like growth factor binding protein 3)	3486
IL-1α (interleukin 1 α)	3552
IL-1β (interleukin 1 β)	3553
IL-1ra (interleukin 1 receptor antagonist)	3557
IL-1RI (interleukin 1 receptor I)	3554
IL-1RII (interleukin 1 receptor II)	7850
IL-2 (interleukin 2)	3558
IL-2Rα (interleukin 2 receptor α)	3559
IL-2Rγ (interleukin 2 receptor γ)	3561
IL-3 (interleukin 3)	3562
IL-4 (interleukin 4)	3565
IL-4R (interleukin 4 receptor)	3566
IL-5 (interleukin 5)	3567
IL-6 (interleukin 6)	3569
IL-6R (interleukin 6 receptor)	3570
IL-7 (interleukin 7)	3574
IL-8 (interleukin 8)	3576
IL-9 (interleukin 9)	3578
IL-10 (interleukin 10)	3586
IL-11 (interleukin 11)	3589
IL-12p40 (interleukin 12 p40 homodimer)	3593
IL-12p70 (interleukin 12 p70 heterodimer)	3592 and 3593
IL-13 (interleukin 13)	3596
IL-13Rα1 (interleukin 13 receptor α 1)	3597
IL-15 (interleukin 15)	3600
IL-16 (interleukin 16)	3603
IL-17A (interleukin 17A)	3605
IL-17E (interleukin 17E)	64806
IL-18 (interleukin 18)	3606
Insulin	3630
IP-10 (IFN-γ–induced protein 10 kDa)	3627
I-TAC (interferon-inducible T-cell α chemoattractant)	6373
KGF (keratinocyte growth factor)	2252
Leptin	3952
LIF (leukemia inhibitory factor)	3976
L-selectin	6402
Lymphotactin	6375
MCP-1 (monocyte chemotactic protein 1)	6347
MCP-2 (monocyte chemotactic protein 2)	6355
MCP-3 (monocyte chemotactic protein 3)	6354
MCP-4 (monocyte chemotactic protein 4)	6357
MDC (macrophage-derived chemokine)	6367
MIF (migration inhibitory factor)	4282
MIG (monokine induced by IFN-γ)	4283
MIP-1α (macrophage inflammatory protein 1 α)	6348
MIP-1β (macrophage inflammatory protein 1 β)	6351
MIP-1δ (macrophage inflammatory protein 1 δ)	6359
MIP-3α (macrophage inflammatory protein 3 α)	6364
MIP-3β (macrophage inflammatory protein 3 β)	6363
MIP-4/PARC (macrophage inflammatory protein 4, pulmonary and activation-regulated chemokine)	6362
MMP-1 (matrix metalloproteinase 1)	4312
MMP-2 (matrix metalloproteinase 2)	4313
MMP-3 (matrix metalloproteinase 3)	4314
MMP-7 (matrix metalloproteinase 7)	4316
MMP-8 (matrix metalloproteinase 8)	4317
MMP-9 (matrix metalloproteinase 9)	4318
MMP-10 (matrix metalloproteinase 10)	4319
MMP-13 (matrix metalloproteinase 13)	4322
MPIF-1 (myeloid progenitor inhibitory factor 1)	6368
MPO (myeloperoxidase)	4353
NAP-2 (neutrophil activating peptide 2)	5473
NGAL (neutrophil gelatinase-associated lipocalin)	3934
NT3 (neurotrophin 3)	4908
NT-proBNP (N-terminal prohormone of brain natriuretic peptide)	4879

(Continued)

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TABLE E1. *(Continued)*

Protein	Entrez gene ID
OPG (osteoprotegrin)	4982
OPN (osteopontin)	6696
PAI-1 active (plasminogen activator inhibitor 1 active)	5054
PAI-1 total (plasminogen activator inhibitor 1 total)	5054
PAPP-A (pregnancy-associated plasma protein A)	5069
PD-1 (programmed death 1)	5133
PDGF-AA (platelet-derived growth factor AA)	5154
PDGF-AB (platelet-derived growth factor AB)	5154 and 5155
PDGF-BB (platelet-derived growth factor BB)	5155
PECAM-1 (platelet endothelial cell adhesion molecule)	5175
PEDF (pigment epithelium-derived factor)	5176
PLGF (placental growth factor)	5228
Prolactin	5617
Protein C	5624
P-selectin	6403
RAGE (receptor for advanced glycation end products)	177
RANK (receptor activator of NF-κB)	8792
RANKL (receptor activator of NF-kB ligand)	8600
RANTES (regulated upon activation, normal T cell expressed and presumably secreted)	6352
RBP4 (retinol binding protein 4)	5950
Resistin	56729
SAA (serum amyloid A)	6288
SCF (stem cell factor)	4254
SDF-1 (stromal cell–derived factor 1)	6387
SHBG (sex hormone binding globulin)	6462
TARC (thymus and activation-regulated chemokine)	6361
TFF-3 (trefoil factor 3)	7033
TGF-α (transforming growth factor α)	7039
TGF-β1 (transforming growth factor β 1)	7040
TGF-β2 (transforming growth factor β 2)	7042
TM (thrombomodulin)	7056
TIM-1 (T cell immunoglobulin mucin 1)	26762
TIMP-1 (tissue inhibitor of metalloproteinases 1)	7076
TIMP-2 (tissue inhibitor of metalloproteinases 2)	7077
TNF-α active trimer (tumor necrosis factor α)	7124
TNF-α monomer + trimer (tumor necrosis factor α)	7124
TNF-RI (tumor necrosis factor α receptor I)	7132
TNF-RII (tumor necrosis factor α receptor II)	7133
TPO (thrombopoietin)	7066
TRAIL (TNF-related apoptosis-inducing ligand)	8743
TSLP (thymic stromal lymphopoietin)	85480
TSP-1 (thrombospondin-1)	7057
TSP-2 (thrombospondin-2)	7058
TWEAK (TNF-related and weak inducer of apoptosis)	8742
VCAM-1 (vascular cell adhesion molecule 1)	7412
VEGF (vascular endothelial growth factor)	7422
VEGF-C (vascular endothelial growth factor C)	7424
VEGF-D (vascular endothelial growth factor D)	2277
VEGF-R1 (vascular endothelial growth factor receptor 1)	2321
VEGF-R2 (vascular endothelial growth factor receptor 2)	3791

TABLE E2. NanoString code set probe sequences for genes reported in this study

Gene identifier	Capture probe sequence	Reporter probe sequence
CAMP	CTATAGCACGAAGCACAGCTTCCTTGTA GCTGAGGACCTGGGCAATGATG	GTAGAGGTTAGCATCCGAGGACCGCTGGTTGATGCCAT
CCL20	CAGGAGCAAACTCTTGGTACAGCACATG GTTTTTAGCTCAAAGAACAGAT	GCTTCTGATTCGCCGCAGAGGTGGAGTAGCAGCACTGACATCAAAGCAGC
CXCL1	CTCTATCACAGTGGCTGGCATGTTGCAGGC TCCTCAGAAATATTAACATA	TTCACAATGATCTCATTGGCCATTTGCTTGGATCCGCCAGC
CXCL8 (IL8)	CCGGTGGTTTCTTCCTGGCTCTTGTCCTAG AAGCTTGTGTG	AGCCACGGCCAGCTTGGAAGTCATGTTTACACACAGTGAGATG GTTCCTT
DEFB4A	TATGGCTCCACTCTTAAGGCAGGTAACAGG ATCGCCTATACCA CCAAAAA	CCACAGGTGCCAATTTGTTTATACCTTCTAGGGCAAAAGACTGG ATGACA
DEFB103B	TTTTTATTTCTTTCTTCGGCAGCATTTTCGGC CACGCGTCGAGC ACTTG	CTTTAAGAAGGCATTTCCACACTTTACAACACTCTCGTCATGTTT CAGGG
LCN2	CTGATCCAGTAGTCACACTTCTTTTTCCTAA ACAGGACGGAGGTGACATT	TAATGTTGCCCAGCGTGAACTCGCCGGGCTGGCAACCTGGAACAAAAGTC
RPL13A	TCCTTGCTCCCAGCTTCCTATGTCCCAGGG CTGCC	ATTCTCCGAGTGCTTTCAAGCAACTTCGGGAGGCAGTGACTAA GACCCTT
RPL19	AATCCTCATTCTCCTCATCCATGTGACCTTC TCTGGCATTCGG GCATTGG	TGGCGATCGATCTTCTTAGATTCACGGTATCTTCTGAGCAGCC GGCGCAA
UBC	CACTTCGAGAGTGATGGTCTTACCAGTCAGG GTCTTCACGAAG ATCTGCA	TCCTTGTCTTGGATCTTTGCCTTGACATTCTCAATGGTGTCACT CGGCTC

8.3 Bodenlenz et al. 2016 – Pharm Res (open access)

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DOI 10.1007/s11095-016-1960-y

RESEARCH PAPER

Kinetics of Clobetasol-17-Propionate in Psoriatic Lesional and Non-Lesional Skin Assessed by Dermal Open Flow Microperfusion with Time and Space Resolution

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ABSTRACT

Purpose To evaluate the kinetics of topically applied clobetasol-17-propionate (CP-17) in lesional and non-lesional psoriatic skin when released from a commercially available low-strength cream using *in vivo* dermal open-flow microperfusion (dOFM).

Methods Twelve patients received Dermovate® cream (CP-17, 0.05%) on small lesional and non-lesional skin test sites for 14 days, once daily. On day 1 and 14, dOFM samples were continuously taken in the dermis for 24 h post-dose and analyzed by LC-MS/MS. Probe depths were assessed by 50 MHz ultrasound scanning.

Results Mixed-effects modelling identified skin condition, treatment duration and probe-depth as kinetics determining variables. The time- and depth-resolved intradermal data revealed (i) slower penetration of CP-17 into lesional than into non-lesional skin, (ii) normalized (faster) skin penetration after repeated dosing, and (iii) no CP-17 accumulation within the dermis independently of the skin condition.

Conclusions Intradermal investigation of a highly lipophilic drug released from low-strength cream was successfully performed by using dOFM and timely and spatially, i.e., probe-depth dependent, resolved kinetic data were delivered. These data support the assumption that the thickened psoriatic stratum corneum might act as trap compartment which lowers the skin penetration rate for lipophilic topical drugs.

KEY WORDS dermal pharmacokinetics · lipophilic drug · open flow microperfusion · skin penetration · topical formulation

Electronic supplementary material The online version of this article (doi:10.1007/s11095-016-1960-y) contains supplementary material, which is available to authorized users.

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ABBREVIATIONS

AUC	Area Under the CP-17 concentration time Curve
CP-17	Clobetasol-17-propionate
dOFM	Dermal open flow microperfusion
LLOQ	Lower Limit of Quantification
MD	Microdialysis
SC	Stratum Corneum
TSS	Total Sum Score

INTRODUCTION

Topical corticosteroids play a major role in the therapy of inflammatory skin disorders (1, 2). These topically applied steroids are highly effective, but their delivery into the skin is sometimes rather inefficient (2). Additionally, the role of the stratum corneum (SC) in different skin diseases regarding the kinetics of such topically applied lipophilic drugs remains unclear up to now, because *in vivo* sampling of such drugs is still challenging. Sufficiently sensitive, accurate and well-tolerable *in vivo* sampling methods are not commonly available (3). Several existing *in vivo* sampling methods, such as punch biopsies (4), suction blister (4), or tape stripping (5, 6) are either highly invasive or do not provide any information on the drug concentration from the site of action. Dermal microdialysis (MD) (7) is minimally invasive and capable of directly measuring the rate and extent of drug absorption at or near the site of action in the skin (8), but for intradermal sampling of topically applied drugs,

MD has certain limitations. Typical topically applied drugs are lipophilic and commercially available topical formulations contain potent active ingredients in low concentration, resulting in concentrations of the recovered drug in the MD sample that may even lie below the limit of quantification (9). Furthermore, the pore size of the semipermeable MD membrane restricts sampling of molecules by excluding large molecules. Open flow microperfusion (OFM) (10), in contrast to microdialysis, uses a fully permeable open mesh and has been adapted for dermal sampling (11, 12). It overcomes the limitations of MD when sampling lipophilic topical drugs (11, 13) and is thus capable of assessing topical glucocorticoid kinetics, when released from a commercial low-strength cream, *in vivo*.

Clobetasol-17-propionate (CP-17) was used as study drug. CP-17 is a highly potent corticosteroid for topical treatment of psoriasis (2, 14) and commercially available as cream with low strength (0.05%). Although CP-17 is widely used, data on the penetration behavior of this drug into skin is rare. One study performed by Au *et al.* (15) investigated the feasibility of CP-17 sampling in the skin, but they applied highly concentrated CP-17 in ethanolic solution, because the high lipophilicity of CP-17 (*LogP* = 3.49) precluded the use of a commercial formulation for MD sampling. A study on the penetration behavior of a topical lipophilic drug into healthy and psoriatic lesional skin using tape-stripping followed by skin punch biopsy was performed by Rony *et al.* (unpublished data, presented at Groupe de Métabolisme et Pharmacocinétique Meeting 2011, Paris). Their results revealed diminished drug penetration into psoriatic skin due to accumulation of the drug in the SC. Thus, they assumed that the thickened SC might act as trap compartment of the skin for lipophilic entities. Also in a previous dOFM study in psoriatic patients, using a moderately lipophilic drug, concentrations of the anti-psoriatic drug candidate BCT194 were found to be lower in psoriatic lesional skin than in non-lesional skin (12).

The aim of the current study was to evaluate the kinetics of highly lipophilic topical CP-17 *in vivo* in lesional and non-lesional psoriatic skin when released from a low strength cream and to elucidate the role of the SC in psoriasis by using timely and spatially, i.e., probe-depth resolved kinetic data.

MATERIALS AND METHODS

Topical Treatment

Clobetasol-17-propionate (CP-17) is a highly potent corticosteroid (US class I, Europe class IV) that activates glucocorticoid receptors and exhibits anti-inflammatory and immunosuppressive activities. CP-17 is widely used for topical treatment of psoriasis (14) and it was applied as commercial cream of low-strength (Dermovate® Cream, 0.05% CP-17, GlaxoSmithKline Pharma GmbH, Vienna, Austria).

Dermovate® Cream was applied once daily from day 1 to day 14 to one lesional and one non-lesional test site of 7.7 cm^2 each at a topical dose of 15 mg/cm^2. On days 1 and 14, when dOFM sampling was continuously performed from baseline to 24 h post-dose, the cream remained on the skin and the test site was protected by a non-occlusive dressing. On the days without investigation by dOFM, the topical dose was applied once and excess of cream was removed at approximately 4 h post-dose.

24 h Dermal OFM Sampling

Continuous intradermal sampling was initiated and performed as described in (12, 16), by using CE-certified dOFM materials. The dOFM probe was tested for potential unspecific adsorption of CP-17 and the absence of any adsorptive losses was confirmed.

Prior to probe insertion, the skin was cooled with an sterile ice bag to avoid pain (16). Three linear dOFM sampling probes (Type DEA15001, OD 0.32 mm, 15 mm open-mesh with integrated 0.5 mm insertion needle) were inserted into a lesional test site and three probes into a non-lesional test site. Following insertion, the three adjacent probes were continuously perfused in a push-pull manner with sterile perfusate (physiological saline solution including 1% Human Serum Albumin) at a flow rate of 1 μL/min using a single wearable OFM pump (Type MPP101 with sterile fluidic kit). The dermal interstitial fluid samples were collected hourly from baseline to 24 h post-dose. The dermal OFM setup is shown in Fig. 1.

Patients

The study was approved by the Ethical Committee of the Medical University of Graz and the Austrian health authorities (AGES) and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Twelve psoriatic patients with a diagnosis of stable plaque type psoriasis for at least 6 months and at least two psoriatic plaques on the upper extremities or proximal lower extremities were recruited (Caucasian, 9 men and 3 women, 40.2 ± 5.8 years) and gave written informed consent. The plaque Total Sum Score (TSS; erythema + induration/plaque elevation + scaling) had to be ≥6 with each item scoring 2 or 3 (moderate to severe) with a maximum of 2 for scaling. Subjects were otherwise healthy and screened for eligibility by assessing their medical history, physical parameters and laboratory parameters. Concomitant medication was not allowed, and women had to use contraception.

