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Morphologische, funktionelle und molekulare Charakterisierung der Ösophagusmukosa bei erosiver und nicht-erosiver gastroösophagealer Refluxerkrankung

Habilitationsschrift

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Inhaltsverzeichnis

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1. Abkürzungsverzeichnis

ALI	air liquid interface
% AET	% acid exposure time
DBI	distal baseline impedance
BCH	basal cell hyperplasia
CD	cluster of differentiation
CGRP	Calcitonin gene related protein
DGVS	Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselerkrankungen
DIS	dilated intercellular spaces
EH	esophageal hypersensitivity
ERD	erosive reflux disease
FH	functional heartburn
FICE	flexible spectral imaging colour enhancement
FOXP3	forkhead box transcription factor-3
GABA	gamma amino butyric acid
GERD	gastroesophageal reflux disease
H. pylori	*Helicobacter pylori*
IL-8	interleukin-8
IPCLs	intrapapillary capillary loops
LCA	leukocyte common antigen
MII-pH	multichannel intraluminal impedance and pH analysis
mRNA	messenger ribonucleic acid
NBI	narrow band imaging
NERD	non-erosive reflux disease
PAR2	protease-activated receptor-2
PAF	platelet activating factor

PE	papillary elongation
PPI	proton pump inhibitor
PPI-REE	PPI-responsive esophageal eosinophilia
qPCR	quantitative polymerase chain reaction
RDQ	Reflux Disease Questionnaire
SAP	symptom association probability
SI	symptom index
SP	substance P
SSRI	serotonin reuptake inhibitor
TLESR	transient lower esophageal sphinkter relaxation
TRPV1	transient receptor potential cation channel subfamiliy V member 1
ZO	zonula occludens

2. Einleitung

Die vorliegende Habilitationsschrift stellt eine kumulative Zusammenfassung der wissenschaftlichen Publikationen zur inhaltlichen Thematik der gastroösophagealen Refluxerkrankung aus dem Zeitraum von 2008 bis 2014 dar. Sämtliche Arbeiten sind in der eigenen Arbeitsgruppe der Klinik für Gastroenterologie, Hepatologie und Infektiologie unter der Leitung von Prof. Dr. h. c. Peter Malfertheiner durchgeführt worden.
Die in der Habilitationsschrift präsentierten Arbeiten beinhalten unterschiedliche Projekte zur Charakterisierung von Patienten mit gastroösophagealer Refluxerkrankung und Patienten mit Therapie-refraktären Symptomen beziehungsweise sogenanntem funktionellen Sodbrennen. Ein Teil der Arbeiten fokussiert die Probleme der klinischen Präsentation und funktionellen Charakterisierung der Patienten. Ein weiterer Teil der Arbeiten beschreibt morphologische und molekulare Veränderungen der Ösophagusmukosa bei Patienten mit Refluxerkrankung. Die genaue Analyse morphologischer und funktioneller Veränderungen der Mukosa ermöglichen dabei eine genauere klinische Diagnose und Differentialdiagnose vor allem in der Abgrenzung zum funktionellen Sodbrennen. Die Ergebnisse der molekularen Fragestellungen bieten darüber hinaus plausible Erklärungen von pathophysiologischen Mechanismen in der Mukosa bei gastroösophagealer Refluxerkrankung. Insbesondere die Arbeiten zur Induktion von entzündlichen Veränderungen in der Ösophagusmukosa beschreiben einen pathophysiologischen Ansatz, der über eine kaustische Schädigung der Speiseröhre durch Magensäure hinausgeht und den ösophagealen Keratinozyten in den Mittelpunkt der Entzündungskaskade rückt. Im Fokus steht insbesondere die Funktion des Protease-aktivierten Rezeptors-2 (PAR2), der durch im Refluxat enthaltene Serin-Proteasen aus weiter distal gelegenen Abschnitten des Gastrointestinaltraktes (z. B. pankreatisches Trypsin) aktiviert wird. Die Aktivierung dieses Rezeptors auf ösophagealen Keratinozyten führt zur Freisetzung von Interleukin-8 (IL-8) und trägt zur Initiierung der mukosalen Entzündung bei.

2.1 Gastroösophageale Refluxerkrankung – klinische Definition einer Symptom-basierten Diagnosestellung und epidemiologische Daten

Die gastroösophageale Refluxerkrankung (GERD) ist die häufigste Diagnose, die durch niedergelassene gastroenterologische Fachärzte in den Vereinigten Staaten von Amerika (USA) im Zeitraum der letzten 10 Jahre gestellt wurde [1]. Durch die aktuelle Montreal-Klassifikation wird die gastroösophageale Refluxerkrankung als klinische Diagnose definiert, die durch das Zurückfließen von Mageninhalt in die Speiseröhre hervorgerufen wird und

dabei typische Symptome verursacht, die den Patienten in seiner Lebensqualität signifikant einschränken [2]. Wenn die Diagnose der Refluxerkrankung symptomatisch bei Auftreten von typischen Symptomen (Sodbrennen, saures Aufstoßen) gestellt wird, beträgt die Prävalenz in den westlichen Industrienationen bis zu 20-30% der Einwohner eines Landes. Epidemiologische Daten aus den USA und skandinavischen Ländern belegen, dass jeder fünfte Einwohner dieser Länder unter typischem Sodbrennen als Kardinalsymptom an durchschnittlich 2-3 Tagen in der Woche leidet [3].

Die Empfehlungen sowohl von nationalen als auch von internationalen Leitlinien sehen für das klinische Management vor, bei Auftreten von typischen Symptomen eine empirische Therapie mit einem Protonenpumpenhemmer (PPI; Protonenpumpeninhibitor) zu beginnen und bei unbefriedigendem symptomatischen Ansprechen die Dosierung weiter zu erhöhen [4-6].

Sodbrennen und das Aufstoßen von Mageninhalt werden als sogenannte typische ösophageale Symptome bezeichnet. Daneben werden in der Montreal-Klassifikation atypische beziehungsweise extraösophageale Symptome, wie zum Beispiel chronischer Husten und Laryngitis, definiert. Auch in diesen Fällen ist die Einleitung einer empirischen Therapie mit einem PPI, bei unbefriedigendem Ansprechen ebenfalls in doppelter Standarddosierung, empfohlen. Die Datenlage zur Assoziation einer pathologischer Exposition des Ösophagus mit Mageninhalt und extraösophagealen Symptomen ist jedoch unzureichend und die Studienlage bezüglich des therapeutischen Ansprechens extraösophagealer Symptome auf eine säuresuppressive Therapie uneinheitlich.

In der täglichen Praxis können die korrekte Diagnosestellung einer Refluxerkrankung und damit verbunden auch die Indikationsstellung einer Therapie mit PPI den behandelnden Arzt vor Probleme stellen. Eine Metaanalyse und Übersichtsarbeit aus dem letzten Jahr verdeutlicht dieses Problem eindrücklich. Boeckxstaens und Kollegen konnten in ihrer Arbeit deutlich herausarbeiten, dass der therapeutische Effekt und zusätzliche Gewinn einer Therapie mit PPI im Vergleich zu einer Therapie mit Placebo für die Heilungsrate einer erosiven Ösophagitis erwartungsgemäß hoch ist. Für die symptomatische Refluxerkrankung ist das therapeutische Ansprechen bei Patienten mit ERD im Sinne der Symptomfreiheit mit bis zu 70% ebenfalls noch sehr gut, bei Patienten mit nicht-erosiver Verlaufsform (NERD) bereits deutlich eingeschränkter (50-55%) und bei atypischen beziehungsweise extraösophagealen Symptomen im Vergleich statistisch vergleichbar mit einer Behandlung mit Placebo [7].

Ein weiteres Problem der Symptom-basierten Diagnose der Refluxerkrankung stellt im klinischen Alltag die große Überschneidung und hohe Querschnittsmenge mit Symptomen aus dem Formenkreis der funktionellen gastrointestinalen Symptome. In bis zu 70% der Fälle besteht eine Überschneidung von Reflux-Symptomen und dyspeptischen

Oberbauchbeschwerden [8, 9] . Eine genaue Anamnese ist für die Diagnostik und die Festlegung des therapeutischen Regimes unterschiedlicher Symptomkomplexe essentiell [10, 11].
Ein besondere Herausforderungen stellen atypische Symptome dar (z.B. chronischer Husten, dentale Erosionen, Laryngitis), die bei einem Teil der Patienten zwar therapiert, jedoch überhaupt nicht mit einer Refluxerkrankung assoziiert sein müssen. Bei einem anderen Teil der Patienten können sie hingegen das einzige Symptom einer zugrunde liegenden Refluxerkrankung darstellen [8, 9, 12]. Neben der Einschränkung in der Lebensqualität des einzelnen Betroffenen [13], ist die Diagnose der GERD mit einer deutlichen Belastung für die Gesundheitssysteme der jeweiligen Länder verbunden. Eine Erhebung aus den USA berechnete für den Zeitraum zwischen 2007 und 2011 für die Behandlung von Patienten mit atypischen Symptomen 5-6-fach erhöhte Kosten im Vergleich zur Behandlung von Patienten mit typischer Symptomatik. Ursächlich konnten die Autoren diese deutlich erhöhten Kosten bei atypischen Symptomen vornehmlich durch eine intensivierte medikamentöse Therapie und wiederholte Konsultation von fachärztlichen Kollegen erklären [14].

2.2 Endoskopische Diagnostik und Klassifikation

Endoskopisch wird eine nicht-erosiven Erkrankung (non-erosive Refluxerkrankung, NERD) von einer erosiven Verlaufsform (erosive Refluxerkrankung, ERD) mit unterschiedlich stark ausgedehnten Erosionen der Mukosa im distalen Ösophagus unterschieden. Die Einteilung des Schweregrads sollte die Ausprägung und Länge der Erosionen berücksichtigen und nach der Los Angeles Klassifikation vorgenommen werden [15]. Bei Vorliegen von Erosionen der Ösophagusmukosa im distalen Ösophagus ist die Diagnose der GERD an sich gesichert. Die Diagnose der NERD wird gemäß den Leitlinien der deutschen Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselerkrankungen (DGVS) als Refluxerkrankung ohne endoskopisch nachweisbare Läsionen definiert. Voraussetzung ist, dass zum Zeitpunkt der endoskopischen Untersuchung typische Reflux-Symptome bestehen, jedoch noch keine Behandlung mit einem PPI eingeleitet wurde [4]. Basierend auf dieser kategorisierenden Einteilung der Refluxerkrankung ist die NERD bei bis zu 60% aller Patienten mit typischen Symptomen die häufigste klinische Verlaufsform der GERD [16, 17]. Die letzten Follow-Up Daten aus der deutschen Beobachtungsstudie (ProGERD) legen jedoch nahe, dass eine so strikte Unterscheidung zwischen NERD und ERD nicht für alle Patienten kategorisch zu treffen ist. Vielmehr stellt die leichte Form der Refluxerkrankung ohne Komplikationen (Stenosen, Blutungen, Ulcera) ein klinisch-endoskopisches Kontinuum

dar und beinhaltet sowohl ein nicht-erosives Erscheinungsbild als auch ein Erscheinungsbild mit gering ausgeprägten Erosionen (Los Angeles Klassifikation A und B) [18].

In einzelnen japanischen Studien wurde versucht, die NERD durch die sogenannte M- und N-Klassifikation endoskopisch mittels Weißlicht-Endoskopie weiter zu charakterisieren [19, 20]. Aufgrund einer hohen Interobserver-Variabilität mit niedrigen Kappa-Werten für die M- und N-Klassifikation [19] konnte sich die M- und N- Klassifikation weder in der klinischen Routine noch die Begrifflichkeit und Definition einer „Minimal Change Ösophagitis“ etablieren und wird in den Leitlinien ausdrücklich nicht empfohlen [4].