Study Design

The study was designed as a single-center, open-label exploratory phase I trial and conducted at the Medical University of Graz, Austria. In the pilot phase, topical CP-17 penetration was investigated in four subjects to identify the quantification

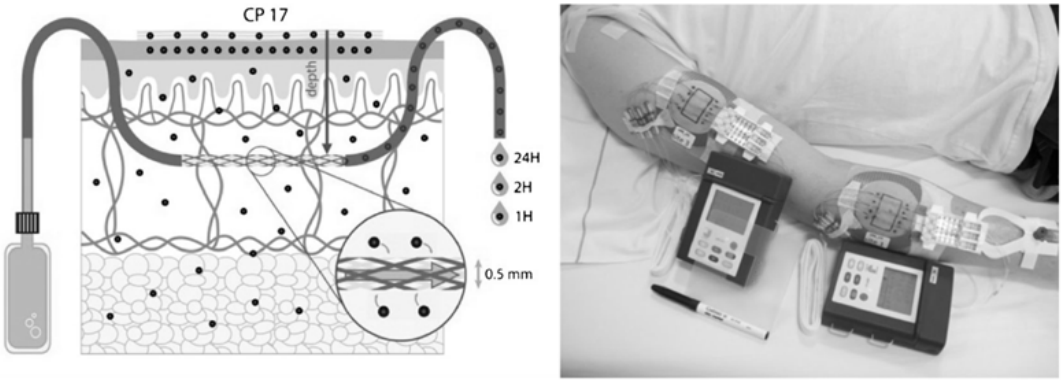

Fig. 1 Left: Scheme of dOFM setup. The membrane-free dOFM probe within the dermis is continuously perfused and delivers interstitial fluid for further analysis. Right: Wearable dOFM setup including the dOFM probe, a wearable push-pull pump, and a unit for sample collection.

limit for CP-17 from dOFM samples. In the main clinical study, eight subjects were dosed with CP-17 from day 1 to day 14, daily. dOFM was performed on day 1 and day 14. Patients were admitted to the clinic at 7 am (day 1). One lesional and one non-lesional site were marked on the skin, and three dOFM probes were inserted into the dermis of each site for continuous sampling as described above. After a run-in period of 60 min to allow the insertion trauma to subside (17), a baseline (pre-dose) sample was collected. At 11 am ($t=0$), Dermovate® cream was applied to each site, as described above. dOFM samples were collected hourly, immediately frozen at −80°C and kept at −80°C until analysis. Sampling was terminated 24 h post-dose. Probe depth and the exact position in the skin were assessed in duplicate by 50 MHz ultrasound scanning (DUB-USB, Taberna Pro Medicum, Lüneburg, Germany) as described in detail previously (16). After probe removal, sites were inspected and covered with light dressings. On days 3–13, patients revisited the study center once daily, received the topical dose, left the clinic 1 h post-dose and were asked to remove the excess of cream approximately 4 h post-dose. On day 14, patients were admitted to the clinic in the morning. The same protocol as on day 1 was followed, but with two methodological alterations taking the reinsertion of dOFM to the now previously used test sites into account: First, the probes were shifted by 5 mm relative to their position on day 1. Second, the probe insertion points outside of the application zones were cleaned thoroughly with gauze, followed by slight tape stripping to reduce risk of probe contamination with residual drug in the SC. On day 14, the clinical improvement of CP-17 treated lesions was also evaluated using the plaque Total Sum Score (TSS). The follow-up visit was scheduled between days 18 and 25. An overview of the study days and procedures is given in Fig. 2.

Sample Analysis and Data Management

Baseline sample and six pooled post-dose samples (0–4 h, 4–8 h, 8–12 h, 12–16 h, 16–20 h, 20–24 h) were analyzed to assess intradermal kinetics. CP-17 was measured after liquid-liquid extraction by LC-MS/MS in selected multiple-reaction monitoring (MRM) positive ion mode. Internal standard (CP-17-d3), 15 mM NH_4OH (50 µL), and diethylether/hexane 80/20 (400 µL) were added to 50 µL of standards or samples. The samples were vortexed and centrifuged. Then, the organic layer was removed and evaporated to dryness under vacuum. The residue was redissolved in 30% acetonitrile (20 µL) and subsequently analyzed. The measurement was performed on a U300 Ultimate HPLC system (Dionex) coupled to a TSQ Ultra AM triple quadrupole mass spectrometer (Thermo). The separation was carried out on a Zorbax SB-C18 (0.5 × 35 mm) column (Agilent), with a flow rate of 50 µL/min and 7.5 µL injection volume using gradient elution (eluent A: water 0.1% formic acid; eluent B: acetontrile 0.1% formic acid). The gradient started with 40% eluent B which was linearly increased to 90% within 2.0 min and kept constant for until 4.5 min. The analyte was detected in MRM mode (CP-17: 467.2−446.9 (3 eV), 467.2−372.9 (9 eV), 467.2−354.9 (10 eV), 467.2−277.9 (17 eV), 467.2−262.9 (24 eV); Internal standard: 470.2−372.8 (8 eV), 470.2−354.8 (10 eV), 470.2−277.90 (18 eV), 470.2−262.8 (17 eV). The Lower Limit of Quantification (LLOQ) was 0.35 ng/mL.

For data management, we used an electronic case report form (eCRF) within the OpenClinica Enterprise Edition (OpenClinica, LLC 460 Totten Pond Road, Suite 200, Waltham, MA 02451). OpenClinica had been fully validated and it is 21 CFR Part 11 compliant.

Pharmacokinetic Analysis and Statistics

Only the 8 subjects of the main study were included in the pharmacokinetic analysis. CP-17 concentration profiles were checked for validity, considering the concentration gradient from the skin surface (500 µg/ml) to the dermal interstitial fluid (about 0.5 ng/ml) and the risk of probe contamination with residual topical drug in the SC during reinsertion of probes on day 14. For the pharmacokinetic analysis, the areas under the CP-17 concentration time curves (AUCs) were calculated by

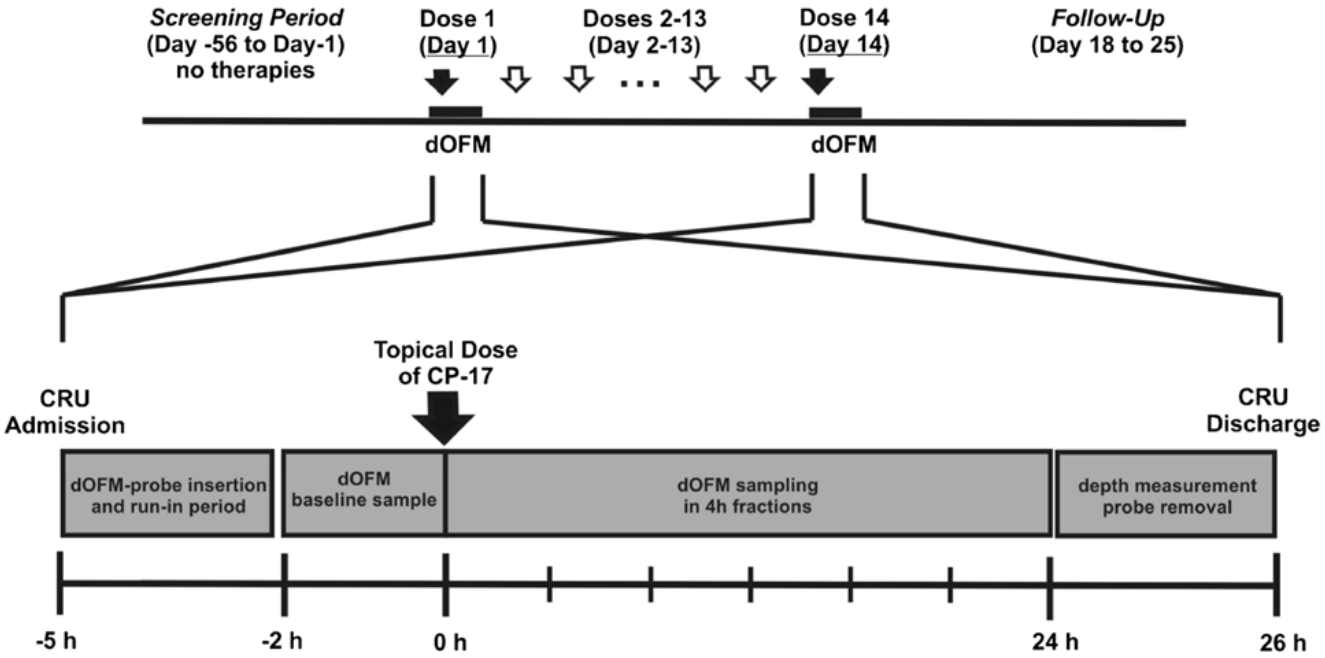

Fig. 2 Study days with treatment regimen and protocol on the days of OFM investigation. CP-17 was dosed as Dermovate® Cream 0.05% once daily from day 1 to day 14. Following the doses on day 1 and day 14 the kinetics of CP-17 were followed in dermal interstitial fluid for 24 h by using dOFM.

cumulating sample concentrations from 0 to 24 h or, in case of a missing sample, by the standard trapezoidal method.

Outliers in CP-17 profiles were excluded when the outlying value was <50% or >200% of the interpolated concentration. All data were expressed as arithmetic mean ± SD. All AUC-values were log-transformed prior to data analysis. The log-transformed AUC-values were tested for statistical significance by means of two-factorial repeated measure ANOVAs using "Time" ("Day 1" *versus* "Day 15") and "Skin site" ("Lesional" *versus* "Non-Lesional") as within-subjects factors. P-values <0.05 were considered statistically significant. In case of a statistically significant ANOVA, paired t-tests were performed to find the significant mean differences. For the interpretation of these post-hoc tests on the raw data (i.e., non-depth corrected data) the significance level was adjusted according to Bonferroni. In order to keep the familywise error rate at 0.05, we adjusted the significance level to 0.05/4 = 0.0125. By applying this Bonferroni adjustment we followed a very conservative approach.

In order to analyze the effect of data treatment, the AUCs were calculated from three different data sets: Data set 1: All CP-17 raw data values below LLOQ were left unchanged; Data set 2: All CP-17 values below LLOQ were set to LLOQ; Data set 3: All CP-17 values below LLOQ were set to LLOQ/2.

The dependency of CP-17 concentration on the depth was identified in a stepwise approach: We (i) plotted the AUC data *versus* probe-depth, (ii) searched for adjacent probes with different depths and counted the pairs for which the AUC-depth relationship was true in a frequency analysis, and (iii) compared the probe with the greatest and the smallest depth per site with respect to the AUC, using paired t-tests. Finally, a linear mixed effects model (iv) was fitted to the data, using Log(AUC) as dependent variable, "Time" and "Skin site" as fixed effects, "Subject" as random effect, and "Probe-depth" as covariate. Model simplification was performed by using likelihood ratio tests, starting with the full model containing all predictor variables.

The open source software package "R" (Version 2.10.1) was used for the statistical analysis.

RESULTS

CP-17 Treatment Effect and dOFM Tolerability

Study procedures and 24 h sampling periods were well tolerated by all subjects. No subjects dropped out and no serious adverse events occurred. Daily topical treatment with CP-17 resulted in a visible improvement of psoriatic skin within 14 days. The plaque Total Sum Score (TSS) for the lesional test site was reduced from 6.3 ± 0.5 on day 1–2.4 ± 1.4 on day 15 ($p < 0.001$) and appeared as a pale rectangular area within the otherwise unchanged psoriatic plaque.

CP-17 Profiling by dOFM

On day 1, CP-17 concentrations in the dermal interstitial fluid of non-lesional skin reached the LLOQ at approximately 10 h post-dose and maximum concentrations were observed at

18 h post-dose (0.07–1.86 ng/ml, mean C_{max} 0.61 ng/ml; Fig. 3a). In lesional skin, the CP-17 levels steadily increased on day 1, but most subjects did not reach the LLOQ during the whole 24 h sampling period (range 0.09–0.42 ng/ml, mean C_{max} 0.19 ng/ml; Fig. 3b).

On day 14, CP-17 baseline concentrations in dermal interstitial fluid of non-lesional skin were not quantifiable. CP-17 levels showed a steep increase resulting in a peak at around 10 h (0.43–4.1 ng/ml, mean C_{max} 1.00 ng/ml; Fig. 3a). In lesional skin, the CP-17 levels were already quantifiable at baseline (pre-dose), were then transiently dropping to levels below LLOQ after dosing before moderately increasing to a peak at 18 h (0.13–1.53 ng/ml, mean C_{max} 0.68 ng/ml; Fig. 3b).

The cumulated quantities (AUC 0–24 h, Table I) after one day of treatment were 0.854 ± 0.422 ng and 2.142 ± 1.993 ng in lesional and non-lesional skin, respectively ($p = 0.033$). This difference was not statistically significant considering the Bonferroni-adjusted significance level of $p = 0.0125$. Corresponding values after 14 days of treatment were increased (2.768 ± 2.010 ng and 4.439 ± 4.602 ng) but more similar to each other compared to day 1 ($p = 0.349$). In lesional skin, the AUC-increase compared to day 1 was pronounced but did not reach statistical significance ($p = 0.020$) considering Bonferroni. The increase for non-lesional skin was less pronounced ($p = 0.093$). Table I summarizes the AUCs. Notably, this conventional comparison of AUCs does not yet take the factor probe depth into account.

Conventional data treatment (values < LLOQ set to LLOQ or LLOQ/2) resulted in a significant increase of the mean profiles, in particular for profiles from lesional skin, which diminished the differences (Figure S1). Thus, when values < LLOQ were substituted by LLOQ/2, the difference found between lesional skin and non-lesional skin on day 1 as well as the increase in lesional skin from day 1 to day 15 did not reach the level of statistical significance ($p = 0.057$ and $p = 0.053$, Suppl. Table S2). When substituted by LLOQ, the level of significance was clearly missed ($p = 0.111$ and $p = 0.113$, Suppl. Table S3).

Evaluation of Influential Parameters

The dependency of AUCs from depth was indicated by a plot of AUCs *versus* probe-depth (Fig. 4). The AUCs of adjacent probe triplets are clearly related by depth (i.e., negative slopes only), while the remaining AUC variability is attributed to the individuals as such (i.e., the inter-subject variability of the skin barrier).

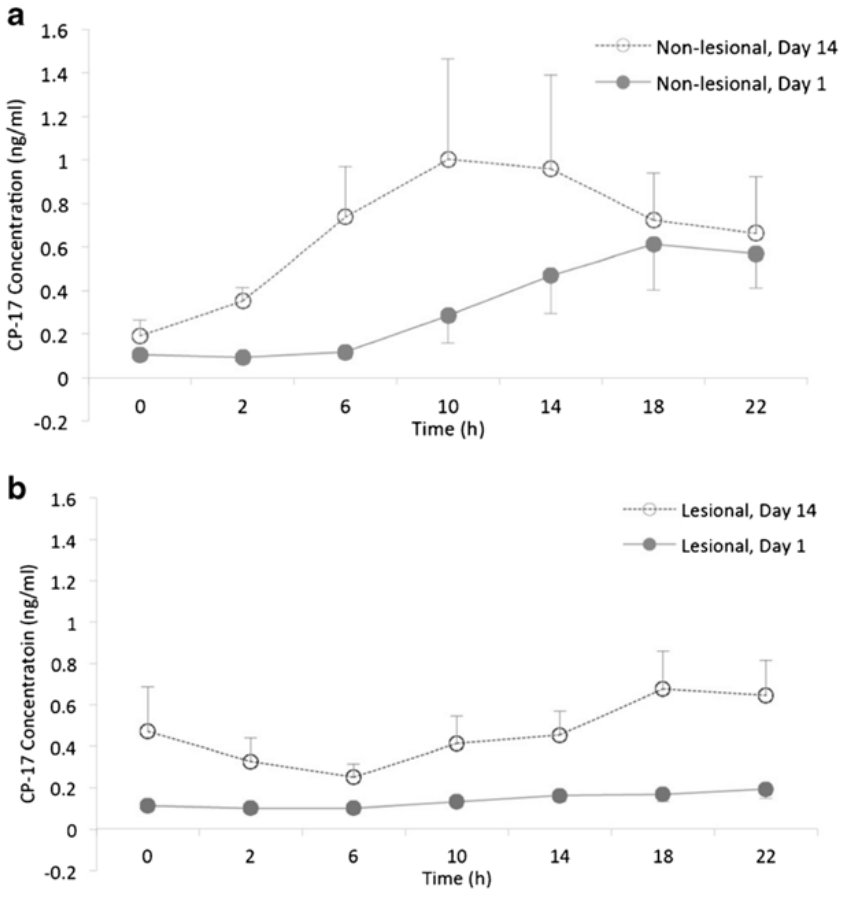

Fig. 3 Mean CP-17 concentration profiles from baseline to 24 h post-dose on the Day 1 (after 1st dose) and Day 14 (after 14th dose). (**a**) Non-lesional skin profiles. (**b**) Lesional skin profiles. Data are mean ± sem.

Table I AUCs Derived from Unmodified Data ind. ANOVA Post-hoc Test on AUC and Log(AUC)

Group	Time	N	Variable	Median	Mean	SD	Day 1 *vs.* Day 14	L *vs.* NL
L	**Day 1**	8	AUC	0.732	**0.854**	*0.422*	***p* = 0.020**	***p* = 0.033**
L	**Day 14**	8	AUC	2.071	**2.768**	*2.010*		*p* = 0.349
NL	**Day 1**	8	AUC	1.705	**2.142**	*1.993*	*p* = 0.093	
NL	**Day 14**	8	AUC	2.521	**4.439**	*4.602*		

Frequency analysis showed that in 23 out of 30 cases (77%), the deeper probe showed a lower AUC than the more superficial probe. The frequency increased to 89% when the depth-difference was larger than the ultrasound measurement error of 0.2 mm. The paired *t*-test showed a statistically significant difference in AUC ($p < 0.001$) between deeper (2.017 ± 2.689 ng*h/ml) and more superficial probes (2.938 ± 3.024 ng*h/ml).

Finally, the linear mixed effects model identified (i) skin type (lesional *versus* non-lesional, $p = 0.0158$), (ii) time (day 1 *versus* day 14, $p < 0.0001$) and (iii) probe depth ($p < 0.0001$) as statistically significant predictor variables for AUC. The following equations summarize the result of the modelling process:

Non-lesional skin at Day 1:

$$\mathrm{AUC} = \exp(1.725\text{-}1.737 * \mathrm{Depth}) \quad (1)$$

Non-lesional skin at Day 14:

$$\mathrm{AUC} = \exp(2.554\text{-}1.737 * \mathrm{Depth}) \quad (2)$$

Lesional skin at Day 1:

$$\mathrm{AUC} = \exp(1.370\text{-}1.737 * \mathrm{Depth}) \quad (3)$$

Lesional skin at Day 14:

$$\mathrm{AUC} = \exp(2.199\text{-}1.737 * \mathrm{Depth}) \quad (4)$$

Figure 5 provides a plot of these equations and their fit to the underlying AUC data. This representation shows the differences in AUC between lesional and non-lesional skin both after the 1st dose and the 14th dose, and the AUC increase by daily topical dosing due to the faster absorption.

DISCUSSION

The intradermal CP-17 concentration profiles assessed in this study revealed a significantly higher penetration of CP-17 into non-lesional skin than into lesional psoriatic skin. We also found that penetration into lesional skin increased with skin healing and that CP-17 did not significantly accumulate in the skin regardless of the skin condition. Thus, by using dOFM, we were able to identify kinetic differences rather than measuring the difference in total quantities only, as has been done in previous studies using classical sampling methods (4, 6). Additionally, we identified dOFM probe depth as a relevant variable to be considered in future topical pharmacokinetic studies with dOFM or dermal microdialysis.

dOFM proved to be suitable for sampling of highly lipophilic drugs because the large openings in the dOFM probe allowed sampling of the total drug concentration without any adsorptive losses. This study provided a CP-17 profile when released from a topically applied low strength cream (0.05% CP-17). In-depth analysis of the AUCs revealed that rather minor differences of 0.2 mm in dOFM probe depths already influence the AUCs observed for CP-17. The identification of probe depth as a relevant variable indicated that the overall methodological variation of dOFM is low. Although the influence of probe depth on kinetics has

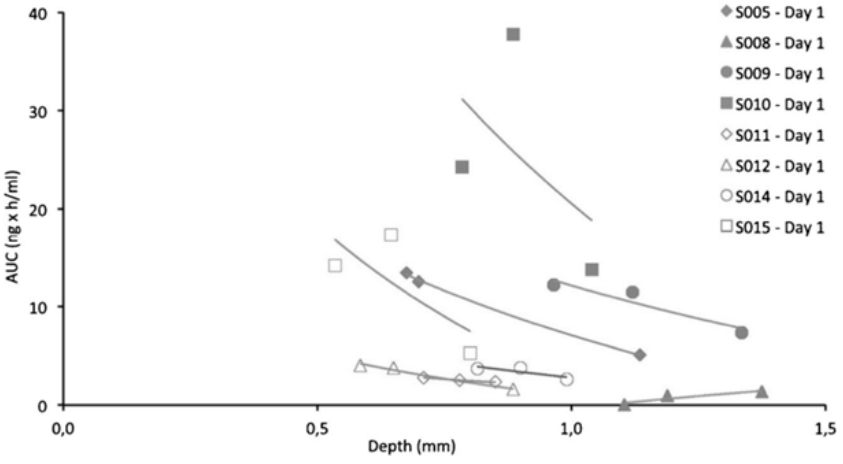

Fig. 4 AUC data of non-lesional skin on day 1 plotted *versus* depth. Regression lines are fitted to the AUCs of the 3 adjacent probes in non-lesional skin for each subject. The illustration indicates a relationship between the AUCs and probe depth in 7 of 8 subjects (negative slopes) and also indicates that most of the variability is due to inter-subject variability of CP-17 penetration.

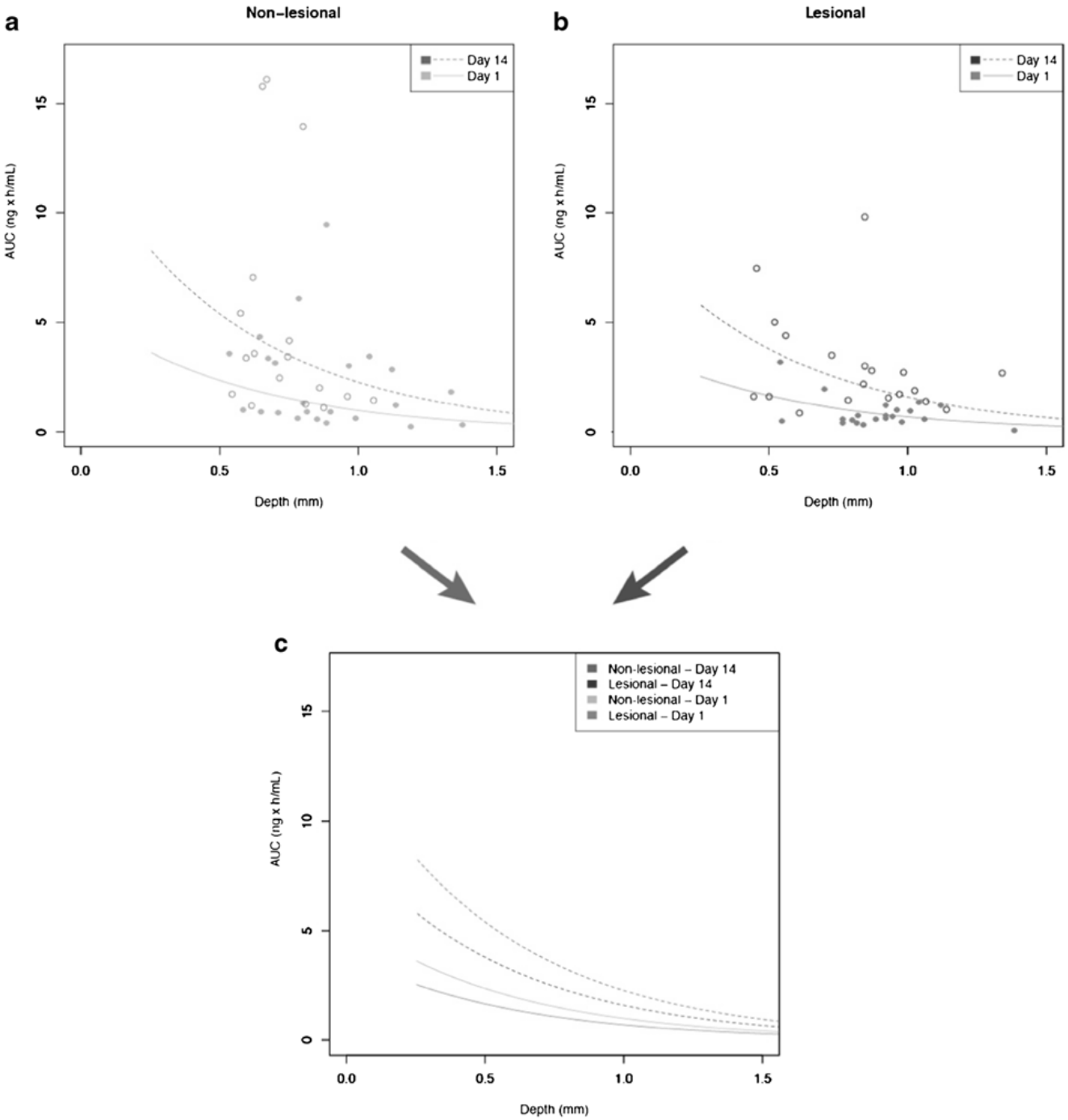

Fig. 5 AUC0-24 h for CP-17 in dOFM samples on the days 1 and 14 as a function of depth. (**a**) AUCs of non-lesional skin and (**b**) AUCs of lesional skin; (**c**) All four AUC regression lines from both non-lesional and lesional skin on both day 1 and day 14 (see Eqs. 1–4).

repeatedly been considered in dermal MD studies (15, 18), no significant effect has been published yet. Methodological differences or the different distribution of predominantly water soluble compounds which have been investigated in these dermal MD studies could have prevented the identification of any impact of probe depth variations. One methodological study found that different depths of the MD probes yielded different AUCs (19) but the depths differences were rather large (1 mm) and thus not representative for standardized use of probes in clinical studies. In contrast, our study assessed the impact of small inevitable depth differences occurring during probe insertion in standardized topical PK studies. We found a statistically significant impact of these small depth differences on the measured CP-17-profiles and this study provided a concentration *versus* depth profile for this glucocorticoid. A similar

depth-dependent profile has been shown in the past for topical hydrocortisone by using radio-labelling and slicing methodology (20).

These results indicate that future dOFM/MD studies which are comparing the PK of topical formulations, like topical bioequivalence studies, should consider that (i) probe depths should be precisely measured (by e.g., full length scanning of each probe using a wide angle ultrasound device), (ii) a potential impact of probe depth should be evaluated and (iii) any impact should be adequately taken into account in the representation of the PK outcome variables.

We investigated the kinetic differences of drug concentrations in lesional and non-lesional skin. These kinetic results shed light on the skin penetration properties of a high-potent topical glucocorticoid and on the role of the SC in psoriasis for the absorption of topically applied lipophilic drugs. The penetration of CP-17 into the dermis after the 1st dosing on day 1 was slow as expected and reached C_{max} at 18 h in non-lesional skin. Interestingly, the penetration into lesional skin was even slower and the measured dermal CP-17 concentrations were significantly reduced. This is in agreement with a previous dOFM study that investigated the penetration behavior of the moderately lipophilic topical drug candidate BCT194 (12). One reason for the lower drug levels measured by dOFM in the dermis could be the distance between the dOFM probe and the SC barrier in the inflamed and thus highly blood-perfused dermis, but lower drug levels in lesional skin were also found by tape stripping and skin punch biopsy from *in vitro* and clinical studies (Rony *et al.*, unpublished data presented at Groupe de Métabolisme et Pharmacocinétique - Meeting 2011, Paris). Rony *et al.* showed that 70–80% of the penetrated dose has been recovered in the SC in healthy skin, while in psoriatic skin at least 95% of the penetrated dose has been recovered in the SC. Less than 1% of the applied dose reached the deeper skin layers, such as epidermis and dermis, which is the site of drug action. The authors concluded that in psoriasis the hyperkeratosed SC seems to absorb higher amounts of the topical drug and therefore acts as a trap compartment. However, this study assessed drug penetration and distribution only at one single point in time and thus does not allow the investigation of transdermal penetration kinetics to reveal the role of SC in topical drug release. Nevertheless, their data and the results from our study are in contrast to the common assumption that diseased skin is more permeable for topical drugs. Recent reviews of *in vitro* (21) and *in vivo* studies (22) concluded that permeation through damaged skin is modestly increased at least for hydrophilic compounds, but studies on psoriatic skin are scarce. One cited study found that psoriatic and unaffected skin showed similar permeability to hydrocortisone, a glucocorticoid less lipophilic than CP-17 (23). Based on this study it has been suggested that psoriasis might not have a penetration barrier defect or that the penetration of lipophilic compounds is not influenced by damaged skin (22). In conclusion, our dOFM data clearly showed that the permeability of the SC barrier is not impaired for topical drug penetration in psoriasis but may instead act as a trap compartment and thus impact the penetration rate for many of the predominantly lipophilic topical drugs.

The low baseline levels on day 14 of our study indicate that, irrespective of the skin condition, CP-17 does not significantly accumulate in the dermis following repeated dosing. Following the 14th dose, the dermal CP-17 concentrations increased significantly faster in lesional skin, but also somewhat faster in non-lesional skin compared to day 1. For lesional skin, the significantly increased absorption on day 14 can be explained by the ongoing healing of the skin leading to a partial normalization (thinning) of the thickened psoriatic SC. Faster penetration into non-lesional skin on day 14 can be explained by the well-known side effect of prolonged topical glucocorticoid therapy that leads to steroid atrophy of the skin (24–26). Noteworthy, the intradermal CP-17 levels on day 14 did not return to baseline after 24 h due to the fact that the cream on this study day has not been removed after approximately 4 h but remained on the skin.

The clinical effects of a glucocorticoid therapy were confirmed by the significantly decreased total sum scores (TSS) for the treated sites, which were also well demarked as white zones within the plaques. This observation is consistent with the well-known skin blanching response of topical corticosteroids (27).

The intradermal sampling of CP-17 was challenging as indicated by the low concentrations where C_{max} did not exceed the 3-fold LLOQ. In the explorative data analysis we either accepted values below LLOQ or substituted them by a standard value (LLOQ or LLOQ/2) and the results demonstrated that standardized raw data treatment should be used with caution in particular for slow penetrating drugs, as it may bias the data.

A major challenge was the cleaning of the probe insertion sites on day 14 to prevent the contamination of the probes by residual concentrations in the SC. The increased baseline and initial post-dose values in lesional skin on day 14 indicated that the cleaning measures were not sufficient. Therefore, in studies including insertion of probes in topically treated skin, the skin cleaning procedure should be further improved, or Band-Aids should be used during the treatment phase to prevent a carry-over of the drug to skin outside the treatment site when the volunteers are at home. We found considerable inter-subject variability regarding the penetration and the dermal drug levels especially in psoriatic lesions. High inter-subject variability in skin permeability has also been observed clinically (13).

Our study has provided intradermal kinetic data of a topical drug *in vivo* in humans with a time and depth related resolution, when released from a low-strength product. This allows experimental verification of mathematical

physiological models for topical drug penetration (28, 29). To support these models, further topical drugs with different lipophilicity and protein binding properties should be investigated. The dOFM approach is well tolerated by healthy subjects and psoriatic patients and it recovers the total drug concentration directly at the site of action. This approach may have a general utility across the entire range of topical dermatological drug products, including early head-to-head testing of formulations in humans when dosed in low quantities as well as head-to-head topical bioequivalence studies of generic *versus* reference listed drug products. Moreover, since dOFM samples represent diluted but otherwise unfiltered dermal interstitial fluid, dOFM samples also include intradermal biomarkers which can be analyzed based on novel highly sensitive low-volume cytokine assays. Hence, future studies can be performed as combined pharmacokinetic and pharmacodynamic studies.

CONCLUSION

In conclusion, the dermal pharmacokinetics of highly lipophilic CP-17 released from a commercial low-strength cream in patients *in vivo* was successfully assessed by dOFM and time as well as depth (i.e., spatially) resolved kinetic data were delivered. These data showed that CP-17 does not significantly accumulate in the dermis following repeated topical dosing and revealed a reduced penetration rate of the high-potent glucocorticoid into hyperkeratosed psoriatic skin. Therefore, our data support the assumption that the thickened SC of psoriatic skin acts as trap compartment for lipophilic topical drugs. In terms of methodological advancements our study shows the utility of dOFM to support the clinical development of new drugs by facilitating studies with short treatment duration and low drug amount at early stages with limited preclinical prerequisites. Demonstrating the high spatial resolution of the dOFM sampling probes, our study provides essential information for the proper design and interpretation of topical bioequivalence studies.

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Letter to the Editor

Secukinumab distributes into dermal interstitial fluid of psoriasis patients as demonstrated by open flow microperfusion

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Abbreviations: dOFMdermal open flow microperfusion; dISF, dermal interstitial fluid; TSS, total sum score; PASI, psoriasis area and severity index.

Key words: dermal open flow microperfusion – monoclonal antibody – pharmacokinetics – psoriasis – secukinumab

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Background

Secukinumab (AIN457), a recombinant high-affinity fully human monoclonal antibody, selectively targets and neutralizes IL-17A. Secukinumab has been shown to be clinically effective (1,2) with a rapid, strong and sustained efficacy and a favourable safety profile in patients with moderate-to-severe plaque psoriasis (3–5). Secukinumab is administered by subcutaneous injection, which is typical for the most recent antibody therapies. For psoriasis therapy, the distribution of secukinumab into lesional and non-lesional skin is of particular interest to provide local activity at the target site. Currently available methods to measure antibody concentrations in the skin (e.g. punch biopsies, suction blisters) can only be used for single time-point measurements as they are rather invasive and burdensome (6). Other methods such as dermal microdialysis are of limited use for large antibody molecules due to limited pore size of clinical membranes (7).

Dermal open flow microperfusion (dOFM) offers an alternative sampling method characterized by minor invasiveness and the ability to continuously sample large and lipophilic molecules directly from the dermal interstitial fluid (dISF) (Fig. 1a) (8,9). Calibration with a reference substance (e.g. sinistrin) or a reference-independent procedure (e.g. No-Net-Flux) allows absolute quantification (S10-12).

Questions addressed

We determined the concentration of secukinumab directly in the skin of healthy subjects and in lesional and non-lesional skin of plaque psoriasis subjects to assess the ability of a single 300 mg subcutaneous dose to neutralize its target, IL-17A, in the skin.

Experimental design

This study (ClinicalTrials.gov: NCT01539213) was carried out at the Medical University of Graz, Austria. It was approved by the local ethics committee and the Austrian Agency for Health and Food Safety (AGES) and was conducted according to GCP and the Declaration of Helsinki. Written informed consent was obtained from each subject before study start.

Eight healthy subjects and eight psoriasis subjects were included (Table S1). We first established the quantification of secukinumab with dOFM in the skin of healthy subjects using a No-Net-Flux procedure and sinistrin as an external reference substance, and by validating the quantification with suction blisters and punch biopsies. dOFM with sinistrin as a reference was then used for lesional and non-lesional skin in psoriasis subjects.

At least three dOFM probes were inserted into the healthy skin of each subject on Days 1, 8 and 15 to collect dISF samples; additional probes were used in lesional psoriasis plaques (Fig. 1b). Sinistrin was administered as a primed-continuous intravenous infusion, and dOFM sampling was performed for up to 14 hours. Blood samples

Experimental Dermatology

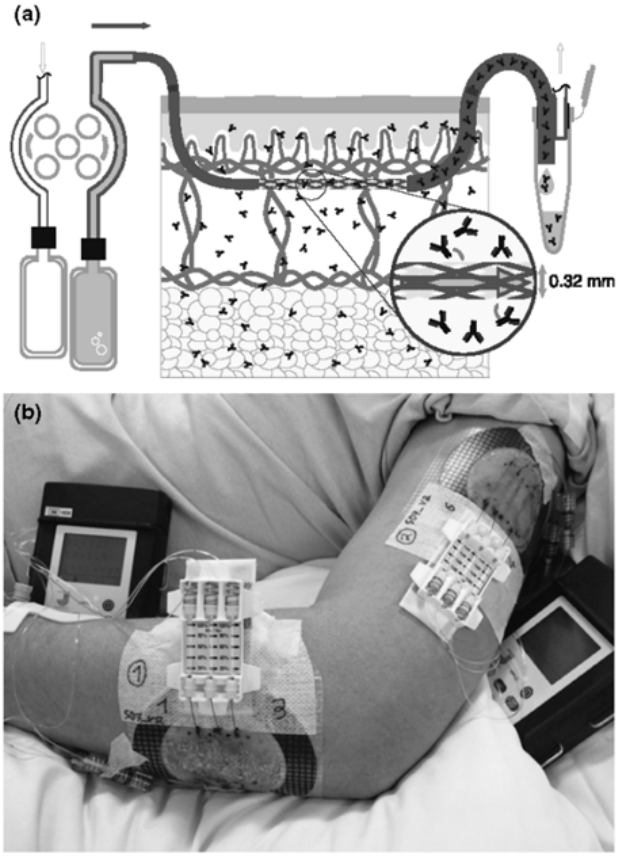

Figure 1. dISF sampling using dOFM. (a) Scheme of the dOFM sampling system. The dOFM probe is placed in the dermis and continuously perfused with a sterile perfusate. Direct exchange between perfusate and dermal interstitial fluid takes place through the macroscopic openings of the probe. The samples collected for the subsequent analysis contain all molecules (e.g. antibodies) that are present in the dISF. (b) Picture of the clinical dOFM set-up. Set of three parallel probes, each with a sampling unit and a perfusion pump placed in lesional skin (lower arm) and non-lesional skin (upper arm) of a psoriasis subject.

for secukinumab and sinistrin quantification in serum were collected at least hourly. After sampling on Day 1, each subject received a single subcutaneous dose of 300 mg secukinumab. Biopsy and suction blister samples were collected in healthy subjects on Day 15.