Die Weiterentwicklungen der hochauflösenden Zoom-Endoskopie und Kontrast-verstärkenden optischen Verfahren (Narrow Band Imaging, NBI (Olympus); Flexible Spectral Imaging Colour Enhancement, FICE (FUJIFILM) (siehe Abbildung 1)) bietet die Möglichkeit, Veränderungen der Mukosa und Submukosa verbessert darzustellen. Insbesondere gelingt es, die Strukturen des Epithels und der in der Submukosa verlaufenden Gefäße kontrastreicher und in hoher Auflösung beziehungsweise vergrößert mittels optischer oder elektronischer Vergrößerung (Zoom) darzustellen. Bei Patienten mit NERD kann man so vermehrte, verlängerte, verstärkt torquierte und dilatierte Gefäße der Ösophagusmukosa als sogenannte „intrapapillary capillary loops“ (IPCLs) erkennen (Abbildung 1) [21, 22]. Diese Veränderungen korrespondieren mit typischen histopathologischen Veränderungen der Mukosa bei GERD und entsprechen der Elongation der epithelialen Papillen mit den darin verlaufenden submukösen Gefäßen (siehe auch: 2.5 pathophysiologische Veränderungen der Ösophagusmukosa). Aufgrund der spezialisierten und zeitaufwendigen Anforderungen an eine hochauflösende Zoom-Endoskopie sowie aufgrund der Tatsache, dass diese Techniken bisher nur in spezialisierten Zentren eingesetzt werden, haben sich diese in der endoskopischen Differentialdiagnostik von mukosalen Veränderungen bei der NERD bisher ebenfalls nicht in der klinischen Routine durchsetzen können.

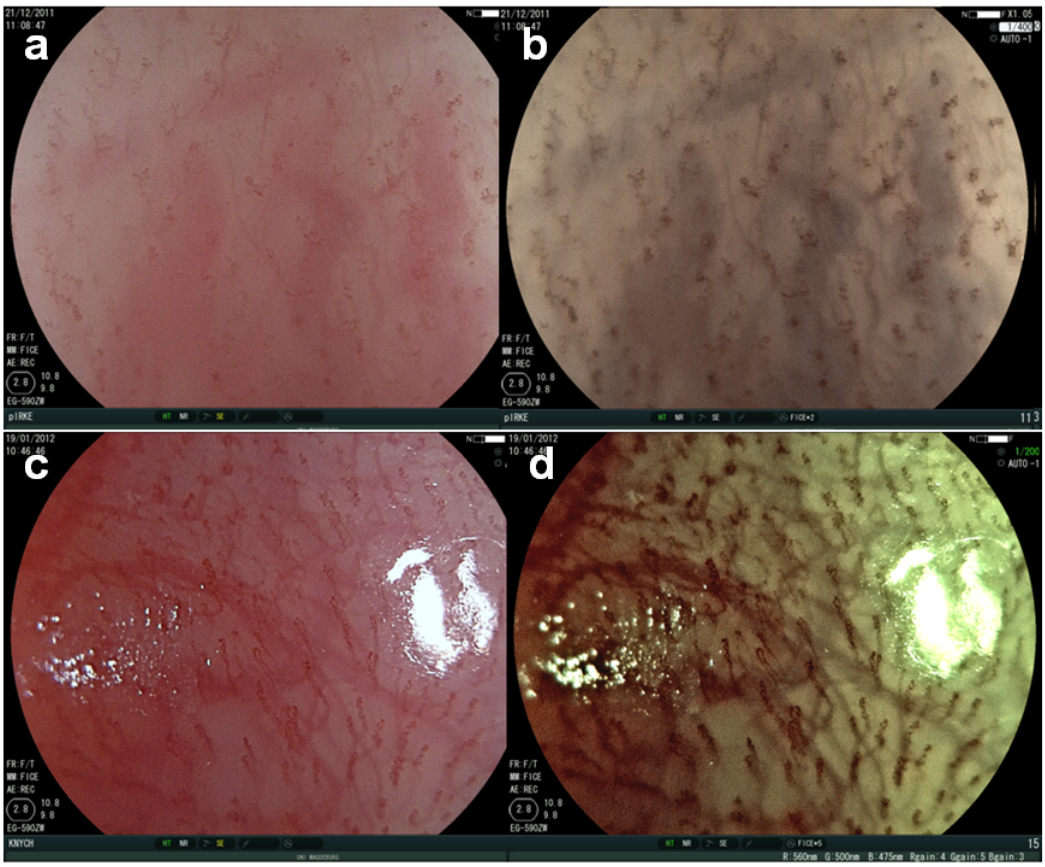


Abbildung 1: IPCLs (intrapapillary capillary loops) in der Ösophagusmukosa bei Patienten mit NERD (c + d) im Vergleich zu einem asymptomatischem Probanden (a + b). Endoskopische Darstellung mittels hochauflösender Zoom-Endoskopie und Kontrast-verstärkenden Verfahren (FICE, FUJIFILM EG-590 ZW, EPX-450 HD Videoprozessor).

Bei endoskopisch- und Symptom-basierter Diagnose stellen die Patienten mit NERD die Subgruppe mit dem schlechtesten Ansprechen auf PPI dar. Ursächlich hierfür ist die eingeschränkte diagnostische Genauigkeit von typischen Symptomen für das tatsächliche Vorliegen einer GERD kritisch zu diskutieren. In der bisher größten, kontrollierten Studie traten typische Symptome (Sodbrennen und Regurgitation) nur bei 49% der Patienten auf, bei denen die Refluxerkrankung objektiv durch den Nachweis einer pathologischen Säureexposition des Ösophagus durch eine 48-Stunden BRAVO© pH-Metrie gesichert wurde. In der gleichen Studie wurde gezeigt, dass bei 23% der Patienten typische Symptome angegeben wurden, bei denen eine Refluxerkrankung mittels BRAVO© pH-Metrie objektiv ausgeschlossen werden konnte [23]. Es machte dabei keinen Unterschied, ob die Symptome von einem klinisch tätigen oder niedergelassenen Facharzt oder von einem Allgemeinmediziner erfasst wurden beziehungsweise die Symptome durch einen speziellen Fragebogen (Reflux Disease Questionnaire, RDQ) erfasst wurde.

In einer kürzlich publizierten Metanalyse zum Ansprechen auf eine PPI-Therapie wurde herausgearbeitet, dass das therapeutische Ansprechen auf eine 4-wöchige säuresuppressive Therapie bei Patienten mit NERD lediglich 49% beträgt, wenn sich die Diagnose lediglich auf das Vorhandensein typischer Symptomen und einer unauffälligen endoskopischen Untersuchung gestellt wird. Wird die Diagnose der NERD bei typischen Symptomen zusätzlich durch eine pathologische pH-Metrie mit erhöhter Säureexposition des distalen Ösophagus bestätigt, so ist das symptomatische Ansprechen auf eine antisekretorische Therapie vergleichbar mit Patienten mit ERD [24].
Die eigenen Daten zu dieser Fragestellung finden sich in der zusammenfassenden Darstellung der Ergebnisse zur Habilitationsschrift. Vergleichbar mit den Daten der Meta-Analyse konnten wir in einer prospektiven Untersuchung bei PPI-naiven Patienten belegen, dass eine Therapie mit PPI über 4 Wochen bei Patienten mit NERD vergleichbare symptomatische Ansprechraten erreicht werden wie für Patienten mit ERD, wenn die Diagnose der Refluxerkrankung basierend auf dem Nachweis eines pathologischen gastroösophagealen Refluxes mittels pH-Metrie gestellt wird [25].

Nur bei einem geringen Teil der Patienten mit typischen Symptomen der Refluxerkrankung liegt eine Zylinderepithelmetaplasie des Ösophagus (Barrett-Metaplasie) vor. Während die Diagnose einer Barrett-Metaplasie durch die US-amerikanischen Leitlinien durch das Auftreten von Becherzellen und einer spezialisierten intestinalen Metaplasie definiert wird [26], unterscheiden die britischen Empfehlungen eine Barrett-Metaplasie mit spezialisierter intestinaler Metaplasie von einer Barrett-Schleimhaut mit gastraler Metaplasie [27]. Da das Karzinomrisiko für eine Zylinderepithelmetaplasie ohne Becherzellen in großen Studien bisher nicht abschließend beurteilt werden konnte, definiert die deutsche Leitlinie die Diagnose eines Barrett-Ösophagus analog der Empfehlung der amerikanischen Fachgesellschaft durch das Auftreten von Becherzellen als sogenannte spezialisierte intestinale Metaplasie des Ösophagus. Dennoch wird beim erstmaligen Nachweis einer gastralen Metaplasie in einer Zylinderepithelmetaplasie der Speiseröhre eine endoskopische und bioptische Kontrolle innerhalb eines Jahres empfohlen [4]. Die endoskopische Einteilung und Beschreibung der endoskopischen Ausdehnung sollte analog den Prag-Kriterien erfolgen, die sowohl die zirkuläre Ausdehnung als auch die maximale Länge von Zylinderepithelzungen berücksichtig (C & M Kriterien) [28]. Ein Risiko-adaptiertes Vorgehen der Therapie und endoskopischen Überwachung richtet sich nach dem Vorliegen von möglichen dysplastischen Veränderungen in der Barrett-Schleimhaut. Zusätzlich ist die Assoziation mit höhergradigen dysplastischen Veränderungen mit der endoskopischen Länge des Barrett-Segmentes assoziiert, was aktuell in den Empfehlungen der britischen

Gesellschaft für Gastroenterologie festgehalten ist und die Länge des Überwachungsintervalls mit bestimmt [27].
Insgesamt wurde das Risiko der malignen Progression einer Barrett-Schleimhaut ohne Dysplasien durch Studien der letzten Jahre deutlich nach unten korrigiert (Inzidenz bei nicht-dysplastischer Barrett-Schleimhaut 0,12-0,33%) [29, 30].
In der Erarbeitung der wissenschaftlichen Arbeiten zur Habilitationsschrift wurden Fragestellungen zur Barrett-Metaplasie ausdrücklich ausgegrenzt.

2.3 Funktionsdiagnostik der gastroösophagealen Refluxerkrankung

Die Durchführung einer gastroösophagealen Funktionsdiagnostik zur Objektivierung eines pathologischen gastroösophagealen Refluxes ist durch die unterschiedlichen Leitlinien erst nach Versagen einer Therapie mit PPI, also bei sogenannten PPI-refraktären Symptomen, empfohlen [4, 5]. Unterschiedliche apparative Methoden stehen zur Verfügung, wobei die Wahl der entsprechenden Methode immer vom jeweiligen Zentrum, den lokalen Verfügbarkeiten und Zugang zu diesen diagnostischen Möglichkeiten sowie auch der klinischen Fragestellung abhängig ist [31].
Es gilt in der Fragestellung an die Funktionsdiagnostik, generell zwei klinische Szenarien für die Diagnose einer Refluxerkrankung zu unterscheiden. Bei bereits zuvor gesicherter GERD und inkompletten Ansprechen auf eine Therapie stellt sich die Frage der Therapieoptimierung. Die aus meiner Sicht weitaus wichtigere Fragestellung im klinischen Alltag an die Funktionsdiagnostik ist es, bei PPI-refraktären Symptomen die Diagnose einer Refluxerkrankung zu sichern beziehungsweise auszuschließen, wenn diese bis dahin nicht objektiviert und gesichert worden ist. Bedeutend als hartes objektives Kriterium für die Diagnose der GERD ist zum einen die Messung einer möglichen pathologischen Exposition der Speiseröhre gegenüber Inhalten aus dem Magen. Die weitere wichtige Eigenschaft der Funktionsdiagnostik ist die Möglichkeit, Refluxepisoden und Symptome parallel aufzuzeichnen und zu analysieren, ob eine Assoziation von angegebenen Symptomen und Refluxepisoden besteht [32].
Basierend auf den ROME III Kriterien wird durch eine adäquate gastroösophageale Funktionsdiagnostik und Erfassung der Symptomassoziation die nicht-erosive Refluxerkrankung differenzierter dargestellt und die Entitäten der ösophagealen Hypersensitivät (EH, Esophageal Hypersensitivity) und sogenanntes „funktionelles Sodbrennen“ (FH, Functional Heartburn) definiert (siehe Abbildung 2).
Bei Patienten mit EH besteht eine physiologische Säureexposition des Ösophagus (% acid exposure time), jedoch eine signifikante Assoziation von Symptomen und Refluxepisoden.

Diese Patienten werden im Allgemeinen als Subgruppe der NERD behandelt. Einzelne Studien konnten belegen, dass Patienten mit EH im Vergleich zu den anderen Subgruppen der GERD vermehrt schwach-saure und proximale Refluxepisoden aufweisen [33, 34]. Eine Placebo-kontrollierte Studie belegte für Patienten mit EH eine signifikantes symptomatisches Ansprechen auf eine Therapie mit Citalopram [35].
Patienten mit FH dagegen weisen zwar ebenfalls ein unauffälliges Profil der Refluxepisoden mit fehlender Säureexposition des distalen Ösophagus auf. Die Symptome bei diesen Patienten stehen allerdings in keinem Zusammenhang mit möglichen Refluxepisoden. Damit wird die Diagnose des „funktionellen Sodbrennens“ definiert. Die genauen Mechanismen der Symptomgeneration sind weder für typische Beschwerden bei nachgewiesener GERD beziehungsweise EH noch für Patienten mit FH pathophysiologisch geklärt. Das therapeutische Ansprechen auf eine Therapie mit PPI ist vor allem für Patienten mit FH schlecht. Die besondere Anforderung an den behandelnden Arzt und die Funktionsdiagnostik ist es, diese Differentialdiagnose zu stellen, da die Fortführung einer PPI-Therapie in diesen Fällen kritisch zu hinterfragen und zu diskutieren ist.

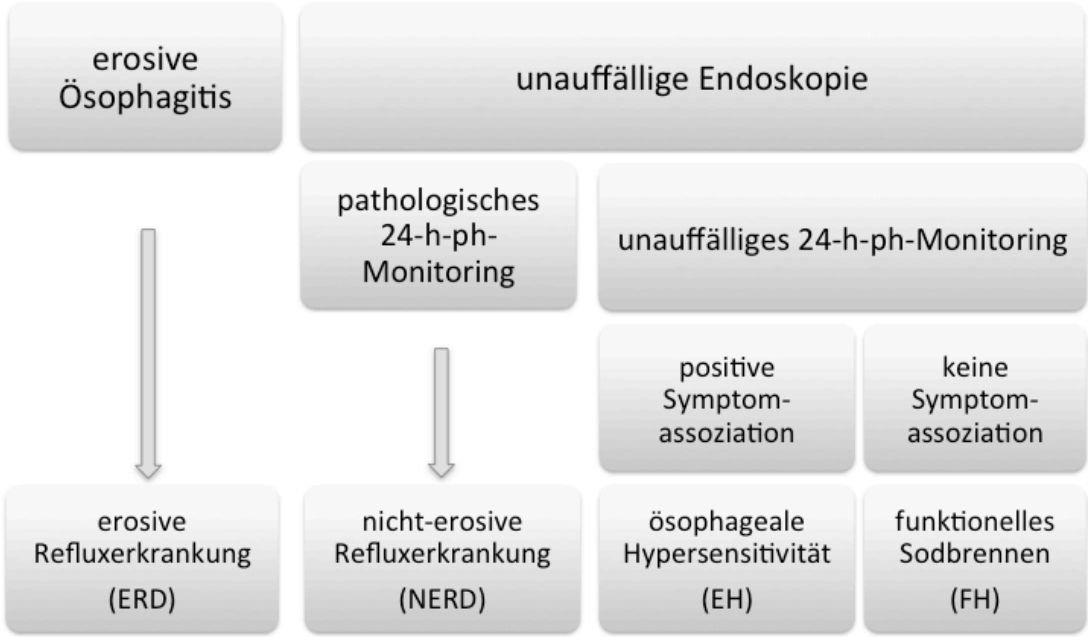


Abbildung 2: Differenzierte Diagnostik der GERD mit PPI-refraktären Symptomen analog den ROME III Kriterien [36]. Basierend auf den Ergebnissen der Endoskopie und der Funktionsdiagnostik erfolgt die Differenzierung zwischen ERD, NERD, EH und FH (Abbildung in Analogie zu [31]).

Neben der klassischen Katheter-basierten 24 Stunden pH-Metrie, bietet die „Katheter-freie“ Kapsel pH-Metrie (BRAVO© Kapsel) die Möglichkeit einer verlängerten Analysedauer bis 96

Stunden, was mit gesteigerter Sensitivität und Spezifität sowie mit verbesserten Toleranz und Compliance der Patienten verbunden ist [37, 38]. Unabhängig von der Methode werden durch die pH-Metrie jeweils nur Veränderungen des pH-Werts im distalen Ösophagus nachgewiesen. Das beste diagnostische Kriterium für einen pathologischen gastroösophagealen Reflux und damit für die Diagnose einer Refluxerkrankung stellt die prozentuale Exposition des distalen Ösophagus gegenüber sauren Bestandteilen im Refluxat über 24 Stunden dar (% acid exposure time < pH4, % AET). Werte über 4,2 % des Analysezeitraums sind als pathologische Säureexposition definiert und sind anderen Parametern der Analyse beispielsweise der Berechnung des DeMeester-Scores überlegen [39].

Die kombinierte intraluminale Impedanz- und pH-Analyse (MII-pH) ermöglicht es, durch Veränderungen des elektrischen Widerstands zwischen 8 Impedanzelektroden auf dem Katheter retrograde Bolusbewegungen in der Speiseröhre zu erkennen und zwischen flüssigen auch gasförmige Anteile zu unterscheiden. Eine Refluxepisode wird durch einen Abfall des Impedanzniveaus um >50% definiert, der als retrograde Bolusbewegung von distal nach proximal verfolgt werden kann. Zusätzlich auf dem Katheter vorhandene pH-Elektroden charakterisieren den pH-Wert und unterschieden so saure Episoden von schwach-sauren und schwach alkalischen Refluxepisoden (siehe Abbildung 3) [40-42].

In Anlehnung an die Berechnung der Symptomassoziation in der klassischen pH-Metrie (Symptomindex, SI; Symptom Association Probability, SAP) wurden auf die MII-pH Analyse identische mathematischen Modelle zur Assoziation von sauren und nicht-sauren Refluxepisoden und individuellen Beschwerden übertragen und werden in identischer Weise angewandt wie in der pH-Metrie (siehe Abbildung 4) [43].

Die MII-pH bietet durch die Detektion aller auftretenden Refluxepisoden die Möglichkeit der Analyse auch unter einer säuresuppressiven Therapie mit PPI. In MII-pH Untersuchungen, die ohne und unter säuresuppressiver Therapie durchgeführt wurden, konnte gezeigt werden, dass sich die Anzahl aller Refluxepisoden durch eine PPI-Therapie nicht verändert. Vielmehr kommt es zu einer veränderten Qualität der Refluxepisoden von überwiegend sauren Refluxepisoden ohne Therapie hin zu nicht-sauren Refluxepisoden unter PPI-Therapie [44]. Durch MII-pH Analysen von Patienten mit PPI-refraktären Symptomen konnte nachgewiesen werden, dass auch nicht-saure Refluxepisoden für die Generation von Symptomen pathophysiologisch eine Rolle spielen. In unabhängigen Studien konnte durch zwei Arbeitsgruppen gezeigt werden, dass bei Patienten mit persistierenden Symptomen, diese auch mit nicht-sauren Refluxepisoden assoziiert sein können [45, 46].

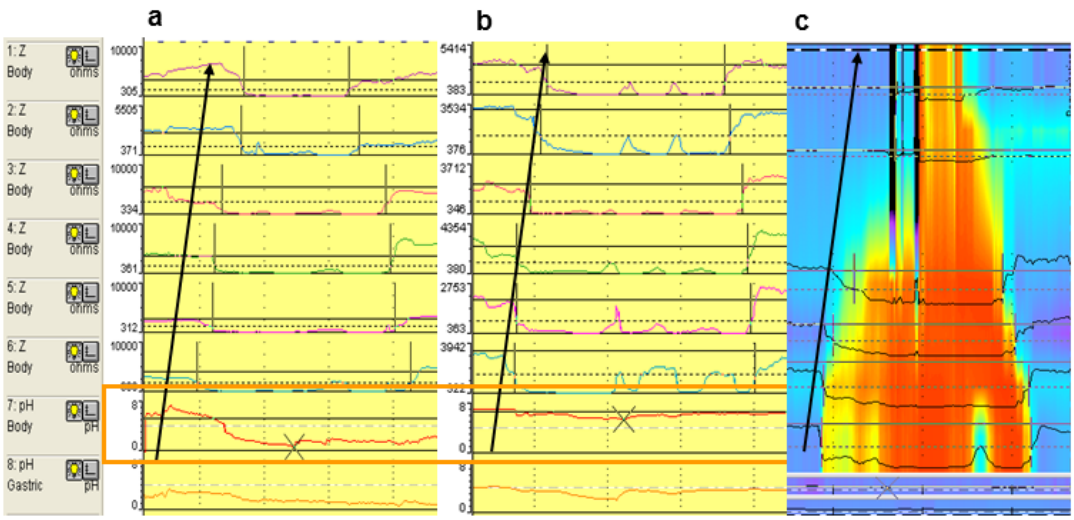


Abbildung 3: **kombinierte intraluminale Impedanz- und pH Metrie (MII-pH) (aus [47]).** Detektion der Refluxepisoden als retrograde Bolusbewegung durch einen retrograden Abfall des Impedanzniveaus (Pfeile). Über die simultane Aufzeichnung des pH Wertes (gelber Kasten, X) erfolgt die Qualifizierung der Refluxepisode in sauer (**a**) oder schwach-sauer (**b**). Die Darstellung der Impedanzänderung durch Fehlfarben erleichtert im Zweifel die Visualisierung und Nachweis der Bolusbewegung (**c**).

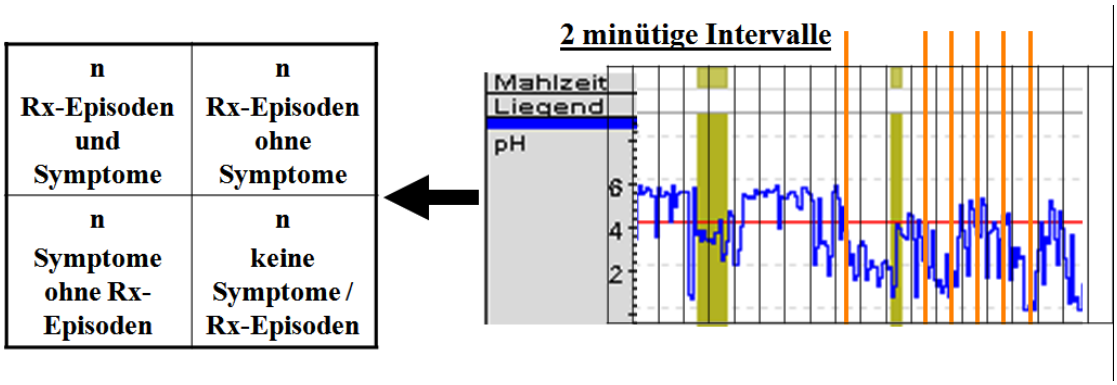


Abbildung 4: Berechnung der Symptom-Assoziation mittels Symptom-Assoziation-Probability (SAP) (aus [31]). Durch eine Software erfolgt die Einteilung der gesamten Aufzeichnung in 2-minütige Intervalle. Die Berechnung der der Wahrscheinlichkeit, dass in diesem Zeitfenster ein Symptom (orange) mit einer Refluxepisode (Abfall <pH 4) assoziiert ist (*RX* Reflux) erfolgt über eine 4-Felder-Tafel.

Über die Analyse von Refluxepisoden hinaus ermöglicht die Analyse des Impedanzniveaus, Rückschlüsse auf die Eigenschaften der Ösophagusmukosa zu schließen. Da die Impedanzelektroden der Schleimhaut direkt anliegen, haben Veränderungen der mukosalen Integrität, die mit Veränderungen der Permeabilität und Leitfähigkeit der Mukosa

einhergehen, Einfluss auf die Höhe des basalen Impedanzniveaus. Eine eigene Arbeit zu dieser Technik in der Differenzierung von Patienten mit FH und GERD ist Gegenstand der in der Habilitationsschrift aufgeführten Publikation IV [48-50].