Samples were analysed for secukinumab by a competitive ELISA and for sinistrin by a validated enzymatic method. Details can be found in Appendix S1.

Results

In healthy subjects, mean secukinumab serum concentrations were 36.1 ± 10.5 μg/ml (Day 8) and 35.0 ± 10.5 μg/ml (Day 15). Measured with sinistrin as reference, secukinumab concentrations in dISF were 7.8 ± 2.7 μg/ml (Day 8) and 8.0 ± 3.2 μg/ml (Day 15) (Fig. 2a). Punch biopsies and suction blisters yielded similar concentrations of 10.4 ± 4.0 μg/ml and 6.9 ± 2.3 μg/ml, respectively ($P > 0.05$) (Fig. 2b).

In psoriasis subjects, the mean secukinumab serum concentrations were 21.1 ± 4.3 μg/ml (Day 8), 21.2 ± 4.9 μg/ml (Day 15) and 17.8 ± 5.1 μg/ml (Day 22). Secukinumab concentrations in dISF were 8.3 ± 3.4 μg/ml in non-lesional skin and 6.8 ± 2.7 μg/ml in lesional skin on Day 8 ($P > 0.05$) measured with sinistrin as reference. On Day 15, secukinumab concentrations were slightly lower, 6.4 ± 3.4 μg/ml in non-lesional skin and 5.7 ± 1.8 μg/ml in lesional skin ($P > 0.05$) (Fig. 2c). Tables S2/S3 show individual secukinumab concentrations, Appendix S1 and Fig. S1 show details of the sinistrin reference. Secukinumab No-Net-Flux data are shown in Figs S2 and S3.

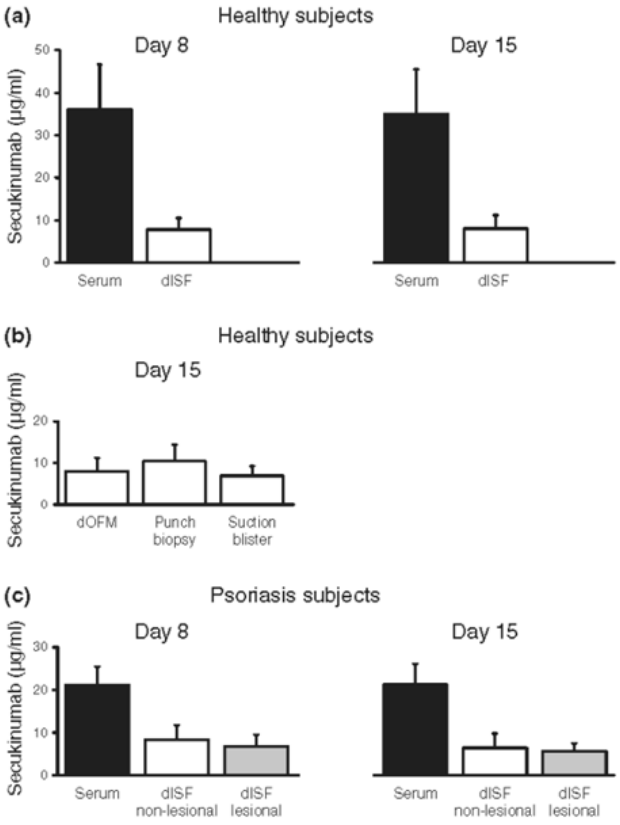

Figure 2. Secukinumab concentrations (mean ± SD) after a 300 mg subcutaneous dose. (a) Serum and healthy skin dISF concentrations in healthy subjects on Day 8 and Day 15. (b) Concentrations determined by dOFM, punch biopsy and suction blister in the skin of healthy subjects on Day 15. (c) Serum, non-lesional skin and lesional skin dISF concentrations in psoriasis subjects on Day 8 and Day 15.

After a single dose of secukinumab 300 mg, the mean TSS (total sum score related to a single plaque) dropped significantly from 6.5 ± 0.8 to 2.6 ± 1.1 ($P < 0.0001$) from Day 1 to Day 22. The mean PASI (psoriasis area and severity index) decreased significantly by 58% from Day 1 to Day 22 ($P < 0.0001$). There were no serious adverse events.

Conclusions

In this study, we found similar serum concentrations of secukinumab on Day 8 and Day 15 postdosing of secukinumab 300 mg on Day 1. Secukinumab concentrations in the skin were also relatively consistent over time. This suggests similar secukinumab pharmacokinetics in blood and skin without skin-specific retention. Overall, there were lower mean secukinumab serum concentrations in psoriasis subjects compared to healthy subjects which might have been caused by higher body weights with accompanying higher clearance (S13).

Absolute secukinumab concentrations determined by suction blister and punch biopsy in healthy subjects corresponded to dOFM results. The 28% to 39% dISF secukinumab concentrations relative to serum in psoriasis subjects suggest a somewhat higher distribu-

tion of secukinumab in the skin of psoriasis subjects compared to 23% in healthy subjects. These concentrations in skin are consistent with tissue concentrations of other therapeutic antibodies, for example relative to plasma 28% of an antibody were found in the synovial fluid of patients with rheumatoid arthritis (S14).

The clinical efficacy of the measured secukinumab concentrations is supported by a significant decrease in PASI as well as TSS of the target plaque. The mean secukinumab concentration (46 nM) measured on Day 8 in dISF from lesional skin indicates a clear molar excess of secukinumab molecules compared to the mean level of IL-17A molecules detected at baseline in the same plaques (0.31 pM, with a maximum of 2.28 pM) (S15).

In summary, dOFM can readily be used to quantify dISF concentrations of therapeutic antibodies such as secukinumab in the skin of healthy subjects as well as psoriasis subjects. We were able to verify levels of secukinumab in the dISF of psoriasis subjects as early as one week after secukinumab injection in quantities that appear sufficient to completely neutralize IL-17A in skin.

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Conflict of interests

This study was funded by Novartis. FP, CC, RW, YC, CL and GB are employees and shareholders at Novartis. All other authors have no conflict of interest to disclose.

Author contribution

CD, FP, MB, BA, RW, TRP, CL, PS and GB conceptualized and designed the study; CD, MB, BA, KIT and JKM performed the clinical experiments; CC and MR were in charge of sample analysis; CD, MB, YC and PS analysed the data; FP, RW, YC, CL and GB interpreted the data; CD drafted the manuscript; and all authors reviewed and revised the manuscript.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Appendix S1. Supplementary Material and Methods, Results and References.
Figure S1. Validation of sinistrin reference.
Figure S2. Secukinumab No-Net-Flux in healthy subjects.
Figure S3. Secukinumab No-Net-Flux in psoriasis subjects.
Table S1. Study population - Demographic summary.
Table S2. Subject individual secukinumab concentrations in healthy subjects.
Table S3. Subject individual secukinumab concentrations in psoriasis subjects.

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Supplementary Material and Methods

Study design

This study was a single-center, open-label, exploratory study (ClinicalTrials.gov Identifier: NCT01539213) to evaluate the distribution of subcutaneously administered secukinumab in the dISF of healthy subjects and psoriasis subjects. It was carried out at the Clinical Research Center at the Medical University of Graz, Austria. The study was approved by the local ethics committee and the Austrian Agency for Health and Food Safety (AGES) and conducted according to GCP and the Declaration of Helsinki. Written informed consent was obtained from each subject before study start.

The study was divided into two parts: In part I, we established the quantification of secukinumab in healthy subjects by using a No-Net-Flux procedure and also by using sinistrin as an external reference substance. In part II the established methodology was applied to investigate the dermal secukinumab concentrations in psoriasis subjects.

Participants

Eight healthy subjects and eight psoriasis subjects were included (Table S1). Eligibility criteria for patients were a diagnosis of chronic plaque psoriasis of at least 6 months. Furthermore, patients had to have at least one plaque suitable for sampling with dOFM that had a Total Sum Score (TSS) of at least 6. Each item of the TSS, namely erythema, plaque thickening and scaling, needed to show at least moderate severity (score of 2).

Study drug

Secukinumab (Novartis Pharma AG, Basel, Switzerland) was provided as 150 mg of lyophilized powder in a syringe. The lyophilizate was reconstituted with 1 ml sterile water for injection. 300 mg of secukinumab were administered in the abdominal region via two subcutaneous injections of 1 ml each.

Reference substance

Sinistrin (Inutest 25%; Fresenius Kabi Austria GmbH, Linz, Austria) is a metabolically inert polysaccharide that distributes equally between all extracellular body compartments at steady-state. On dOFM study days an intravenous bolus was administered followed by a continuous infusion until the end of the sampling period.

dOFM set-up

The dOFM probe (DEA15001; Joanneum Research, Graz, Austria) is a flexible, linear, membrane-free sampling probe characterized by a 15 mm sampling area with macroscopic openings which allow free exchange of large molecules. The probe is CE-certified for use in humans and continuous sampling of up to 48 hours. For a scheme of the dOFM sampling system see Figure 1a. The dOFM probe is inserted into the skin using a 0.7 mm insertion needle and perfused at 1 µl/min using the wearable dOFM pump (8). The dermal placement of each dOFM probe was verified by a 50 MHz ultrasound device (DUB-USB75; Taberna Pro Medicum, Lueneburg, Germany). The perfusate was physiological saline (ELO-MEL isoton; Fresenius Kabi, Graz, Austria) with 1% human serum albumin (Albunorm 20%; Octapharma, Vienna, Austria). Samples were collected hourly, stored at 4°C to 8°C till the end of study day, finally pooled and frozen at -80°C. Since dOFM results in a diluted but otherwise unchanged sample of the interstitial fluid, we applied a No-Net-Flux procedure and a calibration procedure with a reference substance for quantification.

Interventions

In part I, three dOFM probes were inserted into the arm of each healthy subject on Days 1, 8 and 15. At the end of Day 1 each subject received a single subcutaneous dose of 300 mg secukinumab. Sinistrin was administered as a primed-continuous intravenous infusion and dOFM sampling was performed for up to 14 hours on each study day. On Day 8, six additional probes were inserted on the arm or leg for a sinistrin No-Net-Flux procedure. On Day 15, nine additional probes were used for a secukinumab No-Net-Flux procedure. Biopsy and suction blister samples were collected on Day 15. Blood samples for secukinumab quantification in serum were collected on Days 1, 8 and 15 and for sinistrin quantification repeatedly over the 14 hours sampling periods.

Each psoriasis subject in part II of the study had three probes additionally placed in lesional skin for continuous dOFM sampling on Days 1, 8 and 15 (Figure 1b). Sinistrin No-Net-Flux, punch biopsies and suction blister procedures were not performed in psoriasis subjects to avoid additional stress. Additional blood samples were collected at the follow up visit on Day 22.

No-Net-Flux procedure

We used two dOFM No-Net-Flux procedures for precise and direct quantification in the dISF (S10-12), one for secukinumab and one for sinistrin. For each analyte five different concentrations were perfused through the dOFM probes. For secukinumab the perfusate concentrations were 0, 5, 10, 15 and 20 µg/ml and for sinistrin 0, 125, 250, 375 and 500 µg/ml. Probes were perfused in ascending order for two hours each and one sample was generated every other hour. For data evaluation gain or loss (C_{out}-C_{in}) was plotted against the perfusate concentration (x-axis). A linear regression analysis using least-squares estimation was performed. The intersection of the regression line with the x-axis marks the analyte concentration in the dISF (Figure S2).

Calibration strategy

For quantification of secukinumab with the reference substance sinistrin, an intravenous bolus (0.05 g per kg of body weight) of sinistrin followed by a continuous infusion (0.25 mg/min per mg/l of creatinine clearance) was administered on Days 1, 8 and 15, to achieve steady-state concentrations of ~250 µg/ml in serum.

The equal distribution between serum and dISF was verified by the sinistrin No-Net-Flux on Day 8 in healthy subjects, with a serum to dISF ratio of 1.04 and a 90% confidence interval between 0.96 and 1.13 (Figure S1).

The relative recovery (RR) of sinistrin is defined as the ratio of the sinistrin concentration in the dOFM samples to the sinistrin concentration in the dISF. RR of sinistrin was determined for each dOFM sample by using the actual serum concentration as a surrogate for the dISF concentration. The mean RR of secukinumab of all dOFM samples was determined based on the secukinumab No-Net-Flux procedure results of Day 15 and used to calculate the proportionality factor between the RR of sinistrin and

secukinumab. The RR of sinistrin of each individual sample divided by this factor was used for quantification of secukinumab in the samples from healthy subjects as well as lesional and non-lesional skin of psoriasis subjects.

Suction blisters

Applying a negative pressure to the skin leads to the formation of a blister filled with ISF (S16). Three half-spherical blisters (~0.2 ml each) were generated by applying controlled suction (-300 mm Hg for about 2 hours) to the skin at selected sites on the arm. A medical suction device (LSU 4000; Laerdal, Vienna, Austria) connected to the barrels of three standard disposable 2 ml syringes was used.

Punch biopsies

Skin biopsies were taken from the lower back of healthy subjects using 4 mm skin punches (samples of full thickness skin) with prior local anesthesia.

To convert punch biopsy results (total skin tissue, skin biopsy homogenate, concentrations in µg/g) to dISF concentrations (µg/ml) we used a value of 110 ml dISF per kg skin tissue (S17).

Clinical endpoints TSS and PASI

The severity of the psoriatic target lesion was assessed by a Total Sum Score (TSS) which scores each trait of the target plaque, namely erythema, plaque thickening and scaling, from 0 to 4.

Additionally, the mean disease score was assessed using psoriasis area and severity index (PASI), which also takes the percentage of affected surface body area into account (S18).

Sample analysis

Secukinumab was analyzed using a validated ELISA method. The method used a purified, non-neutralizing, anti-idiotype anti-secukinumab antibody (Novartis Pharma AG, Basel, Switzerland) coated on microtiter plates. Serum samples (calibration samples, quality controls or regular samples) and biotin-labeled secukinumab samples were

simultaneously incubated and competed for binding to the anti-idiotypic anti-secukinumab antibody. Unbound material was removed by washing. Bound biotinylated secukinumab was detected by incubating horseradish peroxidase conjugated to streptavidin with O-phenylenediamine dihydrochloride as enzymatic substrate. Lower limit of quantification was 76 ng/ml for dOFM samples and blister fluid and 80 ng/ml for serum.

The biopsies were washed, weighted, a constant amount of lysis buffer (T-PER, Pierce Biotechnology, Rockford, IL, USA) was added and the dermal tissue was homogenized by a Polytron dispenser (PT1200E; Kinematica, Luzern, Switzerland). After centrifugation, the secukinumab concentration was determined in the supernatant with the same assay as for serum.

Sinistrin concentration was determined with an adapted enzymatic in-vitro test for analysis of glucose using 4 sub-steps with photometric detection of NADPH at 340 nm. The method was validated according to the ICH Q2(R1) guidelines. Lower limit of quantification was at 75 µg/ml for serum and 12.5 µg/ml for dOFM samples.

Statistical analysis

To compare secukinumab and sinistrin levels a fixed effects model was fitted to the log-transformed secukinumab and sinistrin concentrations by visit. Observed matrix or site and subject were included as fixed effects. The PASI and TSS data were also analyzed by a fixed effects model with visit as a fixed effect and baseline as continuous covariate. P-values less than 0.05 were considered to indicate statistically significant differences.

Supplementary Results

Validated calibration strategy

In healthy subjects the steady-state of sinistrin in serum was achieved within approximately 90 minutes with individual concentrations ranging from 253 µg/ml to 286 µg/ml on Days 1, 8 and 15. RR of sinistrin was 16% ± 3% and showed high reproducibility between probes, sites, study visits and among individuals. Steady-state sinistrin concentrations in serum of psoriasis subjects ranged from 256 µg/ml to 285 µg/ml on the Days 1, 8 and 15. With 15 ± 3% each, the RR of sinistrin was similar in non-lesional and lesional skin ($p>0.05$) with a high reproducibility of the RR of sinistrin between probes, sites, study visits and among individuals.

The average relative concentration of secukinumab determined by No-Net-Flux in the dISF of healthy subjects on Day 15 was 23% of the serum concentration (Figure S2). This corresponds to an absolute secukinumab concentration in the dISF of 8.0 µg/ml.

In one healthy subject, the No-Net-Flux procedure was not performed due to a local reaction after subcutaneous dosing of secukinumab. The data of another healthy subject was excluded due to inexplicably high concentrations of secukinumab in the dOFM No-Net-Flux samples.

The RR of sinistrin was 2.45-fold higher than the RR of secukinumab. Secukinumab concentrations in the skin of healthy subjects and psoriasis subjects were subsequently determined by using sinistrin as an external reference substance and were based on a correction factor of 2.45.

Safety

No serious adverse events or any discontinuation due to adverse events were recorded during the entire study period. All occurring adverse events were mild ones. One healthy subject was excluded from the No-Net-Flux procedure due to an injection site reaction and erythema after subcutaneous secukinumab injection, even though injection site reactions were rare in the two pivotal phase 3 trials (3).

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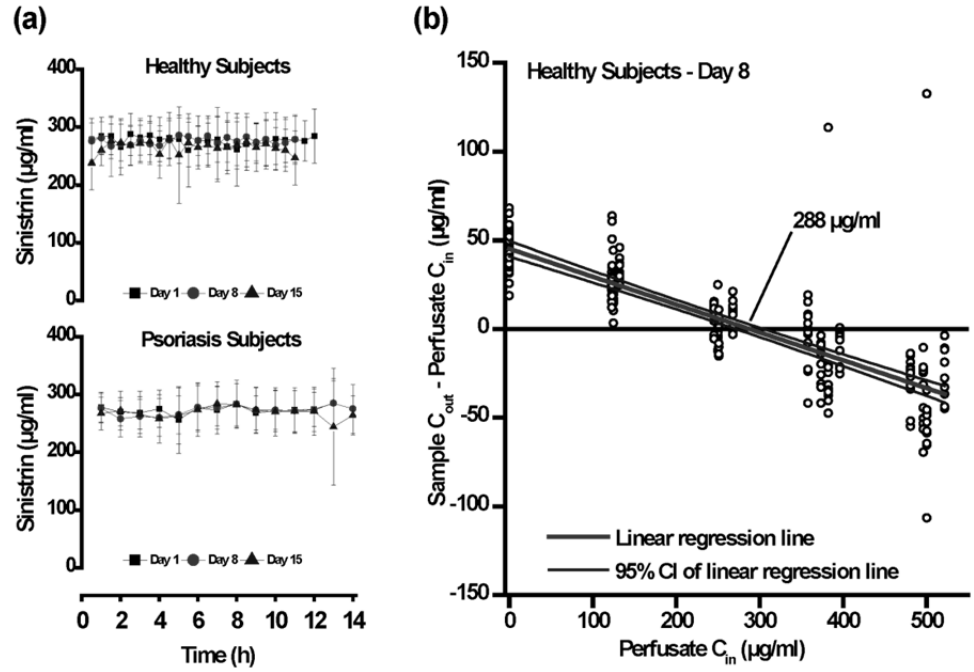

FIGURE S1: Sinistrin is equally distributed between serum and dermal ISF after primed-continuous intravenous infusion.

(a) Sinistrin serum concentrations (mean ± SD) are plotted over time. They reach a steady state in the range from 253 to 286 µg/ml in healthy volunteers and from 256 to 285 µg/ml in psoriatic patients. On Day 8, a mean serum concentration of 277 µg/ml was reached in healthy volunteers.

(b) No-Net-Flux plot for sinistrin in dermal ISF on Day 8 in healthy subjects. Gain or loss in the sample concentration is plotted against the perfusate concentration. The intersection with the linear regression line (R^2=0.66) at the concentration of 288 µg/ml depicts the concentration of sinistrin in the dermal ISF which is comparable to that in serum.

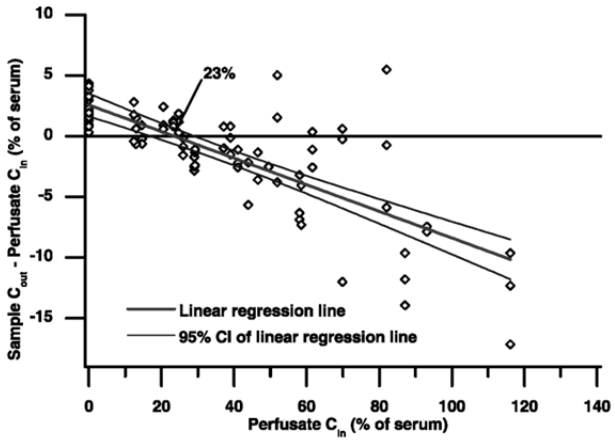

FIGURE S2: Secukinumab concentration in the dISF of healthy subjects on Day 15 was 23% of the mean serum concentration. No-Net-Flux plot of secukinumab in dISF on Day 15 with data points from six healthy subjects. Gain or loss in the sample concentration is plotted against the perfusate concentration. The intersection of the linear regression line (R^2=0.59) with the x-axis at a perfusate concentration of about 23% represents the concentration of secukinumab in the dISF relative to the serum concentration.

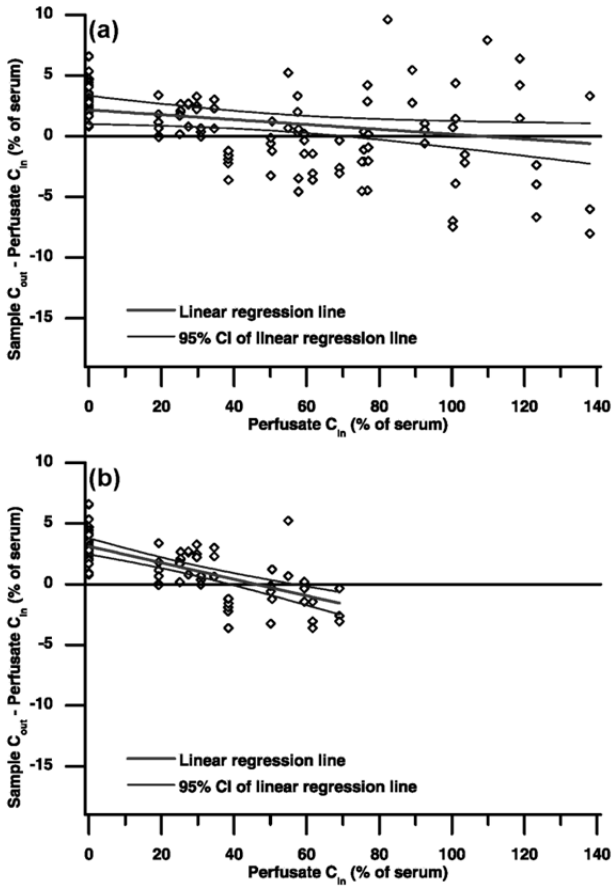

FIGURE S3: No-Net-Flux plot of secukinumab concentrations from eight psoriasis subjects at Day 15.

(a) Data from all five perfusate concentrations are shown. Partly uninterpretable results were obtained because no stable losses were achieved when using concentrated secukinumab perfusates higher than the tissue concentration. Thus linear regression analysis (R^2=0.03) failed to show linear relationship. Consequently these data were not included into further data processing.

(b) The removal of the perfusates with the two highest secukinumab concentrations would result in a reasonable linear regression line (R^2=0.41) and in an intersection point that is a little higher than that found in healthy volunteers. However, these data were not included into further data processing.

Table S1:

Study population - Demographic summary

		Healthy Subjects n=8	Psoriasis Subjects n=8
Age (years)	**Mean ± SD**	26 ± 6	39 ± 8
	Range	19 - 38	29 - 50
Sex (male)	**n**	6	8
Caucasian	**n**	8	8
Weight (kg)	**Mean ± SD**	78 ± 12	94 ± 7
	Range	59 - 91	86 - 106
Height (cm)	**Mean ± SD**	178 ± 9	182 ± 4
	Range	163 - 186	178 - 189
BMI (kg/m^2)	**Mean ± SD**	24.5 ± 2.3	28.4 ± 2.7
	Range	22.2 - 29.0	25.0 - 32.7
TSS[(1)]	**Mean ± SD**	n/a	6.5 ± 0.76
	Range	n/a	6 - 8

[(1)] TSS of the dOFM target plaque at Day 1

TABLE S2:

Subject individual secukinumab concentrations (µg/ml) in healthy subjects

Healthy Subjects	Day 8 Serum	Day 8 ISF dOFM	Day 15 Serum	Day 15 ISF dOFM	Day 15 ISF Punch biopsy	Day 15 Blister fluid Suction blister
1	24.0	4.4	18.6	4.5	7.5	3.3
2	43.0	8.2	36.9	6.4	18.2	7.1
3	39.9	8.5	43.0	8.3	10.0	6.9
4	39.2	11.0	45.1	9.8	8.1	11.3
5	51.5	12.2	42.1	15.0	13.7	7.3
6	25.7	5.8	27.3	6.1	5.9	5.4
7	42.4	7.0	43.6	7.2	11.3	6.1
8	23.0	5.3	23.0	6.9	8.6	7.7
Mean ± SD	**36.1 ± 10.5**	**7.8 ± 2.7**	**35.0 ± 10.5**	**8.0 ± 3.2**	**10.4 ± 4.0**	**6.9 ± 2.3**

Supporting Information Tables S1-3 Dragatin et al.

TABLE S3:

Subject individual secukinumab concentrations (µg/ml) in psoriasis subjects

Psoriasis Subjects	Day 8 Serum	Day 8 ISF dOFM non-lesional	Day 8 ISF dOFM lesional	Day 15 Serum	Day 15 ISF dOFM non-lesional	Day 15 ISF dOFM lesional
1	19.8	7.8	4.9	19.8	10.1	6.2
2	21.5	8.2	5.5	18.3	8.3	4.2
3	18.9	6.9	6.8	18.6	4.1	5.3
4	17.3	11.4	7.5	29.9	4.9	5.5
5	19.9	5.6	5.6	15.0	4.0	5.6
6	16.9	5.5	12.6	20.5	3.6	9.7
7	29.8	15.2	7.4	27.1	12.2	4.6
8	25.0	6.2	3.9	20.8	3.9	4.0
Mean ± SD	**21.1 ± 4.3**	**8.3 ± 3.4**	**6.8 ± 2.7**	**21.2 ± 4.9**	**6.4 ± 3.4**	**5.7 ± 1.8**

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original article

Diabetes, Obesity and Metabolism 17: 1166–1172, 2015.

ORIGINAL ARTICLE

Bioavailability of insulin detemir and human insulin at the level of peripheral interstitial fluid in humans, assessed by open-flow microperfusion

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Aims: To find an explanation for the lower potency of insulin detemir observed in humans compared with unmodified human insulin by investigating insulin detemir and human insulin concentrations directly at the level of peripheral insulin-sensitive tissues in humans *in vivo*.

Methods: Euglycaemic-hyperinsulinaemic clamp experiments were performed in healthy volunteers. Human insulin was administered i.v. at 6 pmol/kg/min and insulin detemir at 60 pmol/kg/min, achieving a comparable steady-state pharmacodynamic action. In addition, insulin detemir was doubled to 120 pmol/kg/min. Minimally invasive open-flow microperfusion (OFM) sampling methodology was combined with inulin calibration to quantify human insulin and insulin detemir in the interstitial fluid (ISF) of subcutaneous adipose and skeletal muscle tissue.

Results: The human insulin concentration in the ISF was ~115 pmol/l or ~30% of the serum concentration, whereas the insulin detemir concentration in the ISF was ~680 pmol/l or ~2% of the serum concentration. The molar insulin detemir interstitial concentration was five to six times higher than the human insulin interstitial concentration and metabolic clearance of insulin detemir from serum was substantially reduced compared with human insulin.

Conclusions: OFM proved useful for target tissue measurements of human insulin and the analogue insulin detemir. Our tissue data confirm a highly effective retention of insulin detemir in the vascular compartment. The higher insulin detemir relative to human insulin tissue concentrations at comparable pharmacodynamics, however, indicate that the lower potency of insulin detemir in humans is attributable to a reduced effect in peripheral insulin-sensitive tissues and is consistent with the reduced *in vitro* receptor affinity.

Keywords: adipose tissue, clinical trial, insulin analogues, pharmacodynamics, pharmacokinetics

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Introduction

Insulin detemir was the first clinically available representative of a new type of long-acting soluble insulin analogue [1–3]. Insulin detemir is a modified human insulin in which threonine in position B30 is deleted and a fatty acid at B29 is acylated to lysine [1,4,5]. The prolonged hypoglycaemic action and stable glycaemia of insulin detemir are believed to be based on di-hexamer formation and albumin binding in the depot and in plasma [1,3,5–7]. A multitude of studies in both humans [6–9] and animals [2,10] have shown that insulin detemir is able to stimulate glucose uptake and to suppress endogenous glucose production with a slower onset and without pronounced peak activity compared with NPH insulin. Further studies showed a reduced subject variability in pharmacodynamics [11,12] which might be associated with the albumin binding of insulin detemir [13], a reduced risk of hypoglycaemia and reduced weight gain in animal studies [14,15] and in patients with type 1 and 2 diabetes [5,16–19]. The flat time–action profile and the possibility of insulin administration only once a day has been demonstrated in several studies [20,21] and is based on a protracted action attributable to albumin binding. Beside these clinical advantages, insulin detemir has shown a lower molar potency relative to unmodified human insulin and thus insulin detemir is formulated at a fourfold higher molar concentration in commercially available preparations [3]. The lower molar potency of insulin detemir in humans has been explained by altered receptor binding and receptor dissociation, but also by plasma albumin binding which reduces the transcapillary transport to the target tissues [3,8,22,23]. Because of its slow absorption into the circulation and the fact that >98% of circulating insulin detemir is bound to albumin [24], only a minimal amount is available for the transcapillary transport to the target tissues [1,3]; however, the actual concentrations at the target sites have never been measured in humans, because no standard method for measuring insulin detemir directly at tissue level has been available to date. Even sampling of this predominantly albumin-bound insulin by the semi-permeable membranes in human microdialysis [25] is difficult.

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Membrane-free open-flow microperfusion (OFM) provides direct access to the interstitial fluid (ISF) of human tissue *in vivo* and substance sampling is not limited by size, protein binding, or lipophilicity [26–28]. The suitability of OFM for the *in vivo* sampling of human insulin and insulin detemir has recently been shown in rats [29]. By using OFM, together with a validated reference substance for calibration, insulin and other peptides can be reliably quantified. We therefore established the polysaccharide inulin as an exogenous reference substance for insulin quantification and subsequently used inulin to quantify insulin detemir and human insulin in peripheral insulin-sensitive tissue in humans. The aim of the present study was to investigate the relationship between pharmacodynamic and interstitial pharmacokinetic steady state, after intravenous (i.v.) administration of insulin detemir and human soluble insulin to explain the observed lower molar potency of insulin detemir in humans.

Materials and Methods

Open-Flow Microperfusion

Open-flow microperfusion has previously been described in detail [26,28,30–32]. Briefly, a membrane-free minimally invasive probe is inserted into the tissue of interest creating a direct access to the interstitium for analyte sampling. The probe is continuously perfused with a sterile isotonic solution which partially equilibrates with the surrounding ISF and provides a diluted but unfiltered sample of the ISF (Figure 1). By choosing a reference substance with similar exchange behaviour as the analyte, the 'relative recovery' (RR) and thus the dilution factor can be calculated. The RR value is subsequently used to determine the absolute interstitial analyte concentration.

Study Population and Clinical Trial

The study was conducted according to the declaration of Helsinki, followed Good Clinical Practice guidelines, and was approved by the local ethics committee of the Medical University of Graz (ClinicalTrials.gov NCT02162407). Written consent was obtained from all participants before inclusion in this study.

The study included 6 healthy male participants [mean ± standard deviation (s.d.) age 27.3 ± 5.7 years, BMI 23.8 ± 1.1 kg/m^2] in the inulin validation study and 11 healthy male participants (mean ± s.d. age 26.8 ± 5.1 years, BMI 23.8 ± 1.5 kg/m^2) in the clamp visits. After fasting overnight, participants arrived at 07:00 hours at the clinical research centre. They received local anaesthesia with procaine (Novanaest®, Gebro Pharma GmbH, Fieberbrunn, Austria) and then two OFM probes were placed in the adipose tissue of the abdominal region and another two OFM probes were inserted in the tissue of the rectus femoris muscle.

Inulin as Reference Substance

Inulin is a polysaccharide with a molecular weight which is similar to that of human insulin. Inulin is known to be metabolically inert and it readily distributes between serum and the peripheral tissue ISF after constant i.v. infusion [33]. At steady state, the serum inulin concentration reflects the interstitial inulin concentration. Thus, the serum inulin concentration can be used to calculate the RR for substances of similar molecular size in the ISF. Inulin has previously been used as a reference substance for insulin in microdialysis [33,34].

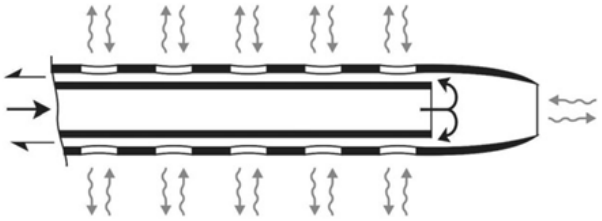

Figure 1. Schematic representation of the open-flow microperfusion (OFM) double lumen probe. After insertion into the tissue, the OFM probe is continuously perfused with a sterile isotonic perfusate via the inner lumen and the sample is simultaneously withdrawn via the perforated outer lumen. The perfusate passes by the macroscopic openings (0.5 mm) and leads to partial equilibration with the surrounding interstitial fluid in the tissue.

To verify that inulin distributes evenly between serum and ISF, we performed an inulin validation study using the no-net-flux approach [25,26,31]. One i.v. cannula was inserted into the forearm to collect serum samples. A vein in the contralateral arm was used for an i.v. inulin bolus (50 mg/kg, Inutest® Fresenius Pharma Austria GmbH, Graz, Austria), followed by a constant inulin infusion starting 2 h before the insulin infusion in order to achieve steady state. The individual inulin infusion rates [mean ± standard error of the mean (s.e.m.) 0.37 ± 0.02 mg/kg/min, range 0.31–0.47 mg/kg/min] were calculated using the Cockroft formula [35] to yield steady-state serum levels of ~250 mg/l. OFM probe perfusion started with 0.5 µl/min using an isotonic perfusate (Krebs-Ringer with 2 mmol/l glucose) and OFM sampling started after 150 min. Each of the OFM probes was perfused with three different perfusate concentrations. One perfusate contained no inulin, whereas the other two perfusates had an inulin concentration randomly chosen from four possible concentrations (125, 250, 375 or 500 mg/l). Serum inulin and OFM samples were collected in 60-min fractions for a period of 9 h. Inulin was quantified in the OFM samples and absolute interstitial inulin concentrations were determined by using linear regression analyses.