Der Zusammenhang zwischen Säureexposition, Mukosaschädigung und Generation von Symptomen ist bisher nicht abschließend aufgeklärt. So scheint die Säureexposition nicht alleinig als Auslöser von Beschwerden von Bedeutung zu sein [45, 51, 52]. Anderen Bestandteilen des gastroösophagealen Refluxates wird eine wichtige Rolle bei der Entstehung sowohl von Mukosaläsionen als auch von Symptomen bei GERD eingeräumt. Dies ist insbesondere bei PPI-refraktären Symptomen ein wichtiger Ansatzpunkt für die pathophysiologische Erklärung von mukosaler Entzündung und Symptomgeneration.
Ein möglicher Mechanismus stellt die Aktivierung spezifischer Rezeptoren in der Ösophagusschleimhaut durch Trypsin oder andere Proteasen aus Magen- und/oder Dünndarmsekreten dar, und ist als Hypothese Ansatzpunkt der experimentellen Arbeiten, die in der Habilitationsschrift dargelegt werden. In dieser Arbeit fokussieren wir die Interaktion von luminalen Bestandteilen des Refluxates mit spezifischen Rezeptoren der ösophagealen Mukosa. Die Keratinozyten des ösophagealen Plattenepithels scheinen dabei eine essentielle Bedeutung für die Initiierung der mukosalen Entzündungsreaktion bei Patienten mit GERD einzunehmen. Die Hypothesen und Schlussfolgerung dieser Arbeit liefern die pathophysiologischen Erklärungsansätze, die in zwei hochrangig publizierten Übersichtsarbeiten vertieft diskutiert werden (siehe auch Abbildung 13; Publikation X) [53-55].

2.4 Pathophysiologische Grundlagen der Refluxerkrankung und Entwicklung alternativer therapeutischer Ansätze

Die grundlegende pathophysiologische Voraussetzung für gastroösophagealen Reflux sind sogenannte transiente Relaxationen des unteren Ösophagussphinkters (transient lower esophageal relaxations, TLESRs). Diese Relaxationen sind nicht mit Schluckakten assoziiert und treten bei Patienten mit GERD vermehrt auf. Während dieser transienten Relaxationen kommt es zum Übertritt von Mageninhalt in die Speiseröhre [56, 57]. Das gleichzeitige Vorliegen einer axialen Hernie, Übergewicht oder die Einnahme von Medikamenten, die mit dem Muskeltonus der glatten Muskulatur interagieren, begünstigen das Auftreten von TLESRs und vermehrten Refluxepisoden [58, 59].
Eine säuresuppressive Therapie mit PPI kann das Auftreten von TLESRs und Refluxepisoden nicht verhindern, sondern ändert lediglich den pH-Wert und damit die

Qualität des Refluxates. Wie bereits oben angeführt, wird die Anzahl der Refluxepisoden über 24 Stunden durch eine PPI-Therapie nicht beeinflusst [44].
Aufgrund des hohen klinischen Stellenwerts von PPI-refraktären Beschwerden wurden in den letzten Jahren verschiedene medikamentöse Wirkstoffe in der Entwicklung verfolgt, deren pharmakologische Ansätze auf eine Reduktion der TLESRs zielten [60]. Als „Refluxinhibitor“ kann Baclofen, als $GABA_B$-Agonist, die Anzahl der TLESRs und Refluxepisoden bei Patienten mit GERD um bis zu 40% reduzieren. Eine breite Anwendung ist jedoch durch häufige zentralnervösen Nebenwirkungen eingeschränkt, so dass der Einsatz nicht allgemein empfohlen werden kann [61, 62]. Weiterentwicklungen von Substanzen mit agonistischer Wirkung am $GABA_B$-Rezeptor sind das R-Enantiomer Arbaclofen plarcabil [63] und das ausschließlich peripher wirksame Lesogaberan [64], deren Effizienz in klinischen Studien (Phase IIb) belegt wurde.
Obwohl beide Präparate zu einer signifikanten Reduktion der Anzahl der TLESRs sowie Refluxepisoden bei einem tolerablen und deutlich verbesserten Nebenwirkungsprofil führen, wurde ihre Weiterentwicklung durch die pharmazeutischen Firmen aufgrund eines als zu gering eingestuften klinischen Benefits gestoppt.
Auch die pharmakologische Entwicklung einer Substanz mit einer metabotrophen, antagonistischen Wirkung am Glutamat-5-Rezeptor (mGlu5) führte zu einer signifikanten Reduktion sowohl von sauren als auch von nicht-sauren Refluxepisoden bei Patienten mit Therapie-refraktären Beschwerden [65-67]. Aufgrund einiger Fälle mit hepatischen Nebenwirkungen in der Phase II Studie wurde die Entwicklung dieses Produktes ebenfalls nicht weiter verfolgt.

Eine weiterer Mechanismus, der die Refluxerkrankung und Exposition des unteren Ösophagus mit Mageninhalt begünstigt, sind Beobachtungen während der pH-Metrie, die zur Definition der sogenannten „Acid Pocket“ geführt haben. Hierbei handelt sich um einen Bereich im proximalen Fundus und Kardia des Magens, dessen Mukosa verstärkt Säure sezerniert und als Reservoir für sauren gastroösophagealen Reflux angenommen wird [68-70] . Obwohl die Therapie mit PPI zwar Einfluss auf die Ausdehnung und auf die Azidität dieses Bereichs nimmt, scheint die Säuresekretion in diesem Bereich weniger durch PPI beeinflussbar als im restlichen Magen und wird nur in geringen Maßen durch Nahrungsaufnahme gepuffert und neutralisiert [68]. Bei Vorliegen einer axialen Hernie befindet sich der Bereich der „Acid Pocket“ zu größeren Anteilen auch oberhalb des Zwerchfells und begünstigt das Auftreten von sauren Refluxepisoden. Dieser Mechanismus scheint vor allem für postprandiale Refluxepisoden von pathophysiologischer Bedeutung zu sein.

Neuere Untersuchungen mittels Szintigraphie, MII-pH und MII-Manometrie konnten zeigen, dass Alginate in Kombination mit einem Antazidum in einer bestimmten pharmakologischen Formulierung (Gaviscon Dual) pathophysiologisch an der „Acid Pocket“ angreift und eine potente therapeutische Option darstellen kann. Dabei kommt es durch das Antazidum zu einer reduzierten Azidität der „Acid Pocket“ vor allem in den proximalen Anteilen des Magens. Zum anderen kommt es durch Kontakt mit der Magensäure zur Ausbildung eines gelartigen Schaums um den Speisebrei herum, der sowohl die Anzahl an Refluxepisoden im Allgemeinen als auch die proximale Ausdehnung zu reduzieren scheint. [71-73].

2.5 Pathophysiologische Veränderungen der Ösophagusmukosa

Die Mukosa von Patienten mit GERD weist charakteristische Veränderungen des Plattenepithels auf, die auch lichtmikroskopisch zu beschreiben und charakterisieren sind. Eine Hyperplasie der Basalzellschicht wurde bereits in den 1970er Jahren von Ismail-Beigi histomorphologisch beschrieben [74]. Hopwood und schließlich Tobey konnten in elektronenmikroskopischen Untersuchungen die Dilatation der Interzellularspalten (dilated intercellular spaces, DIS) im Spatium spongiosum als charakteristische Veränderungen bei Patienten mit GERD, sowohl bei ERD als auch bei Patienten mit NERD, beschreiben [75-77]. Neben der Dilatation der Interzellularspalten finden sich als charakteristische Veränderungen bei Patienten mit GERD eine verbreiterte Basalzellschicht (Basalzellhyperplasie) und elongierte Papillen mit darin enthaltenen submukösen Gefäßen (Papillenelongation). Diese Veränderungen können auch in der Lichtmikroskopie erkannt und unterschiedlichen Schweregraden zugeordnet werden (siehe Abbildung 5).

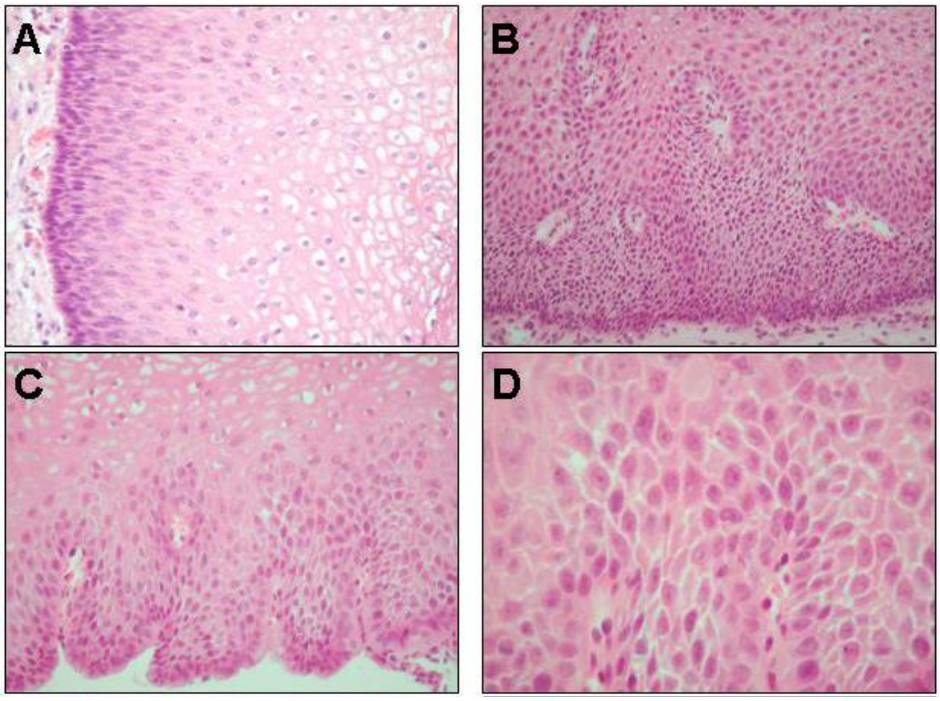


Abbildung 5: Histomorphologische Veränderungen der Ösophagusmukosa von Patienten mit GERD (Kandulski et al. NOVA Publishing 2010, siehe auch Publikation V, [78]). Exemplarische Darstellung von unauffälliger Mukosa (**A**), Basalzellhyperplasie (**B**), Papillenelongation (**C**) und erweiterter Interzellularspalten (**D**) bei Patienten mit GERD.

Obwohl wiederholte Versuche unternommen wurden, unterschiedliche histopathologische Scoring-Systeme für Veränderungen der Mukosa bei GERD zu entwickeln, konnte keine dieser Klassifikationen in der klinischen Routine zur standardisierten histopathologischen Beurteilung der Mukosa implementiert werden [79-82].

Als funktionelles Korrelat der veränderten Morphologie findet sich eine gestörte Integrität mit erhöhter Permeabilität der Mukosa bei Patienten mit GERD. Im Tiermodell konnte gezeigt werden, dass die Ösophagusschleimhaut auf Exposition mit Medien sauren pH-Werts mit der Ausbildung von DIS reagiert. Funktionell sind diese Veränderungen mit einem reduzierten transepithelialen Widerstand assoziiert [76, 83-85]. Die Ergebnisse aus dem Tiermodell konnten in Teilen auf das humane System übertragen werden. Bei gesunden Probanden, konnten Farré et al. durch Perfusion des Ösophagus mit Lösungen sauren pH-Werts eine Erweiterung der Interzellularspaten induzieren. Funktionell gingen diese morphologischen Veränderungen der Mukosa ebenfalls mit einem reduzierten transepithelialen Widerstand und einer erhöhten Permeabilität einher [49].
Veränderungen der mukosalen Integrität haben Einfluss auf die Leitfähigkeit und die elektrischen Feldeigenschaften der Mukosa und sind mit Veränderungen des intraluminalen

Impedanzsignals assoziiert. Direkte Messungen der mukosalen Impedanz mittels spezieller Katheter aber auch Analysen des basalen Impedanzniveaus der Mukosa während der MII-pH erlauben Rückschlüsse auf die Integrität der Ösophagusmukosa [49, 86]. Die eigenen Arbeiten zu dieser Methodik in der klinischen Anwendung zur Differentialdiagnose von FH und GERD sind Gegenstand dieser Habilitationsschrift (Publikation IV, [50]).