Pharmacokinetics and Pharmacodynamics

The clamp study was performed on three different visits as a three-period crossover trial to investigate the pharmacokinetics and pharmacodynamics of insulin detemir and human insulin. Participants received either human insulin (Actrapid® 100 IU/ml, Novo Nordisk, Bagsværd, Denmark) i.v. at a constant rate of 6 pmol/kg/min or insulin detemir at a constant rate of 60 or 120 pmol/kg/min during a period of 8 h. Euglycaemia (5 mM) was maintained by variable glucose infusion. On each study day, one forearm was kept in a thermoregulated box (55 °C) to obtain arterialized venous blood samples for tight glucose control and to obtain serum samples for inulin

indicate a differential hepatic effect, but the present study was not designed to investigate hepatoselectivity of insulin detemir.

Similarly to earlier pharmacodynamic observations, we also found that the time to steady-state GIR was much shorter for human insulin [10,43]. For both insulin types the time to steady-state GIR did not correspond to the time to steady state of insulin in serum, which indicates that peripheral ISF could be more relevant for stimulating glucose uptake than serum and should therefore be used for insulin measurements. We did not find a proportional increase in the GIR with the high insulin detemir dose, although insulin detemir serum and muscle ISF concentrations were doubled. It is questionable whether results from the present study can be extrapolated to more physiological conditions because the very high insulin detemir doses administered i.v. in this experimental trial are not comparable with subcutaneous bolus doses during treatment trials. Steady-state levels of insulin are usually not obtained in clinical trials and the importance of transendothelial transport of insulin might vary with the concentrations given.

This first study of insulin detemir concentrations at the interstitial level in humans shows a highly effective retention in the vascular compartment according to insulin detemir's degree of plasma albumin binding. The lower insulin detemir potency in humans, however, can be explained by a reduced effect at peripheral target cells in line with the known reduced *in vitro* receptor affinity. Our findings add substantial information to the understanding of the pharmacological properties of human insulin and acylated insulin analogues. Moreover, this report describes the validation and utilisation of clinical *in vivo* sampling methodology and thus may serve as a guide for more informative studies of drugs at their target in humans.

Acknowledgements

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Conflict of Interest

The authors declare that there are no conflicts of interest that might bias their work. L. V. J. is employed by Novo Nordisk A/S, Denmark.

M. B. contributed to the preparation, conduct of the study, acquisition of data, data analysis and manuscript preparation. M. E., L. S., L. V. J., J. P., G. A. B., A. W., T. P. contributed to the design, conduct, data analysis and manuscript preparation. B. A. and S. I. M. contributed to the data analysis, interpretation and manuscript preparation. All authors approved the final manuscript.

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Clinical applicability of dOFM devices for dermal sampling

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Background: Sampling the dermal interstitial fluid (ISF) allows the pharmacokinetics and pharmacodynamics of dermatological drugs to be studied directly at their site of action. Dermal open-flow microperfusion (dOFM) is a recently developed technique that can provide minimally invasive, continuous, membrane-free (thus unfiltered) access to the dermal ISF. Herein, we evaluate the clinical applicability and reliability of novel wearable dOFM devices in a clinical setting.
Methods: Physicians inserted 141 membrane-free dOFM probes into the dermis of 17 healthy and psoriatic volunteers and sampled dermal ISF for 25 h by using wearable push-pull pumps. The tolerability, applicability, reproducibility, and reliability of multiple insertions and 25 h continuous sampling was assessed by pain scoring, physician feedback, ultrasound probe depth measurements, and 25 h-drift and variability of the sodium relative recovery.
Results: Insertion pain was moderate and decreased with each additional probe. Probe insertion was precise, although slightly deeper in lesional skin. The wearable push-pull pump enabled uninterrupted ISF sampling over 25 h with low variability. The relative recovery was drift-free and highly reproducible.
Conclusion: dOFM sampling devices are tolerable and reliable for prolonged continuous dermal sampling in a multiprobe clinical setting. These devices should enable the study of a wide range of drugs and their biomarkers in the skin.

Key words: open-flow microperfusion – *in vivo* – human – interstitial fluid – cutaneous – pharmacokinetics – pharmacodynamics – tolerability – push-pull – bioequivalence

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VARIOUS METHODS have been developed for pathophysiologic and pharmacological *in vivo* investigations in the skin (1, 2). Traditional methods such as biopsies and suction blister provide samples directly from the living dermis of the skin. However, the invasive nature of those methods and the limited information they provide means they are not optimal for use in clinical studies involving volunteers (3). Therefore, an *in vivo* method that provides direct access to dermal target tissue, is less invasive and provides more information would be of great benefit for clinical pharmaceutical research (3); that is, evaluation of drug penetration and bioequivalence, and optimization of formulations. The ideal sampling method and devices for clinical dermatological trials should be (i) compatible with medical device regulations, (ii) safe and tolerated by volunteers, (iii) reliable with regard to results obtained from the site of drug action, and (iv) data-rich to enable trials with high statistical power in as few subjects as possible (see Table 1).

According to these criteria, the best-performing method to date has been dermal microdialysis (4–9), with its minimally invasive dialysis probes for continuous intradermal sampling. However, current dermal microdialysis still suffers a number of drawbacks: (i) the probe's semipermeable membrane limits the range of substances that are accessible with respect to size and lipophilicity (1, 2, 4, 10), (ii) there is no wearable multi-channel pump that enables sampling from several probes in parallel over a longer period (24 h or more) in humans, and (iii) there is no pump that exploits the advantages of high-cut-off dermal microdialysis probes ($\geq$100 kDa), which have been shown to benefit from a push-pull pump to

TABLE 1. Criteria for the ideal sampling technique

For the volunteer	For the clinical scientist ('user')	
Safe	Tissue-specific placement in target tissue e.g. the dermis	True concentrations, suitable for quantification
No pain	Multiple use in parallel	Sufficient sample volume
Non- or minimally invasive	Multiple and/or paired test sites	No discrimination of analytes, i.e. total matrix for bioanalysis (unchanged ISF composition)
No scars	Continuous or repeated sampling	Preclinical use in vivo/ex vivo in addition to human in vivo
No pigment changes	Long application (and observation) times	
Access to normal bathroom visits during experiment	Low methodological variability	

prevent volume loss during perfusion and to yield higher recoveries of larger molecules (11).

To address these issues, a minimally invasive continuous sampling method has been developed that allows direct *in vivo* access to target tissue interstitial fluid: open-flow microperfusion (OFM) (12–14). OFM utilizes macroscopically fenestrated probes (i.e. it is membrane free), which expands the range of accessible analytes to include very large and lipophilic substances. This increased sampling capacity has the potential to be of significant benefit in dermal pharmacokinetic-pharmacodynamic (PK/PD) sampling; particularly in studies of topical drugs and formulations, because both the drug itself (generally lipophilic) and its biomarkers (high molecular weight) can be assessed simultaneously. Therefore, the OFM concept has been adapted for intradermal sampling (dermal OFM, or dOFM) and its feasibility demonstrated in a PK/PD trial (15). The devices have been developed further to medical device standards and have recently received CE certification for human use.

The aim of this exploratory study was to evaluate the applicability and tolerability of the dOFM devices in healthy and lesional skin sites on 17 volunteers. Continuous dermal sampling over 25 h with subsequent investigation of the variability of the sampled sodium concentration (also) provided data on sampling reliability and reproducibility.

Materials and Methods

dOFM devices and standard procedures

The dOFM probe (DEA15001; Joanneum Research) is a CE-certified, flexible, linear, membrane-free sampling probe with a 15 mm braided (stent-like) sampling section (outer diameter 0.32 mm). The probe has (i) an anti-adsorptive inner coating, (ii) visible surface marks for exact positioning below the testing area, (iii) minimal-volume luer connectors and (iv) a 0.5 mm diameter needle bonded to the probe for sterile probe insertion in humans.

The wearable multi-channel push-pull pump (MPP101, Joanneum Research) enables perfusion of several sampling probes in mobile subjects. Its multi-channel design with two peristaltic pump heads provides variable perfusion modes, including push, pull, or both. The pump generates a flow rate ranging from 0.1 to 10 µL/min, which is independently adjustable for push and pull, and is used with sterile 10 mL perfusate bags and a sterile push-pull tubing kit with minimal-volume connectors. All parts are CE certified for human use (Fig. 1). The design enables all known protocols for quantitation (zero flow, no net flux, recirculation, and suction (14)) with simultaneous operation of up to six probes of any type (OFM or MD).

The sampling unit (SCS001, Joanneum Research) connects sampling capillaries directly to the probe outlets. The sampling unit enables fractionized collection of dOFM samples in a

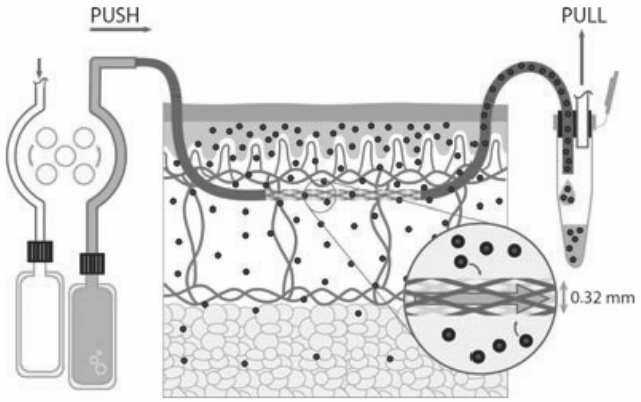

Fig. 1. Schematic drawing of a dOFM sampling system. The membrane-free dOFM probe is permeable to all molecules present in the interstitial milieu of the target tissue. After insertion, the probe's inlet is connected to a pump for continuous perfusion with a sterile perfusate (push), and the outlet to a sampling vial that is evacuated by the pump to control the sampling rate from the probe (pull). This push-pull set-up enables volume-controlled interstitial fluid sampling from the dermis for subsequent analysis.

drug. Thus, the use of several such wearable pumps per subject will maximize the output of paired data from each study volunteer, and will also help to include sampling sites for proper control, as has been demanded for studies on inflammatory markers (25, 26). The pump's push-pull design and features may also prove useful for high molecular weight cut-off microdialysis to prevent perfusate loss (11) as well as for quantitation of absolute levels of analytes in the dermis or other tissues, because the pump supports all protocols known in OFM or microdialysis for quantitation purposes (no-net-flux, retrodialysis, suction/ultrafiltration, zero flow, and recirculation).

A key advantage of the dOFM probe is that it should provide reliable access to all biochemical entities and drugs, even if they are very large and/or lipophilic, because the probe has no membrane, and includes an anti-adsorptive coating. This avoids the need for prior testing and specific perfusates (6). Thus, dOFM may facilitate sampling studies on skin pharmacology and physiology, but may also be used in other peripheral tissues. In future studies, we aim to explore the method's limits by sampling a wide range of substances from small, lipophilic topical drugs to large inflammatory biomarkers and – even larger – therapeutic antibodies with a molecular weight beyond 100 kDa.

The dOFM technique and the sampling devices presented here may represent a powerful new tool for testing skin pharmaceuticals because they can be used right through from the preclinical to the clinical phase. The method should provide PK/PD data in the preclinical phase when used *in vivo* in animals or *ex vivo* in human skin (27). As demonstrated in this study, dOFM is safe and well tolerated by volunteers and thus can be carried forward into the clinical phase, where it may enable very early PK/PD studies, in which very small doses of the drug are applied topically just above the intradermal probes.

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Conflict of interest: M. Bodenlenz, C. Höfferer, T. Birngruber, L. Schaupp and J. Priedl have filed patents (pending) associated with the evaluated devices.

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Research Paper

Dermal PK/PD of a lipophilic topical drug in psoriatic patients by continuous intradermal membrane-free sampling

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ABSTRACT

Background: Methodologies for continuous sampling of lipophilic drugs and high-molecular solutes in the dermis are currently lacking. We investigated the feasibility of sampling a lipophilic topical drug and the locally released biomarker in the dermis of non-lesional and lesional skin of psoriatic patients over 25 h by means of membrane-free dermal open-flow microperfusion probes (dOFM) and novel wearable multi-channel pumps.

Methods: Nine psoriatic patients received a topical p-38 inhibitor (BCT194, 0.5% cream) on a lesional and a non-lesional application site once daily for 8 days. Multiple dOFM sampling was performed for 25 h from each site on day 1 and day 8. Patients were mobile as dOFM probes were operated by a novel light-weight push–pull pump. Ultrasound was used to verify intradermal probe placement, cap-LC–MS/MS for BCT194 and ELISA for TNFα analysis.

Results: dOFM was well tolerated and demonstrated significant drug concentrations in lesional as well as non-lesional skin after 8 days, but did not show significant differences between tissues. On day 8, TNFα release following probe insertion was significantly reduced compared to day 1.

Conclusions: Novel membrane-free probes and wearable multi-channel pumps allowed prolonged intradermal PK/PD profiling of a lipophilic topical drug in psoriatic patients. This initial study shows that dOFM overcomes limitations of microdialysis sampling methodology, and it demonstrates the potential for PK/PD studies of topical products and formulations in a clinical setting.

1. Introduction

A critical stage in the development of topical drugs is the provision of evidence for effective penetration of the skin's barrier layer, the stratum corneum, and evidence for the efficacy at the intended target site of action, the dermis, in clinical trials [1–3]. Traditional methods in skin research such as punch or shave biopsies, suction blister [4] and tape stripping [5,6] provide limited kinetic information [1,7]. Furthermore, biopsies and blisters are too invasive for multiple or repetitive application in volunteers, and tape stripping does not provide information from the dermis being the site of action. Progress was made with the introduction of the minimally invasive, tissue-specific continuous approach of microdialysis (MD) [8,9] into dermatological research in 1991 [10] and then with the comprehensive evaluation of dermal microdialysis (DMD) for topical drug sampling [11,12]. MD proved to be a versatile, safe and valuable tool for pharmacokinetic (PK) and pharmacodynamic (PD) studies [7]. Hence, MD and DMD have also gained attention as tools for drug testing by authorities [1,3].

However, MD has some limitations when sampling lipophilic, protein-bound and high-molecular drugs [1,7,13–17]. Due to non-specific membrane binding and size exclusion, these compound classes show low or no recovery in MD. These shortcomings particularly affect research on the PK/PD of anti-inflammatory drugs for topical treatment of skin disease. These topical drugs are designed as lipophilic entities to provide skin-selectivity [18,19], and the associated markers (cytokines) are of high

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molecular weight (MW). Thus, many dermatological drugs and PD markers pose unique challenges for MD sampling [20].

To overcome these limitations, we developed a novel type of dermal probe based on open-flow microperfusion (OFM). OFM probes are free of a dialysing membrane and thus provide a direct access to the tissue milieu [21] in order to recover molecules regardless of MW or lipophilicity. OFM has been repeatedly used as an alternative to MD in adipose and muscle tissue to study protein and hormone concentrations [22,23]. Previously, we have successfully used dermal OFM (dOFM) in rat skin *in vivo* [24], but have not, until now, used dOFM in healthy or diseased human skin, or for PK/PD sampling of a lipophilic substance.

Furthermore, there is a lack of pumps enabling informative and efficient MD or OFM studies in a clinical setting. Clinical PK/PD studies of slowly permeating dermatologicals require sampling periods exceeding the typical duration of 5–10 h [5] and thus require a wearable pump. Bioequivalence studies of formulations require parallel operation of several probes [16,25] and thus a multichannel pump. OFM probes and high-MW MD probes require rate-controlled perfusate supply ('push') and ideally also rate-controlled sample withdrawal ('pull') for stable sample volume retrieval [26] and maximum analyte recovery [27]. With the aim to enable longer sampling periods and simultaneous operation of several probes, we also designed a wearable multichannel push–pull pump.

The purpose of this initial clinical study was to explore the technical feasibility of prolonged and multiple dOFM sampling of a lipophilic topical drug and a high-MW inflammatory marker in non-lesional and lesional skin of psoriatic patients.

2. Materials and methods

2.1. Lipophilic topical drug

BCT194 (Novartis Pharma AG, Switzerland) is a lipophilic ($\log P = 3.1$) investigational entity designed as a topical anti-psoriatic drug to inhibit the p38-pathway and thus the intradermal release of pro-inflammatory cytokines, such as the high-MW inflammatory mediator TNFα (51 kDa as trimer). BCT194 0.5% cream was supplied in 10 g tubes by Novartis Pharma AG and stored at 4 °C.

2.2. Dermal OFM probe

The dermal OFM probe had a linear design and was manufactured from PTFE-Tubing (OD 0.4 mm, ID 0.2 mm, Art. Nr. S 1810-02, Bohlender GmbH, Grünsfeld, Germany). A helical slit was cut at a length of 15 mm to serve as an exchange area between inner lumen and surrounding tissue (Fig. 1). This tubing was directly combined with a small needle (40 mm long, outer diameter 0.5 mm) by a crimping technique to form an easily implantable, minimally invasive probe. The probe was sterilised by ethylene oxide at the Medical University of Graz.

2.3. In vitro feasibility and adsorption tests

To test adsorption behaviour of dermal probes for BCT194, two BCT194 solutions (0.2 ng/ml) and 1 ng/ml BCT194 in ELO-MEL + 0.5% BSA) were pumped through 80 mm PTFE tubing used to manufacture the dermal probes. The outflow was sampled in sealed tubes (LoBind tubes, 200 µl, Eppendorf, Germany) and quantified for BCT194. The same test was done with 80 cm of Tygon® tubing (Saint-Gobain Performance Plastics, F), which has often been attached to the probe outlet for sampling in a pull manner.

2.4. Wearable dermal OFM set-up

A wearable light-weight push–pull 6-channel micropump was designed enabling simultaneous rate-controlled perfusion of six probes in push or three probes in push–pull.