Neben morphologischen und funktionellen Veränderungen ist die Schleimhaut des Ösophagus von Patienten mit GERD durch ein proinflammatorisches Zytokinmilieu charakterisiert. Sowohl in Biopsien von Patienten mit GERD als auch in Biopsien und isolierten Keratinozyten aus unterschiedlichen Tiermodellen konnte eine verstärkte Expression von Interleukin-8 (IL-8), IL-1ß und anderen proinflammatorischen Zytokine nachgewiesen werden [87-92]. Diese molekularen Veränderungen zeigen eine enge Korrelation mit dem endoskopischen Schweregrad bei ERD und histopathologischen Veränderungen der Mukosa.
Arbeiten, die die Schleimhaut im Rahmen einer erfolgreich durchgeführten Anti-Refluxchirurgie untersuchten, konnten regrediente Befund dieser molekularen Veränderungen im postoperativen Follow-Up beschreiben [87, 89, 93-95].
Die Induktion eines proinflammatorischen Zytokinmilieus der Mukosa sind zeitlich sehr früh und deutlich vor dem Auftreten mikroskopischer oder gar makroskopischer Veränderungen nachweisbar. In einigen experimentellen Studien konnte gezeigt werden, dass plattenepitheliale Keratinozyten durch Kontakt mit Mageninhalt zur Sekretion proinflammatorischer Zytokine angeregt werden [53, 92]. Dies wiederum führt durch die Eigenschaften als Chemokine zu einer verstärkten Migration und Infiltration von neutrophilen Granulozyten in die Mukosa. In einem Rattenmodell zur Untersuchung der zeitlichen Abfolge dieser spezifischen Veränderungen konnten Souza et al. die Sekretion von IL-8 durch mukosale Keratinozyten als initiales Ereignis beobachten, das im zeitlichen Verlauf zur Infiltration von Lymphozyten und Leukozyten aus der Blutbahn in die Schleimhaut führt. Diese entzündlichen Veränderungen gehen einer proliferativen Antwort des Epithels mit den charakteristischen Veränderungen (Basalzellhyperplasie, Papillenelongation, erweiterte Interzellularspalten) voraus (siehe Abbildung 13) [54, 55].

Über Jahrzehnte hinweg wurden die entzündlichen Veränderungen der Ösophagusmukosa pathophysiologisch durch eine Säure-bedingte, direkte Schädigung des Plattenepithels erklärt. Die Infiltration von Entzündungszellen in die Mukosa wurde als sekundäres Ereignis interpretiert, um den Abbau geschädigten Zellmaterials und Zelldetritus zu übernehmen. Es bestand das Dogma einer kaustischen Verletzung der Mukosa, die an der Oberfläche beginnend, zum Teil ulzerierend in die tiefere Schichten der Schleimhaut vordringt und eine

proliferativen Antwort des Epithels als regenerative Antwort induziert [96]. In diesem pathophysiologischen Modell führen Wasserstoffionen des sauren Magensafts sowie im Refluxat enthaltenes Pepsin zur Degradation von Zell-Zellkontakten zu einer Zerstörung des Epithels mit einer sekundären Induktion von entzündlichen Veränderungen [97, 98]. Die so destruierten Zell-Zell-Kontakte würden ein Voranschreiten des epithelialen Schadens in tiefere Schichten begünstigen. Die Funktionen der ösophagealen Keratinozyten und der Einfluss anderer Bestandteile im gastroösophagealen Refluxat blieben in diesem Modell der Pathophysiologie lange Zeit unbeachtet. Ebenfalls unklar bleibt in diesem Modell, warum auch die Exposition von luminalen Bestandteilen nicht-sauren pH-Werts zu Symptomen führen kann.
Die in der Habilitationsschrift dargelegten Arbeiten zur Pathophysiologie und Beteiligung des Protease-aktivierten Rezeptor-2 [53] unterstreichen diesen Paradigmenwechsel im Verständnis der entzündlichen Veränderungen in der Mukosa von Patienten mit Refluxerkrankung. Die erarbeiteten Ergebnisse und entwickelten Thesen weisen alternative therapeutische Ziele in der Behandlung insbesondere von Patienten mit PPI-refraktären Symptomen auf [99].

3. Aufgabenstellung der Habilitationsschrift

Die in der Habilitationsschrift dargestellten Arbeiten beschäftigen sich mit der klinisch-funktionellen Charakterisierung von Patienten mit GERD mittels moderner Funktionsdiagnostik und anhand morphologischer und molekularer Veränderungen der Ösophagusmukosa. Dabei spielt die Abgrenzung der nicht-erosiven Refluxerkrankung zu Patienten mit funktionellem Sodbrennen in der Differentialdiagnose eine zentrale Fragestellung.
Des Weiteren stellte die molekulare Charakterisierung der Entzündung des gastroösophagealen Überganges und der Ösophagusschleimhaut bei Patienten mit GERD einen inhaltlichen Schwerpunkt der Arbeiten.

Im Einzelnen wurden folgende Teilaspekte bearbeitet:

- klinische Charakterisierung und Untersuchungen zum therapeutischen Ansprechen auf eine Standardtherapie mit PPI, basierend auf moderner gastroösophagealer Funktionsdiagnostik;
- morphologische und funktionelle Veränderungen der Mukosa von Patienten mit GERD in Abgrenzung zu Patienten mit funktionellem Sodbrennen;
- molekulare Charakterisierung von Veränderungen der Tight Junctions und desmosomalen Komponenten der Mukosa von Patienten mit GERD;
- molekulare Charakterisierung entzündlicher Veränderungen der Mukosa von Patienten mit GERD und experimentelle Untersuchungen zur Bedeutung des Protease-aktivierten Rezeptor-2 (PAR2) für die Pathogenese der gastroösophagealen Refluxerkrankung.

4. Darstellung der Ergebnisse

4.1 Untersuchungen zur klinischen Charakterisierung, therapeutischem Ansprechen und gastroösophagealer Funktionsdiagnostik

Wie einleitend betont, ist die korrekte Diagnose der Refluxerkrankung für die Einleitung einer adäquaten Therapie und des Therapieerfolges essentiell. Die Diagnose der Refluxerkrankung und die Indikation zur Einleitung einer Therapie mit PPI sollte nach den Empfehlungen von nationalen und internationalen Leitlinien zunächst basierend auf dem Vorhandensein typischer Symptome gestellt werden [4-6]. Epidemiologische Daten belegen, dass neben typischen Symptomen der gastroösophagealen Refluxerkrankung vor allem funktionelle Beschwerden und Symptomkomplexe in der westlichen Allgemeinbevölkerung gehäuft auftreten. Eine genaue Differenzierung der verschiedenen Symptome, die Differentialdiagnose und Therapie ist vor allem bei chronischen Verläufen schwierig [9, 10]. Die wissenschaftliche Fragestellung der unter Publikation I aufgeführten Arbeit zielte zunächst auf eine Charakterisierung von Patienten anhand der prädominanten Symptomen, mit denen diese sich in ambulanter Behandlung in der gastroenterologischen Sprechstunde der Klinikambulanz befanden [8]. Im speziellen zielte die Erfassung der dominanten Symptome auf die Differenzierung von Symptomen aus dem Bereich der funktionellen gastrointestinalen Symptomen, die durch die aktualisierten ROME III Kriterien definiert wurden [36, 100, 101]. Die Diagnose der Refluxerkrankung erfolgte basierend auf der

Montreal-Klassifikation bei Vorliegen typischer Symptome und wurde endoskopisch in ERD, NERD und Barrett-Ösophagus unterschieden [2]. Die Schwere der Reflux-Symptome wurde mittels dem validierten Fragebogen zur Refluxerkrankung (Reflux Disease Questionnaire, RDQ) objektiviert [102, 103]. Zur Evaluierung der dyspeptischen und funktionellen Symptome entwickelten wir einen vereinfachten Fragebogen analog den Empfehlungen der ROME III Konsensus Gruppe. Beide Fragebögen wurden während der Visite in der Kliniksambulanz durch die Patienten ausgefüllt.
Gezielt durch die Items der Fragebögen erfasst, gaben in unserem Kollektiv bis 70% der Patienten mit NERD an, zusätzlich unter dyspeptischen Beschwerden und abdominellen Schmerzen zu leiden. Die Ergebnisse unserer Studie finden sich in Erhebungen aus den Vereinigten Staaten bestätigt, die eine bedeutende Querschnittsmenge von Reflux-Symptomen mit dyspeptischen Symptomen sowie Symptomen des Reizdarmsyndroms vor allem bei Patienten mit NERD beschreiben [9].
Diese Daten belegen, wie schwierig eine korrekte Diagnose der Refluxerkrankung entsprechend der Montreal-Klassifikation basierend auf Symptomen zu stellen sein kann und wie häufig zusätzliche Symptome aus dem funktionellen Formenkreis in der Differentialdiagnose berücksichtigt werden müssen. Die Ergebnisse liefern einen Erklärungsansatz, warum gerade die Patientengruppe mit NERD in epidemiologischen Studien ein so schlechtes therapeutisches Ansprechen aufweisen [24]. Daten aus der prospektiv durchgeführten DIAMOND Studie belegen darüber hinaus, dass selbst Patienten, bei denen eine Refluxerkrankung durch eine pH Metrie objektiv gesichert wurde, nur in etwa 50% der Fälle unter typischen Symptomen (Sodbrennen, saures Aufstoßen) leiden [23].

In der unter Publikation II aufgeführten Studie haben wir untersucht, in wie weit eine mittels gastroösophagealer Funktionsdiagnostik objektivierte Diagnose der Refluxerkrankung ein erfolgreiches symptomatisches Ansprechen auf eine Therapie mit PPI vorhersagen kann. Prospektiv wurden in dieser Studie Patienten mit typischen Reflux-Symptomen (Sodbrennen und/ oder saures Aufstoßen) eingeschlossen, die bis zum Studieneinschluss keine säuresuppressive Therapie erhalten hatten (PPI naiv). Alle Patienten wurden sowohl endoskopisch als auch funktionell mittels BRAVO© Kapsel pH-Metrie über 48 Stunden untersucht. Bei pathologischer Säureexposition des distalen Ösophagus (% AET >4,2) und/oder erosiven Veränderungen des Ösophagus während der Endoskopie wurde die Diagnose der Refluxerkrankung gestellt und eine Therapie mit PPI initiiert. Wenn die Diagnose der Refluxerkrankung anhand dieser objektiven Kriterien gestellt wurde, zeigte die säuresuppressive Therapie in einfacher Standarddosierung ein exzellentes Ansprechen nach einer Therapiedauer von 4 Wochen. Dabei fanden sich keine Unterschiede zwischen Patienten mit NERD und ERD. Die Daten unserer Studie entsprechen den Ergebnissen einer

Meta-Analyse, in die die bisher publizierten, prospektiven Untersuchungen zum therapeutischen Ansprechen auf PPI eingeschlossen wurden. Insgesamt wurden 54 Studien in der Metanalyse analysiert. Die Meta-Analyse zeigt, dass das therapeutische Ansprechen auf eine Therapie mit PPI für Patienten mit NERD eine Erfolgsrate von lediglich 49% aufweist, wenn die Diagnose der Refluxerkrankung basierend auf typische Symptome und dem endoskopischen Ergebnis gestellt wird (8 Studien). Dies ist vergleichbar mit dem therapeutischen Erfolg einer empirisch begonnen PPI Therapie (12 Studien). Lediglich in 2 Studien wurde die Diagnose der NERD basierend auf einem pathologischen Befund einer pH-Metrie mit einer pathologischen Säureexposition (% AET > 4.2) und einem unauffälligem gastroösophagealen Übergang während der Endoskopie definiert. Vergleichbar mit den Ergebnissen unserer Studie fanden die Autoren der Meta-Analyse ein gutes Ansprechend von Patientenmit NERD und ERD, wenn die NERD entsprechend den aufgeführten Kriterien einer auffälligen pH-Metrie objektivierbar diagnostiziert wurde.

Auch wenn der sogenannte „PPI Test“ als diagnostischer Test im eigentlichen Sinne nicht empfohlen werden kann, ist es aus klinischer Sicht ein pragmatisches Vorgehen und durch die Leitlinien empfohlen, bei typischen Symptomen und bei Fehlen von sogenannten Alarmsymptomen mit einer PPI-Therapie zu beginnen. Aufgrund des begrenzten Zugangs zu einer adäquaten gastroösophagealen Funktionsdiagnostik, eines zum Teil begrenzten Wissens über die Technik und Limitationen der Methodik im Allgemeinen und nicht zuletzt aufgrund einer unzureichenden Abbildung in der Vergütung erscheint es im klinischen Alltag schwer, diese schon früh im diagnostischen Algorithmus der Refluxerkrankung zu implementieren. Sollte das Ansprechen auf eine Therapie jedoch unbefriedigend, legen unsere Daten und die bisher publizierten Arbeiten nahe, frühzeitig die Diagnose der Refluxerkrankung zu objektivieren und zu verifizieren [24, 25] . Entscheidend dabei auch die klinische Fragestellung an die gastroösophageale Funktionsdiagnostik. Sollte die Diagnose der Refluxerkrankung (insbesondere NERD) zum Untersuchungszeitpunkt nicht objektiviert und sicher gestellt worden sein, ist die Durchführung der Funktionsuntersuchung ohne PPI zu empfehlen [5, 40, 41]. Aufgrund der Katheter-freien Technik und der geringen Einschränkung in den Tätigkeiten des Alltags bietet die BRAVO© Kapsel pH-Metrie in dieser Indikation im Vergleich zur konventionellen pH-Metrie eine deutlich verbesserte Patienten-Compliance. Darüber hinaus erhöhen sich durch die verlängerte Analysedauer von 48 Stunden die statistischen Testkriterien deutlich [37, 38, 104].
Wenn in der ambulanten pH-Metrie ein pathologischer gastroösophagealer Reflux ausgeschlossen werden kann, so ist sowohl die Diagnose der Refluxerkrankung als auch die Indikation einer säuresuppressiven Therapie kritisch zu hinterfragen und gegebenenfalls auch zu beenden [105].

Die Entwicklung der kombinierten intraluminalen Impedanz und pH-Analyse (MII-pH) bietet die Möglichkeit, gastroösophageale Refluxepisoden auch unter einer Therapie mit PPI zu untersuchen [31, 41, 42, 44]. Dabei wird eine Refluxepisode als Abfall des Impedanzwertes von distal nach proximal als retrograde Bolusbewegung im Ösophagus definiert (siehe auch Kapitel 2.3; Abbildung 3). Durch zusätzliche pH Elektroden auf dem Meßkatheter werden die Refluxepisoden anhand ihres pH Wertes in saure, schwach-saure und schwach-alkalische Refluxepisoden unterschieden [40].

Ziel der unter Publikation III aufgeführten Studie war es, Patienten mit PPI-refraktären Symptomen mittels MII-pH besser zu charakterisieren. Dabei wurden Patienten eingeschlossen, die unter einer Therapie mit PPI in doppelter Standarddosierung unter fortbestehenden typischen Symptomen litten. Neben der Anzahl und Qualität der gastroösophagealen Refluxepisoden und deren Symptom-Assoziation wurde das pH-Profil des Magens analysiert und hinsichtlich des Auftretens eines sogenannten nächtlichen „Säuredurchbruchs“ (nocturnal acid breakthrough, NAB) analysiert. NAB wird als nächtlicher Abfall des pH-Wertes im Magen unter einer PPI-Therapie in doppelter Standarddosierung definiert [106]. In unserem Studienkollektiv konnten wir einen NAB in 46% der Patienten nachweisen. Refluxepisoden während der Phasen dieser NABs traten jedoch in so gut wie keinem der untersuchten Fälle auf und stellen damit ein extrem seltenes Ereignis dar. Vergleichbare Daten wurden durch Arbeiten der Arbeitsgruppe um Ronny Fass publiziert. Untersuchungen zu Refluxepisoden, Symptomen in Assoziation zum Schlaf konnte die Arbeitsgruppe zuletzt zeigen, dass Refluxepisoden während kurzer Phasen des Ruhens (Naps) deutlich häufiger auftreten als während des nächtlichen Schlafens [107].

Die Patienten in unserer Studie, bei denen ein NAB nachweisbar war, wiesen im gesamten Analysezeitraum insgesamt signifikant häufigere Refluxepisoden auf. Das Auftreten von NAB in unserem Studienkollektiv interpretieren wir als Risikofaktor für schwere und therapierefraktäre Verläufe. Bei Patienten mit NAB und nächtlichen Symptomen beziehungsweise nächtlichem Erwachen wird diskutiert, dass eine zusätzliche Therapie mit einem H_2-Rezeptor-Anatgonisten zur Reduktion der Dauer des nächtlichen pH-Abfalls und auf eine Reduktion der nächtlichen Symptome führen kann [108]. Entsprechende kontrollierte Untersuchungen mit dieser speziellen Fragestellung wurden bisher jedoch nicht durchgeführt.

4.2 Untersuchungen zu morphologischen und funktionellen Veränderungen der Ösophagusmukosa von Patienten mit gastroösophagealer Refluxerkrankung und funktionellem Sodbrennen

Die Ergebnisse der unter Publikation IV [50] aufgeführten Arbeit zeigen, dass die MII-pH die technische Möglichkeit bietet, auch funktionelle Veränderungen der Mukosa abzubilden. Dabei spiegelt die Höhe des basalen intraluminalen Impedanzmusters die Leitfähigkeit der Mukosa wider und kann als Surrogatparameter für die mukosale Integrität interpretiert werden.

In die Studie wurden Patienten mit PPI-refraktären Symptomen eingeschlossen. Bei mindestens einem der geschilderten PPI-refraktären Symptome sollte es sich um ein typisches Symptom (Sodbrennen und/oder saures Aufstoßen) handeln. Bei allen Patienten, die in diese Studie eingeschlossen wurden, wurde die Therapie mit einem PPI zuvor ausgeschlichen und für wenigstens 2 Wochen pausiert, um einen sogenannten „Acid Rebound“ [109] während der Untersuchung zu verhindern. Vor der Durchführung der MII-pH wurde bei allen Patienten eine ÖGD mit Entnahme von Biopsien aus dem Ösophagus (3-5 cm oberhalb des gastroösophagealen Überganges) zur histopathologischen Beurteilung durchgeführt. Bei dem überwiegenden Teil der eingeschlossenen Patienten erfolgten die endoskopische Untersuchung und die Anlage des MII-pH-Katheters am gleichen Untersuchungstag.
In der Analyse der intraluminalen Impedanzmuster wurden Artefakte durch Schluckakte und Refluxepisoden ausgeschlossen und die Höhe des basalen Impedanzniveaus über einen Analysezeitraum von 30 Minuten in liegender Körperposition als sogenannte „basale Impedanz“ gemessen (siehe Abbildung 6).
Bei Patienten mit GERD ist dabei ein signifikant reduziertes intraluminales basales Impedanzsignal (distal baseline impedance, DBI) im distalen Ösophagus nachweisbar, anhand dessen man Patienten mit nachgewiesener Refluxerkrankung von Patienten mit funktionellem Sodbrennen unterscheiden kann. Dabei korreliert ein vermindertes Signal des basalen Impedanzmusters im distalen Ösophagus mit einer gestörten Integrität der Mukosa und mit erweiterten Interzellularspalten in der konventionellen histopathologischen Auswertung mittels konventioneller Lichtmikroskopie.

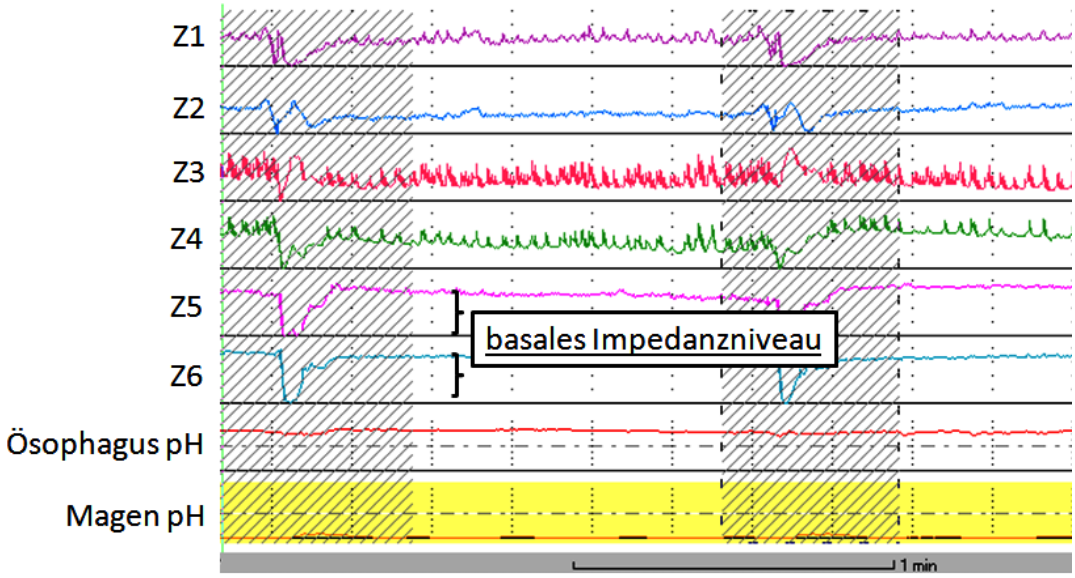


Abbildung 6: Analyse der Baseline Impedance (basales Impedanzniveau, BI) im distalen Ösophagus während der MII-pH [31]. Die Analyse des distalen Impedanzniveaus erfolgt in den beiden distalen Impedanzkanälen (Z5, Z6). Schluck- oder Refluxepisoden werden von der Analyse ausgeschlossen (grau schraffiert). Die Analyse erfolgt in einem Abschnitt der Aufzeichnung mit einem möglichst stabilem Impedanzniveau ohne Artefakte über einen längeren Zeitraum (liegende Position, nachts).

Physikalisch stellt der Messwert der mukosalen Impedanz den Kehrwert der elektrischen Leitfähigkeit der Mukosa dar. Veränderungen der mukosalen Integrität mit Erweiterung der Interzellularspalten führen zu einer erhöhten parazellulären Permeabilität und gehen mit einem reduzierten transepithelialen Widerstand einher [49, 110]. Damit erhöht sich die Leitfähigkeit der Mukosa und das basale Impedanzsignal ist vermindert. Wir konnten in unserer Studie eine Korrelation von erweiterten Interzellularspalten und vermindertem DBI bei Patienten mit GERD nachweisen. Wir diskutieren diese Beobachtung durch eine verbesserte elektrische Leitfähigkeit der Mukosa bei erweiterten Interzellularspalten, da nicht nur eine erhöhte Permeabilität vorliegt sondern vielmehr auch mehr Ladungsträger in der Gegenwart von DIS in der Mukosa zu einer veränderten Leitfähigkeit beitragen.

Bei Patienten mit FH waren keine vergleichbaren Veränderungen des DBI nachweisbar. Das basale Impedanzniveau bei FH war im Vergleich zu Patienten mit GERD signifikant erhöht (siehe Abbildung 7). Mit einem Cut-Off Wert von 2.100 Ohm war es uns in unserem Kollektiv möglich, Patienten mit GERD von FH zu unterscheiden (Sensitivität 78%, Spezifität 75%, PPV 75%, NPV 75%).