In vitro results led to the design of a novel collection unit that couples 20 µl glass capillaries (Minicaps, Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) directly to the probe outlets and thus enables sample collection without contact to any tubing. To achieve pull-sampling, the pump's three pull-channels were used to evacuate the glass capillaries. Sampling concept and devices (Fig. 1) were developed by Joanneum Research, Graz, Austria.

2.5. Perfusate

The perfusate was based on isotonic saline (ELO-MEL isoton, Fresenius Kabi Austria; electrolyte composition in mmol/l: 140 Na^+, 5 K^+, 2.5 Ca^{2+}, 1.5 Mg^{2+} and 108 Cl^-). Individual serum from each subject was added (ELO-MEL + Serum = 4 + 1) [22] to ensure a ~1% albumin content. Serum was obtained on day 1 and day 8 immediately before dOFM application.

2.6. Patients

The study was approved by the Ethical Committee of the Medical University of Graz and the Austrian health authorities (AGES) and was performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Twelve psoriatic patients (part I: 3, part II: 9) having untreated stable plaque psoriasis were recruited (Caucasian; 11 men, 1 woman, 37.7 ± 18.0 years with 12.1 ± 11.5 years of psoriasis) and gave written informed consent. Subjects were otherwise healthy and screened for eligibility assessing their medical history, physical parameters and laboratory parameters. Concomitant medication was not allowed, and women had to use contraception.

2.7. Study design

The study was designed as a single-centre, open-label exploratory phase I trial. In part I, dOFM was investigated in three subjects for 25 h on a single occasion (day 1), and BCT194 was dosed only once. The interim analysis was used to identify an appropriate quantification limit for BCT194 from dOFM samples. In part II, nine subjects were dosed daily from day 1 to day 8. dOFM was done on day 1 and day 8, and skin biopsies were taken as reference.

Patients were admitted to the clinic at 7 am (day 1). Two lesional and two non-lesional sites were chosen for drug application. One lesional and one non-lesional site were used for dOFM sampling, the other two for biopsies. For dOFM application sites (18 mm diameter), dOFM insertion points were marked using a surgical marker. Following skin disinfection, three probes were implanted intradermally in each site by using a needle holder for insertion guidance. Pain relief was provided by cooling the implantation sites with an ice pack prior to probe insertion, thus providing effective anaesthesia [16] and tolerability of multiple dOFM probe insertions as has recently been demonstrated in a study on 17 volunteers using pain scoring (Bodenlenz et al., unpublished data). The insertion needles were immediately removed by using scissors and, to prevent movement, probes were fixed to the skin using adhesive medical tape. One wearable dOFM push–pull sampling unit was mounted close to each of the two sampling sites and connected to three probes each (Fig. 2). Probes were flushed with perfusate at ~2 µl/min for 10 min and their patency verified. Thereafter, flow was set to the nominal perfusion/sampling flow of 0.25 µl/min. After a run-in period of 60 min, allowing the insertion trauma to subside [28], a baseline (pre-dose) sample was

enable informative and efficient clinical trials on the pharmacokinetics and pharmacodynamics of a wide range of topical drugs. More trials are necessary to validate this novel continuous approach against traditional methods.

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RESEARCH PAPER

Comparison of Open-Flow Microperfusion and Microdialysis Methodologies When Sampling Topically Applied Fentanyl and Benzoic Acid in Human Dermis *Ex Vivo*

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ABSTRACT

Purpose To compare two sampling methods—dermal Open-Flow Microperfusion (dOFM) and dermal Microdialysis (dMD)—in an international joint experiment in a single-laboratory setting. Using human *ex-vivo* skin and topically administered Fentanyl and Benzoic Acid, and to guide to researchers in choosing the most efficient method for a given penetrant and give suggestions concerning critical choices for successful dermal sampling.

Methods The dOFM and dMD techniques are compared in equal set-ups using three probe-types (one dOFM probe and two dMD probe-types) in donor skin (n=9) - 27 probes of each type sampling each penetrant in solutions applied in penetration-chambers glued to the skin surface over a time range of 20 h.

Results Pharmacokinetic results demonstrated concordance between dOFM and dMD sampling technique under the given experimental conditions. The methods each had advantages and limitations in technical, practical and hands-on comparisons.

Conclusion When planning a study of cutaneous penetration the advantages and limitations of each probe-type have to be considered in relation to the scientific question posed, the physico-chemical characteristics of the substance of interest, the choice of experimental setting e.g. *ex vivo/in vivo* and the analytical skills available.

KEY WORDS dermal · *ex vivo* · human · microdialysis · open-flow microperfusion

INTRODUCTION

Traditional microdialysis (MD) is a sampling technique, which can be used *ex vivo* as well as *in vivo* and in all organs including the skin-dermal microdialysis (dMD). The technique has existed for dermal use since 1991 (1) and provides chronological, real-time pharmacokinetics of drugs and other substances. Dermal MD is a unique technique for *in vivo* sampling of topically as well as systemically administered drugs at the site of action, e.g. sampling the unbound tissue concentrations in the dermis and subcutaneous tissue Fig. 1. The method has undergone significant development, improvement and validation during the last decade and is a useful and safe tool in pharmacokinetic and pharmacodynamic studies (2). Sampling of large, highly lipophilic and/or protein-bound substances has, however, always been a challenge in MD when using the traditional MD probes and perfusates. Traditional probe membranes have a low MW cut-off, which may result in a negligible concentration of the

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Rapid online-SPE-MS/MS method for ketoprofen determination in dermal interstitial fluid samples from rats obtained by microdialysis or open-flow microperfusion

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Abstract

Pharmacokinetic studies of topical ketoprofen formulations using continuous sampling techniques such as microdialysis (MD) or open-flow microperfusion (OFM) require sensitive assays due to small sample volumes. A simple and easy online-SPE-MS/MS method for ketoprofen analysis was developed for both MD and OFM samples obtained from rat dermal tissue. The quantification range is 25–5000 ng/ml with a limit of detection of 3 ng/ml using only 10 μl sample volume. The method is characterized by a simple setup using a short polymeric SPE column (OASIS HLB) for desalting with 1.5 min run times in combination with a sensitive MS detection in negative ESI MRM mode. An easy sample workup procedure was used which enables high throughput analysis of a large number of samples for pharmacokinetic studies. In addition, a commercial available (fenoprofen) as well as an isotopically labelled (deuterated ketoprofen) standard were investigated as potential internal standards. The method was validated according to FDA guidelines for bioanalytical validation in terms of accuracy, intra-batch and inter-batch precision, linearity, matrix effect, recovery and stability for both internal standards. Accuracies were 98–113% (fenoprofen) and 95–108% (deuterated ketoprofen), intra-batch precision was 2–3% R.S.D. (fenoprofen) and 2–6% R.S.D. (deuterated ketoprofen), and inter-batch precision was 2–6% R.S.D. (fenoprofen) and 3–6% R.S.D. (deuterated ketoprofen) over the entire quantification range. The presented method was applied to dermal interstitial fluid samples obtained in a topical administration study of ketoprofen in rats.

Keywords: Ketoprofen; Interstitial fluid; Microdialysis; Open-flow microperfusion

1. Introduction

Topical application of certain drugs is a good alternative to oral administration for certain active substances, avoiding unwanted systemic side effects. Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID) which is generally very effective for the relief of pain and inflammation. However, if orally administered, it can lead to several adverse effects that primarily involve the gastrointestinal tract and the kidneys [1]. For this reason, much effort is being put into the development of ketoprofen formulations for topical administration which efficiently enhance its transport through the skin into the body [2].

In order to investigate the efficiency of different topical formulations of ketoprofen in vivo, the pharmacokinetics of ketoprofen need to be quantified. Therefore, continuous sampling of dermal interstitial fluid from skin tissue over a longer period is necessary. Microdialysis (MD) and open-flow microperfusion (OFM) are continuous sampling techniques that enable in vivo investigations of events in interstitial fluid (ISF) of various tissues including dermal tissue [3–5].

Microdialysis is a well established technique for the assessment of dermal drug delivery but has certain drawbacks associated with the use of a semipermeable membrane such as low recoveries for large molecules and protein-bound drugs and difficulties with sampling of highly lipophilic drugs [3]. With open-flow microperfusion, analytes are sampled through macroscopic holes which enables direct access to interstitial fluid without a membrane. Sampling of large molecules such

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was successfully applied to an open-flow microperfusion study performed in dermal tissue of rats.

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Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access

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Bodenlenz, Manfred, Lukas A. Schaupp, Tatjana Druml, Romana Sommer, Andrea Wutte, Helga C. Schaller, Frank Sinner, Paul Wach, and Thomas R. Pieber. Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access. *Am J Physiol Endocrinol Metab* 289: E296–E300, 2005. First published March 15, 2005; doi:10.1152/ajpendo.00431.2004.—Insulin's action to stimulate glucose utilization is determined by the insulin concentration in interstitial fluid (ISF) of insulin-sensitive tissues. The concentration of interstitial insulin has been measured in human subcutaneous adipose tissue and skeletal muscle, however, never in parallel. The aim of this study was to compare interstitial insulin levels between both tissue beds by simultaneous measurements and to verify and quantify low peripheral ISF insulin fractions as found during moderate hyperinsulinemia. Nine healthy subjects (27.2 ± 0.8 yr) were investigated. A euglycemic-hyperinsulinemic clamp was started with a primed-constant intravenous insulin infusion of 1 $mU \cdot kg^{-1} \cdot min^{-1}$. For direct access to ISF, macroscopically perforated open-flow microperfusion catheters were inserted in both tissues. During steady-state conditions (9.5 h), interstitial effluents were collected in 30-min fractions using five different insulin concentrations in the inflowing perfusates ("no net flux" protocol). Regression analysis of insulin concentrations in perfusates and effluents yielded the relative recovery and the perfusate insulin concentration, which was in equilibrium with the surrounding tissue. Thus, in subcutaneous adipose tissue and skeletal muscle, the mean ISF-to-serum insulin level was calculated as 21.0% [95% confidence interval (CI) 17.5–24.5] and 26.0% (95% CI 19.1–32.8; $P = 0.14$), respectively. Recoveries for insulin averaged 51 and 64%, respectively. The data suggest that the concentrations of insulin arising in healthy subjects at the level of ISF per se are comparable between subcutaneous adipose and skeletal muscle tissue. The low interstitial insulin fractions seem to confirm reports of low peripheral insulin levels during moderate insulin clamps.

extracellular fluid; euglycemic-hyperinsulinemic glucose clamp; open-flow microperfusion; no net flux; equilibrium method

INSULIN'S ACTION TO STIMULATE GLUCOSE UTILIZATION is determined by the insulin concentration in interstitial fluid (ISF) that bathes insulin-sensitive cells (21). Several studies in animals (1, 2, 7, 12, 14, 21–23) provided evidence demonstrating a close correlation between glucose uptake and the concentration of insulin in lymph fluid. Jansson et al. (10) argued that collection of lymph may not provide correct information of the interstitial insulin concentration in insulin-sensitive tissues and suggested that the insulin concentration should be measured directly in the ISF. This was facilitated in human studies by the use of large-pored membranes in the microdialysis sampling technique (11). Since then, insulin concentrations have been estimated with microdialysis in human subcutaneous ISF (10) and repeatedly in human skeletal muscle tissue (8, 9, 18–20).

To our knowledge, however, comparative measurements of insulin in subcutaneous adipose and skeletal muscle tissue have not been performed to date. Thus it is not known whether there are tissue-specific differences in the interstitial insulin levels. Therefore, to explore insulinemia and potential differences at the level of human insulin-sensitive tissues, the need for paired insulin measurements in human muscle and subcutaneous adipose ISF per se was identified.

Although measuring in ISF, microdialysis, similar to the lymph approach, has been finding significantly lower interstitial insulin levels compared with plasma. The data suggest the existence of an endothelial barrier for insulin (2, 3, 13) in combination with tissue clearance, which leads to lower ISF insulin levels in subcutaneous and muscle tissue. Lately, microdialysis has been used for ISF insulin estimations at physiological plasma insulin concentrations (9). The insulin fraction in healthy subjects' skeletal muscle was found to be very low compared with the plasma concentration during a euglycemic insulin clamp. Low insulin fractions under such moderate hyperinsulinemic conditions had already been reported in 1994 by Castillo et al. (3) from the only known study of peripheral lymph insulin in humans. We argued whether these findings of low insulin fractions in ISF/lymph for moderate hyperinsulinemia could be verified using the beneficial features of direct interstitial access by open-flow microperfusion together with that of the well-known "no net flux" (NNF) approach.

Therefore, the aim of this study was *1*) to investigate ISF insulin levels simultaneously in skeletal muscle and subcutaneous adipose tissue in healthy subjects and *2*) to verify and quantify low peripheral ISF insulin fractions in healthy subjects during moderate hyperinsulinemia.

METHODS

Subjects. Nine healthy volunteers (age 27.2 ± 0.8 yr, body mass index 24.6 ± 0.92 kg/m^2; means ± SE) participated in this study. Their mean fasting plasma glucose concentration was 5.2 ± 0.13 mM, and their fasting plasma insulin averaged 55 ± 15 pM. None was taking any regular medication. Written informed consent was obtained

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after the purpose, nature, and potential risks of the study were explained to the subjects. The experimental protocol was approved by the local ethical committee.

Open-flow microperfusion. The principle of ISF sampling by open-flow microperfusion (OFM) has been described in detail previously (6, 15, 17). Briefly, a perforated (100 holes ~0.5 mm in diameter) conventional catheter (FEP-Teflon; 18 gauge; 48 × 1.3 mm; Angiocath; Beckton-Dickinson Sandy, UT) enables direct access to the ISF. After the insertion of the catheter in the tissue of interest by a steel mandarin, a concentric inlet (Teflon tubing) was introduced to allow for perfusion with a fluid ("perfusate") and simultaneous withdrawal of ISF-enriched effluent perfusate ("effluent"). Continuous flow within the perforated catheter was established using multichannel peristaltic pumps (Minipuls 3; Gilson, Villier-le-Bel, France). Via the macroscopic perforations, substances were exchanged between perfusate and ISF regardless of their molecular size or charge (5, 17); there were no membrane-related effects, since the exchange occured nonselectively in either direction. Figure 1 represents a schematic view of a microperfusion catheter.

The ability of OFM catheters to recover large molecules at reasonable rates of relative recovery was demonstrated in a study of albumin (molecular mass 68 kDa) in muscle and subcutaneous adipose tissue (5), suggesting that the catheters also recover the smaller insulin molecule (5.9 kDa) effectively. Before this study, the sampling system was characterized in vitro regarding potential effects of nonspecific binding of insulin as reported from microdialysis (10, 16, 19). The tests revealed an unchanged passage of insulin concentrations, i.e., no significant insulin binding to OFM material in the presence of 1% albumin in the perfusate.

NNF calibration protocol. The NNF calibration protocol or "equilibrium method" was established in microdialysis by Lönnroth et al. (11). It has been used either for direct quantification of ISF concentrations or for estimations of the relative "recovery" of substances, including that for insulin. In OFM, the protocol has so far been used for direct quantification of small molecules (e.g., glucose) and for human albumin (5). In brief, known concentrations of the substance to be quantified in the interstitial space are added to the perfusate, in concentrations higher and lower than expected in the ISF. Because of permanent exchange across the catheter, a net flux of substance occurs according to the concentration gradient. Thus perfusate concentrations (C_{in}) exceeding that in the ISF become diluted when passing the catheter, whereas others become more concentrated. Quantification of the substance's concentration in the ISF with the NNF protocol means to assess the equilibrium concentration where no net flux (i.e., neither concentration nor dilution of the perfusate) occurs. This is done by linear regression analysis of the C_{in} vs. the associated net loss or net gain [effluent concentration (C_{out}) − C_{in}] as determined from the concentrations in the effluent samples. The concentration at the *x*-intercept of the linear regression line represents the mean interstitial concentration over the sampling period; the line's slope is a direct measure of the mean recovery rate [recovery = −slope = $\Delta(C_{out} - C_{in})/\Delta C_{in}$].

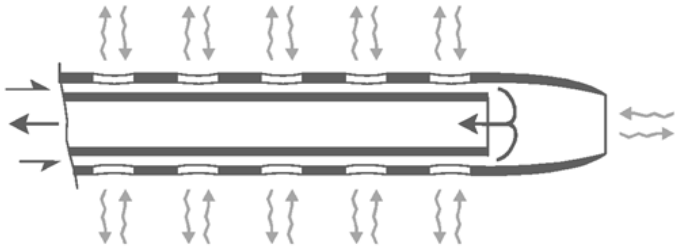

Fig. 1. Schematic representation of a microperfusion double-lumen catheter. Macroscopic perforations (500 μm diameter) allow unrestricted exchange between perfusate and the interstitial fluid in the surrounding tissue. In- and outflow of the perfusate are indicated by dark arrows. Exchange of substances across the perforations is indicated by light gray arrows.

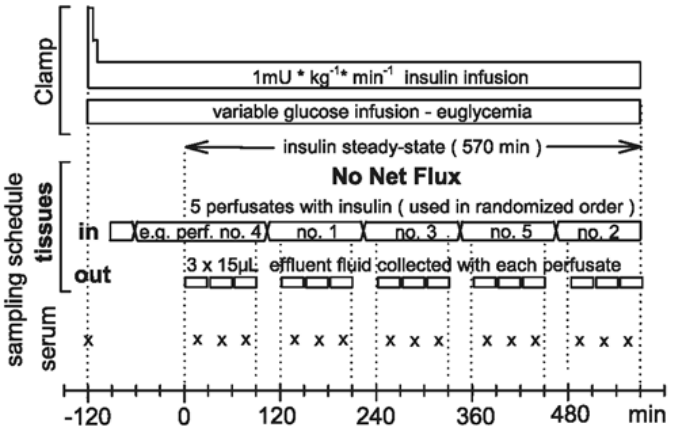

Fig. 2. Time schedule for the euglycemic hyperinsulinemic clamp (*top*) and the no net flux sampling protocol (*bottom*). Catheters in both tissues were perfused with 5 perfusates (in), and 3 samples of effluent (out) were collected per perfusate. Corresponding serum samples were taken (x). Interstitial fluid (ISF) insulin was assessed by linear regression analysis of the concentration differences between out- and inflowing perfusates.