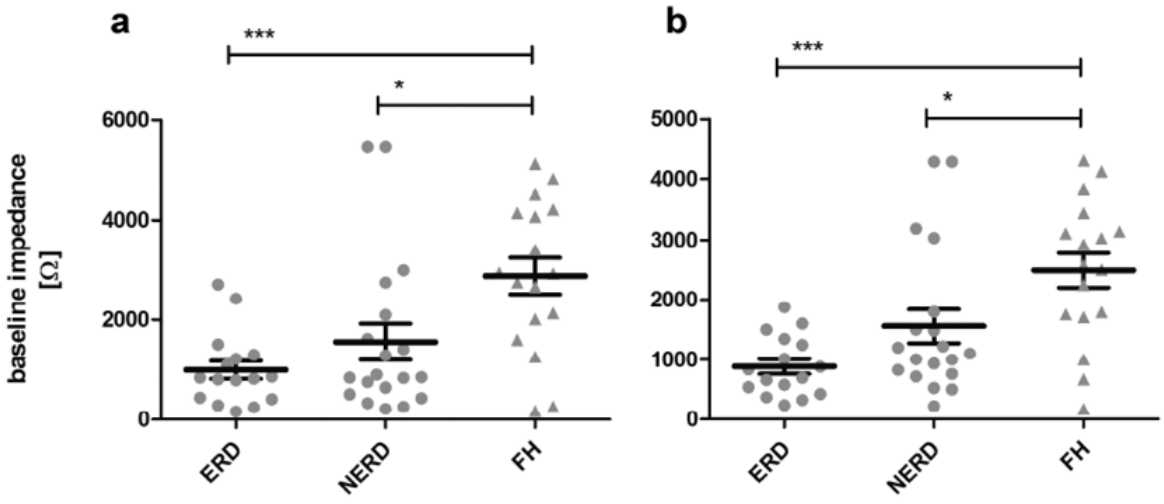


Abbildung 7: Baseline Impedance (BI) der Mukosa im distalen Ösophagus bei Patienten mit ERD, NERD und FH (aus Publikation IV [50]). Panel **a** + **b** stellen die Ergebnisse der Veränderungen der BI für ERD, NERD und FH im distalen Ösophagus dar ((**a**) 3 cm oberhalb des gastroösophagealen Überganges, (**b**) 5 cm oberhalb des gastroösophagealen Überganges).

Die Analyse der MII-pH mit zusätzlicher Auswertung des DBI bietet somit die Möglichkeit, Aussagen über die Funktionalität und Integrität der Ösophagusmukosa zu treffen und sollte als zusätzliche Analyse bei PPI refraktären Symptomen in der Abgrenzung zu FH durchgeführt und als Parameter in der Gesamtbewertung mit einbezogen werden.

Analog zu den Ergebnissen unserer Studie findet sich eine Publikation von André Smout und Kollegen aus den Niederlanden. Die Autoren beschreiben vergleichbare Veränderungen der DBI bei Patienten mit nachgewiesener Refluxerkrankung. Dabei war die gemessene Höhe der DBI im distalen Ösophagus vergleichbar mit den Analysen von Patienten mit GERD in unserem Kollektiv. In identischer Weise konnten die Kollegen ebenfalls eine signifikante Korrelation von vermindertem DBI und erhöhter Säureexposition des distalen Ösophagus (% AET) nachweisen (siehe Abbildung 8).

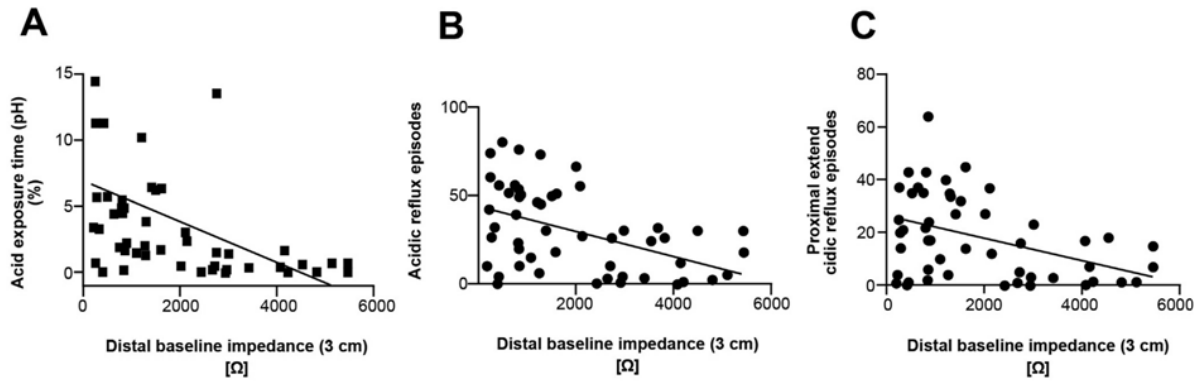


Abbildung 8: Negative Korrelation von DBI bei 3 cm mit AET (A) (r: -0,45, p=0,008), Anzahl der sauren Refluxepisoden (B) (r: -0,45, p=0,001) und Anzahl der proximalen, sauren Refluxepisoden (C) (r: -0,4, p=0,003) (aus Publikation IV [111]).

Eine weitere Arbeit beschreibt Veränderungen des DBI vor und nach erfolgreicher Anti-Reflux-Operation. Die Autoren dieser Publikation konnten Veränderungen des DBI bei Patienten mit GERD herausarbeiten, die mit den Ergebnissen unserer Arbeit vergleichbar sind [112]. Nach Fundoplikatio wurden alle Patienten 6 Monate nach dem operativen Eingriff mittels MII-pH nachuntersucht. Im Follow-Up konnten die Autoren deutlich höhere DBI Werte beschreiben als in der prä-operativen Untersuchung. Diese waren jedoch immer noch auf einem niedrigeren Niveau als im Vergleich zu freiwilligen Probanden ohne Symptome.
In beiden genannten Studien wurden keine Biopsien aus dem distalen Ösophagus zur Evaluierung morphologischer Veränderungen (DIS) der Mukosa entnommen. Aus Langzeitbeobachtungen im Kollektiv des sogenannten LOTUS-Trials ist bekannt, dass die morphologischen Veränderungen in der Mukosa ebenfalls langsam regredient sind und erst nach Jahren eine Restitutio erreichen [113], was sehr gut mit der Assoziation zwischen funktionellen Veränderungen mit vermindertem DBI und morphologischem Korrelat (DIS) übereinstimmt.
Eine Stärke unserer Studie im Vergleich zu den anderen Arbeiten ist insbesondere die funktionelle und diagnostische Abgrenzung zu Patienten mit FH mittels DBI, die in keiner der genannten Publikationen vorgenommen wurde.

Bei PPI refraktären Symptomen ist, wie einleitend ausführlich dargelegt, eine weiterführende Funktionsdiagnostik notwendig. In der als Publikation V [78] aufgelisteten Studie wurde untersucht, in wie weit die Entnahme von Biopsien aus dem distalen Ösophagus mit histopathologischer Analyse definierter Veränderungen (dilatierte Interzellularspalten (DIS), Elongation der intramukosalen Papillen (PE), Hyperplasie des Basalzell-Kompartiments

(BCH) (siehe Abbildung 5) in der Differentialdiagnose von GERD speziell in der Abgrenzung zu funktionellem Sodbrennen weiterführend ist.
In die Studie wurden analog zu Publikation IV [50] Patienten mit PPI-refraktären Symptomen eingeschlossen. Die Medikation mit PPI wurde schrittweise ausgeschlichen und, wie in der bereits aufgeführten Arbeit beschrieben, wenigstens 2 Wochen vor der Untersuchung pausiert. Endoskopisch wurden Biopsien aus dem distalen Ösophagus etwa 3 – 5 cm oberhalb der Z-Linie entnommen.
Basierend auf den Untersuchungsergebnissen der ÖGD und MII-pH wurde eine Einteilung der Patienten in die Gruppen ERD, NERD und FH vorgenommen. In der Gruppe der Patienten mit NERD wurde zusätzlich die Subgruppe mit EH (ösophageale Hypersensitivität) unterschieden.
Die histomorphologische Auswertung der Biopsien erfolgte lichtmikroskopisch durch die fachärztliche Kollegin (PD Dr. med. Dörthe Jechorek) aus dem Institut für Pathologie. Die Beurteilung erfolgte standardisiert an Hämatoxylin-Eosin (HE)-gefärbten histologischen Schnitten. Frau PD Dr. med. Jechorek war bezüglich der endoskopischen Ergebnisse und der Ergebnisse der MII-pH nicht informiert, so dass die histopathologische Analyse verblindet erfolgte. Dabei wurden die Veränderungen der Mukosa (DIS, PE, BCH, Inflammation) semiquantitativ (0=keine Veränderungen, III=schwerste Veränderungen) bewertet.

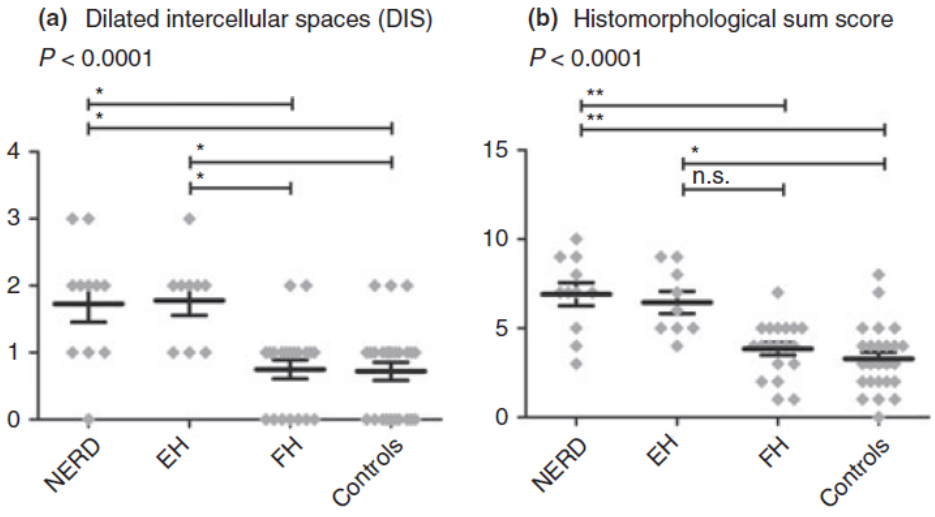


Abbildung 9: Differenzierung von Patienten mit FH von Patienten mit NERD und EH anhand der Analyse von DIS (a) und eines Summen-Scores (DIS + BCH + PE + Inflammation) (aus Publikation V [78]). Mit einer Sensitivität von 85% und einem negativen prädiktiven Wert von 80% war es in unserem Kollektiv möglich, mittels histomorphologischem Summen-Score bei einem Cut-Off von ≥5 Punkten Patienten mit NERD von Patienten mit FH zu unterscheiden.

Basierend auf der Analyse von DIS und eines histopathologischen Gesamt-Scores, berechnet als Summe der einzelnen Parametern (DIS, PE, BCH und Inflammation), konnten wir Patienten mit FH von Patienten mit GERD, insbesondere von NERD unterscheiden (siehe Abbildung 9). Für den histopathologischen Gesamt-Score und einem Punktwert von ≥5 Punkten konnten wir zur Differenzierung von NERD und FH eine Sensitivität von 85% bei einer Spezifität von 63% berechnen.
Basierend auf diesen eigenen Daten sowie auf den publizierten Daten anderer Gruppen [114, 115] empfehlen wir, speziell bei PPI-refraktären Beschwerden in der diagnostischen Aufarbeitung die Durchführung von Biopsien und histopathologische Beurteilung der Ösophagusmukosa durch einen geschulten Kollegen der Pathologie. Aus unserer Sicht besitzt die histomorphologische Beurteilung der Ösophagusmukosa nicht nur zur Abgrenzung zur eosinophilen Ösophagitis beziehungsweise zur PPI-sensitiven ösophagealen Eosinophilie (PPI-REE, PPI-responsive esophageal eosinophilia) einen klinischen Stellenwert [116, 117]. Um diesen Standpunkt zu verdeutlichen, wurden wir zu einem Kommentar in der entsprechenden Fachzeitschrift eingeladen [118]. Die histopathologische Bewertung der Ösophagusmukosa sollte aus unserer Sicht als zusätzliches diagnostisches Werkzeug eingesetzt werden, dessen zusätzlichen Nutzen in der Differentialdiagnose für die komplizierte Refluxerkrankung gemeinsam mit einer adäquaten Funktionsdiagnostik das weitere therapeutische Management beeinflussen kann.