Study protocol. After being fasted overnight, subjects arrived at 0700 and were investigated in a supine position. An intravenous cannula was placed in a dorsal hand vein, and the forearm was kept in a thermoregulated box (50°C) to obtain arterialized venous blood samples for glucose and insulin analysis. A vein in the contralateral arm was cannulated for the infusion of human insulin and glucose. Blood was withdrawn to measure fasting plasma glucose and fasting insulin levels.

At 0730, a hyperinsulinemic-euglycemic clamp was started according to DeFronzo et al. (4), with a primed infusion of human soluble insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) followed by continuous infusion of 1 $mU \cdot kg^{-1} \cdot min^{-1}$, and administration of a variable glucose infusion (20%) to maintain plasma glucose at 5 mM. Potassium chloride solution was added to the glucose solution to prevent hypokalemia.

For the direct access to ISF within tissues, at 0800, two OFM catheters were placed in periumbilical subcutaneous adipose tissue and two in the rectus femoris muscle. A distance of 30 mm was kept between adjacent catheters. After a running-in period of 90 min, OFM sampling of ISF according to the NNF protocol started at 0930 and was continued until the end of the experiment at 1900. Each catheter was perfused with five perfusates (plasma-Krebs-Ringer = 1:5) containing different insulin concentrations. The perfusates were used in randomized order to prevent systematic effects. The perfusion flow rate was set to 0.5 μl/min, yielding 15 μl of effluent in 30-min intervals. In total, 15 samples of effluent fluid were collected per catheter for subsequent insulin analysis (3 samples insulin concentration). The effluent was collected in vials (PCR softtube 0.2 ml; Biozyme Diagnostik, Oldendorf, Germany) that were kept on ice and covered air-proof to prevent evaporation. Perfusion flow rate and sample volume were monitored by weighing the vials before and after sampling. Corresponding to the sampling of OFM effluents from the tissues, serum samples were withdrawn every 30 min for insulin analysis. Figure 2 shows the schedules for the insulin clamp and for perfusion and sampling as implemented in OFM to allow for quantification of ISF insulin by the NNF approach.

Analytical methods. Plasma glucose levels were monitored using a glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA) with a coefficient of variation of 2%.

Serum samples (1 ml), samples of the insulin perfusates (0.5 ml), and the OFM effluents (15 μl) were immediately frozen at −80°C. Insulin was measured using a solid-phase two-site ELISA (Mercodia Ultrasensitive Insulin ELISA; Mercodia, Uppsala, Sweden). The as-

say was validated in-house for 10 μl of ISF matrix, revealing an actual detection limit of 2.52 pM, a lower and an upper limit of quantification of 9 and 120 pM, respectively, and an interrun/assay precision between 2.7% (upper limit of quantification) and 10% (lower limit of quantification).

Calculations and statistics. After insulin analysis, any C_{in} and C_{out} was expressed as a percentage of the corresponding serum insulin concentration. This was done to account for *1*) intrasubject fluctuations of serum insulin and *2*) individually different serum insulin levels. For each of the five C_{in}, the net change of insulin during catheter passage ($C_{out} - C_{in}$) was determined from the three corresponding effluent samples. The mean net change was calculated for each of the five triplicates, and finally the point of no net change (or NNF) was assessed from the five means by first-order least-mean-square linear regression analysis.

The analysis of the data was performed with two different approaches. In the first approach, analysis was done for each catheter separately (Table 1; *analysis I*). Separate analyses accounted for catheter-individual recoveries (slopes) of geometrically identical catheters. Catheter-by-catheter analyses also allowed comparison of the results between adjacent catheters. The arithmetic mean of the adjacent catheters in one tissue bed was considered the subject's specific tissue result. The mean of the nine individual results was taken as the overall result. Significance of differences between both tissue regions and between tissue and serum levels was tested at the $P < 0.05$ level using the nonparametric Wilcoxon's signed-rank test.

In the second approach, the overall ISF insulin fraction and recovery for a tissue bed was assessed from a single regression analysis on the condensed data from all nine subjects (*analysis II*). This approach was known to ignore the catheter- and subject-individual recoveries (i.e., slopes of regression lines) entirely. However, the joint analysis allowed depiction of all the data in a single graph with an average single regression line, and the result obtained was supposed to be less vulnerable for outlying data. The variance of the linear regression and thus of the *x*-intercept was described by the borders of the 95% confidence interval (CI).

RESULTS

Primed-constant intravenous infusion of insulin resulted in serum levels of 379.5 ± 18.8 pM (mean ± SE; range 316.6–517.6) within the predefined steady-state (SS) measurement period (0–570 min). Intrasubject variability of serum insulin was described by an average coefficient of variation of 8.3 ± 1.0% (range 3.7–12.2). Table 1 lists individual SS serum insulin data. Plasma glucose was successfully clamped to euglycemic levels (4.98 ± 0.02 mM) with an average glucose infusion rate of 8.4 ± 0.9 $mg \cdot kg^{-1} \cdot min^{-1}$ (range 3.0–10.5).

The raw data obtained from the NNF procedure already revealed the direction of the insulin net fluxes for the five C_{in}, indicating ISF insulin concentrations above that of *perfusate 2* and below that of *perfusate 4*. Figure 3 depicts all effluent concentrations as profiles over time together with the inflowing concentrations. The exact results for both tissues were obtained on an individual basis, and the overall result was derived as the mean ($n = 9$; *analysis I*). Thus, in subcutaneous adipose tissue and skeletal muscle, the mean ISF-to-serum insulin level was calculated as 21.0 ± 1.8% (95% CI 17.5–24.5) and 26.0 ± 3.5% (95% CI 19.1–32.8), respectively. Both tissue insulin levels were significantly lower than serum insulin ($P < 0.008$). The differences between the tissue beds regarding ISF concentrations and recoveries did not reach statistical significance ($P = 0.14$ and $P = 0.11$, respectively; $n = 9$). All individual results and the means are listed in Table 1.

With the use of nonindividual data analysis (Fig. 4; *analysis II*), ISF insulin in subcutaneous adipose and skeletal muscle tissue was determined as 21.67% of serum (95% CI 18.8–24.5%; $r^2 = 0.988$; $P < 0.001$) and 23.05% (95% CI 20.3–25.8%; $r^2 = 0{,}989$; $P < 0.001$), respectively.

DISCUSSION

The present study is the first to compare ISF insulin levels in human subcutaneous adipose and skeletal muscle tissue. The paired measurements in healthy subjects during SS conditions demonstrate that there is no statistically significant difference of the insulin fractions in either tissue bed. Furthermore, proper implementation of selected approaches enabled reliable quantification of insulin's actual tissue fraction under the conditions of moderate (still physiological) hyperinsulinemia. This fraction was found to be 20–25% of serum. Thus the present study confirms reports of low peripheral insulin fractions under moderate hyperinsulinemia during euglycemic insulin clamps.

To date, concentrations of nonvascular insulin in humans have been published from studies of lymph fluid (3, 13) and studies of tissue ISF with microdialysis (8–10, 18–20). In

Table 1. *Individual results for interstitial insulin from linear regression analyses on insulin no net flux sampling data*

		Interstitial Insulin, %Serum Insulin				Insulin Recovery in Catheter, %	
		Adipose tissue		Skeletal muscle			
Subject No.	Serum Insulin, pM	Catheter A	Catheter B	Catheter C	Catheter D	Adipose tissue A,B mean	Skeletal muscle C,D mean
1	375±10	23.2*	17.7*	17.0*	13.7*	68.3	71.1
2	317±8	27.4 (NS)	21.1 (NS)	20.3*	20.9*	55.7	74.2
3	338±5	34.5*	16.6*	19.6*	34.2*	29.3	47.6
4	387±6	30.0†	24.6*	17.1 (NS)	30.9†	65.1	41.8
5	367±6	20.4 (NS)	17.7†	27.7*	25.6*	61.6	75.4
6	383±3	15.2*	17.6†	20.3*	NA	42.6	80.8(C)
7	366±8	14.5*	5.3*	20.9*	9.7*	41.6	76.3
8	518±7	23.9†	19.8†	68.1 (NS)	26.5†	50.8	40.6
9	365±10	22.8*	25.3*	NA	37.3*	44.5	64.3(D)
Mean±SE	379±19 pM	21.0±1.8%		26.0±3.5%		51.4±4.3%	63.6±5.3%

Data for serum insulin are means ± SE. Interstitial insulin concentration was obtained from the *x*-intercept of the linear regression line in %current serum level. Recovery was obtained from the slope of the regression line. Strength of the linear relationship between insulin's net change in perfusate and the insulin concentration used: *$P < 0.01$ ($r > 0.87$) and †$P < 0.05$ ($r > 0.75$). NS, not significant; NA, not analyzed. Interstitial fluid samples of *catheters 6D* and *9C* were not analyzed, since reddish appearance indicated contamination by noninterstitial fluid.

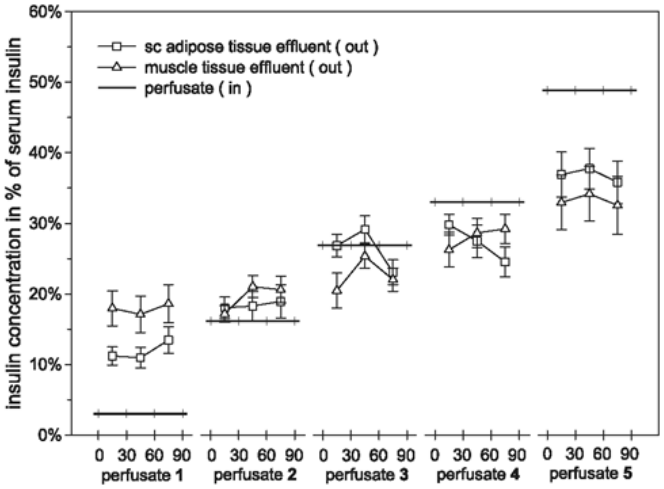

Fig. 3. Average insulin concentrations in catheter effluents (□, adipose tissue; △, skeletal muscle) are presented for each perfusate concentration level. For clarity, the 5 perfusate levels are arranged in ascending order and are drawn as straight lines without error bars. Time scale on *x*-axis is given for the sampling duration of 3 × 30 min for each concentration level. Data are means ± SE; $n = 9$.

1967, Rasio et al. (13) assessed thoracic duct lymph insulin levels and found considerably lower concentrations than in plasma. Investigators speculated that, in ISF of tissues with low capillary permeability, such as striated muscle and adipose tissue, low levels might be expected. In 1994 Castillo et al. (3) found an average insulin fraction of 34% (range 18–60%) in lymph fluid that drained off subcutaneous forefoot tissue of lean and obese men. The availability of high molecular cut-off membranes in the microdialysis sampling technique enabled recovery of insulin from the ISF space of human tissues, leading to estimations of insulin's ISF tissue concentration. Thus, in 1993, Jansson et al. (10) estimated insulin concentrations in subcutaneous adipose tissue. During SS hyperinsulinemia (plasma insulin of ~1,500 and 3,500 pM), measurements yielded subcutaneous insulin fractions of ~58 and 45% compared with plasma. These ISF data suggested that an endothelial barrier for insulin in combination with tissue clearance leads to lower insulin levels and altered kinetics in ISF compared with plasma. Subsequent microdialysis studies measured ISF insulin in skeletal muscle of healthy, obese, and diabetic subjects (8, 18–20) to explore aspects of the delivery of insulin to ISF over the capillary wall. Also in those studies, high plasma insulin levels between ~900 and ~4,000 pM were induced to achieve measurable insulin concentrations in microdialysis effluents. Thus muscle insulin fractions between ~38 and 54% of plasma in healthy subjects were found. Lately, microdialysis-based estimations of ISF insulin were done at physiological plasma insulin concentrations (9). Plasma insulin levels of ~500 pM were induced in healthy subjects by means of a euglycemic-hyperinsulinemic clamp. Starting from a baseline muscle insulin fraction of 48% (of plasma), that fraction was significantly reduced during the moderate hyperinsulinemic clamp to 12% at SS, whereas during an oral glucose tolerance test and similar plasma insulin levels the interstitial fraction remained fairly unchanged (43% of plasma). OFM catheters had once been used for insulin sampling from human muscle (3). However, the effluent insulin concentrations were not calibrated, and thus no insulin concentrations in ISF per se were obtained.

In short, all previous studies with estimations of insulin in ISF per se were based on microdialysis effluents, and estimations were done in subcutaneous adipose tissue as well as in skeletal muscle, but never simultaneously in a study. Because sampling membranes, calibration procedures, glycemia, and insulinemia were different between the studies, comparison between the tissue ISF insulin levels was not possible. Furthermore, quantification in the microdialysis studies was based on small effluent concentrations resulting from the membrane's low (3–11%) insulin recovery, which may add a significant error. These facts may explain some of the variation in the results for the ISF insulin fractions in microdialysis studies (range 12–54% in muscle tissue of healthy subjects). It may also be hypothesized that some of the variation is because of a potential dependency of the resulting tissue fractions on the underlying vascular insulin levels. The relative low tissue fractions found at lower hyperinsulinemic levels provide an indication. We investigated the insulin concentrations in subcutaneous adipose and skeletal muscle ISF per se and performed paired measurements to allow for a comparison. Particular effort was put on the quantification of the actual ISF

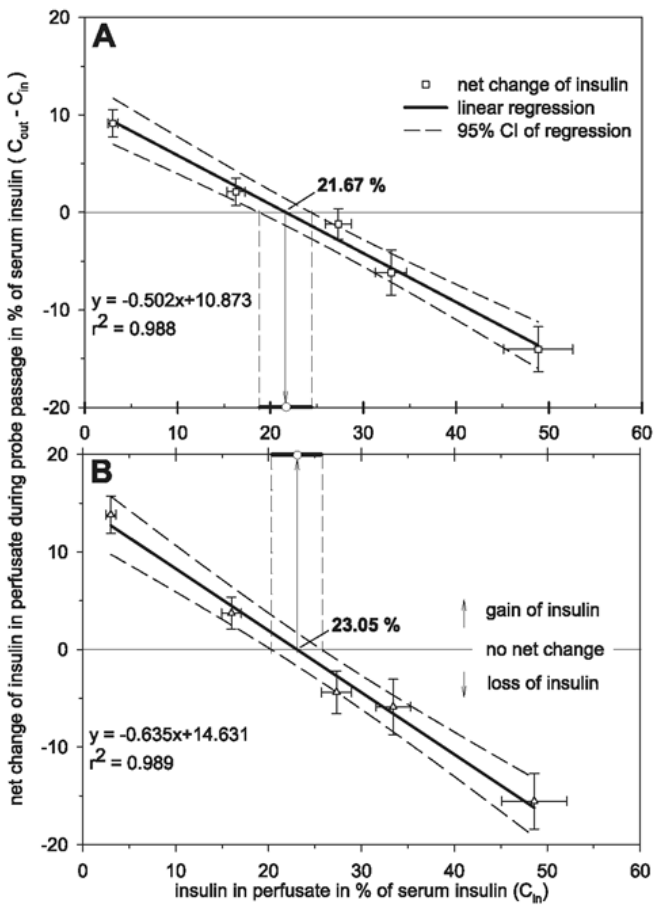

Fig. 4. *Analysis II*: assessment of insulin's ISF fraction by a single regression analysis on the condensed no net flux data for sc adipose (*A*) and skeletal muscle (*B*) tissue. The *x*-intercepts at 21.67% for sc adipose and 23.05% for skeletal muscle tissue represent the ISF fractions of insulin relative to serum. The results of this overall regression analysis match well with the means calculated from the 9 individual results (Table 1). Broken lines show the borders of the 95% confidence interval (95% CI) for the regression. Data are means ± SE; $n = 9$ subjects. C_{out}, effuent concentration; C_{in}, perfusate concentration.

insulin concentration to be able to verify the appearance of low tissue fractions at SS moderate hyperinsulinemia. This was done by combining the open-flow microperfusion technique for access to the ISF and the NNF protocol, thus avoiding the use of reference substances. The catheters' macroscopic openings assured direct access to ISF insulin without interference of a membrane. This led to recoveries of insulin >50% on average, as indicated by the pronounced slopes of the regression lines. The high recovery was fundamental for the reliability of the NNF quantification approach. As had been expected, recoveries varied between catheters; however, the recovery was not a determinant for ISF insulin. Although the mean insulin recovery in the two investigated tissue beds was not equal (12% higher in muscle; maybe because of the different vascularization), the ISF insulin fraction was the same for both tissues. It may be speculated whether this was compensated by other influences (e.g., blood flow, insulin clearance), but this should be investigated in further studies.

The perfusion of two adjacent catheters in each tissue and separate NNF analyses helped to obtain reasonable individual tissue means. The direct comparison of the NNF results (Table 1) reveals considerable differences between some adjacent (muscle) catheters. Differences may arise from inherent methodological uncertainties and biological variability. In our study, the impact of uncertainties in the intercept assessment by regression was low because of the high insulin recoveries. With the use of OFM, the identification of contaminated ISF samples (reddish color, mostly from muscle) was possible, and, by their exclusion, unperceived adulteration of ISF results by plasma concentrations was prevented. We do not know whether morphological inhomogeneities within tissues or random positions of inserted catheters to, e.g., larger blood vessels could have contributed to variation. Finally, the means calculated from the individual catheters in both tissue beds (Table 1) match those from the less outlier-sensitive overall regression analysis (Fig. 4).

In summary, our data suggest that the concentrations of insulin arising in healthy subjects at the level of ISF per se are comparable between subcutaneous adipose and skeletal muscle tissue. Under SS conditions of moderate hyperinsulinemia after a euglycemic insulin clamp, the ISF concentration in both tissue beds averages ~20–25% of serum insulin. The low insulin fractions measured in ISF per se seem to confirm reports of low peripheral insulin levels under these distinct insulinemic conditions.

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