4.3 Molekulare Untersuchungen zu morphologischen Veränderungen, zur Charakterisierung entzündlicher Veränderungen der Mukosa und molekulare Mechanismen für die Pathogenese der gastroösophagealen Refluxerkrankung

In den unter Publikationen VI und VII aufgeführten Arbeiten wurden in einem translationalen Ansatz molekulare Veränderungen von Bestandteilen der Tight Junctions und von desmosomalen Komponenten der Zell-Zell-Kontakte in der Ösophagusmukosa von Patienten mit GERD untersucht.
Die Diagnosestellung der GERD erfolgte in diesen Studien analog der Montreal-Klassifikation basierend auf prädominanten typischen Reflux-Symptomen. Alle Patienten wurden endoskopisch untersucht und Biopsien aus der Ösophagusmukosa 5 cm oberhalb des gastroösophagealen Überganges entnommen.
Für die Analyse der Tight Junctions wurden die Ösophagusbiopsien in unserem Labor unserer Klinik aufgearbeitet und die Genexpression von Claudin-1 und -2, Occludin und Zonula Occludens (ZO)-1 und -2 mittels quantitativer PCR (qPCR) untersucht. Die Analyse

auf Proteinebene erfolgte mittels Immunhistochemie (Publikation VI [119]). Insbesondere für Claudin-1 und-2 konnte eine verstärkte Expression sowohl auf Ebene der mRNA als auch in der Immunhistochemie in Abhängigkeit von GERD dargestellt werden. Immunhistochemisch konnte diese verstärkte Expression vor allem in den basalen und suprabasalen Schichten des ösophagealen Plattenepithels lokalisiert werden, was positiv mit der histomorphologischen Beurteilung der Hyperplasie in diesem Kompartiment korrelierte und als adaptive beziehungsweise regenerative Veränderungen der Mukosa bei Patienten mit GERD zu interpretieren sind. Für Occludin, ZO-1 und ZO-2 konnten keine signifikanten Veränderungen zwischen Patienten mit GERD und Patienten ohne Symptome festgestellt werden.

Analog zu den Veränderungen von Komponenten der Tight Junction erfolgte die Analyse der desmosomalen Komponenten Plakoglobin, Desmoglein-1, -2 und -3 in der Ösophagusmukosa von Patienten mit GERD und Symptom-freien Personen auf Genexpressionsebene und mittels Immunhistochemie (siehe Abbildung 10, Publikation VII, [120]). Auf Ebene der mRNA konnten wir mittels qPCR eine verstärkte Expression aller untersuchten Gene bei Patienten mit GERD feststellen und immunhistochemisch auf Proteinebene bestätigen. Dabei zeigt sich die verstärkte Expression von Desmoglein-3 im Stratum spinosum im Bereich der interzellulären Gaps, wobei Plakoglobin und Desmoglein-1 verstärkt in der Basalzellschicht exprimiert werden (siehe Abbildung 10) und signifikant mit der histomorphologischen Beurteilung von DIS und BCH korrelieren.

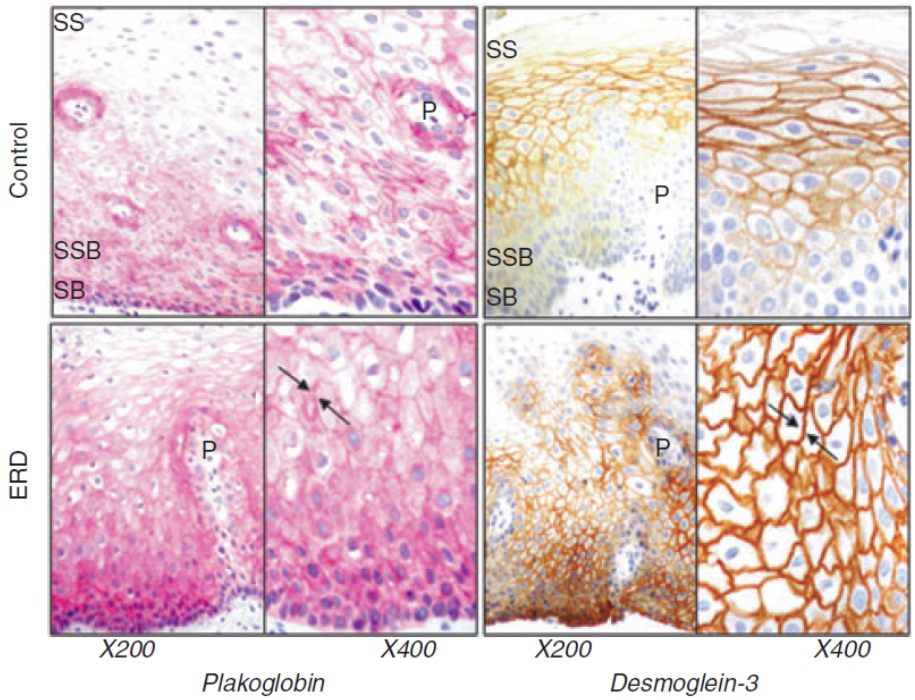


Abbildung 10: Immunhistochemische Darstellung der interzellulären desmosomalen Komponenten Plakoglobin und des intrazellulären „Rückgrades" Desmoglein-3; exemplarisch dargestellt in der Mukosa von Symptom-freien Kontrollen und Patienten mit ERD (siehe **Publikation VII** [120]).

In der Interpretation sind diese Veränderungen als Adaption und regenerative Anpassung der Mukosa an die verstärkte proliferative Komponente im Stratum basale sowie durch die Veränderungen im Rahmen der erweiterten Interzellularspalten zu diskutieren und stehen in molekularer Analogie zu den Veränderungen, die wir lichtmikroskopisch bei Patienten mit GERD darstellen können.

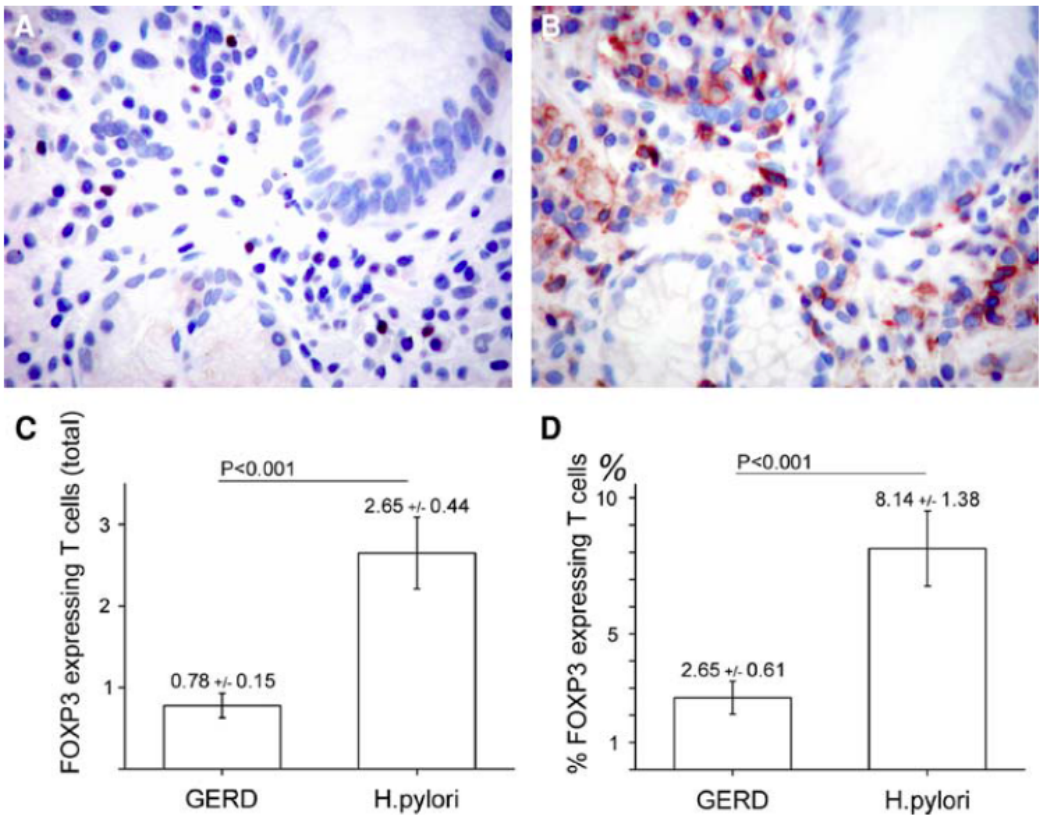
Arbeiten aus den letzten beiden Jahren konnten unsere Ergebnisse in weiten Teilen bestätigen. In der Untersuchung von Liu et al. konnten Veränderungen der desmosomalen Komponente des epithelialen Zellverbandes durch elektronenmikroskopische Analysen bestätigen. Für die Komponenten der Tight Junctions untersuchten die Autoren die Expression von Claudin-1 und -2 ebenfalls mittels Immunhistochemie, die im Gegensatz zu den Ergebnissen unserer Arbeit jedoch nicht verändert waren [121].

Ex vivo konnten unsere Ergebnisse durch die Arbeitsgruppe von Andre Smout bestätigt werden. Analog zu unseren eigenen Untersuchungen fanden die Kollegen Unterschiede in der Genexpression von Proteinen der Tight Junctions in der Schleimhaut von Patienten mit NERD lediglich für Claudin-2. Funktionell und pathophysiologisch scheint der Einfluss der Tight Junctions auf die Integrität der Mukosa *in vivo* (Erfassung durch speziell entwickelten Impedanzkatheter (siehe unten)) und *ex vivo* (Biopsien, Ussing-Chambers) eine untergeordnete Rolle für Patienten mit NERD und ERD zu spielen [122].

Funktionelle Experimente an Ösophagusresektaten konnten belegen, dass die luminale Exposition der Schleimhaut mit Deoxy-Cholsäure oder Trypsin in niedrigen Konzentrationen zunächst zu einer verstärkten Expression von Claudin-1 führen und mit einem erhöhten transepithelialen Widerstand in Ussing-Chambers assoziiert ist.
Erst in höheren Konzentrationen führen Gallensalze und Trypsin zu einer verminderten Expression von Claudin-1, -3 und 4, was mit einer deutlich erhöhten transepithelialer Permeabilität auch von funktioneller Bedeutung zu sein scheint [123].

In der unter Publikation VIII aufgeführten Studie konnten wir zeigen, dass die entzündlichen Veränderungen nicht nur durch proinflammatorische Zytokine gekennzeichnet sind [87, 89] sondern auch durch ein deutlich vermindertes Infiltrat an regulatorischen T-Zellen in der Mukosa des gastroösophagealen Überganges und der gastralen Kardia charakterisiert ist [124]. Während wir bei *H. pylori*-induzierten entzündlichen Veränderungen im Magen sowohl im Antrum als auch an der Kardia eine verstärkte Infiltration regulatorischer T-Zellen finden [125], ist die Anzahl an regulatorischen T-Zellen in der Mukosa des gastroösophagealen Überganges bei Patienten mit GERD vermindert [124].
Eingeschlossen wurden in die Studie insgesamt 70 Patienten, davon 31 Patienten mit einer *H. pylori*-induzierten Gastritis, 22 Patienten mit GERD und 17 asymptomatische Kontrollen. Mit dem Ziel der Charakterisierung der entzündlichen Veränderungen am gastroösophagealen Übergang in der Differenzierung dieser beiden Krankheitsentitäten wurden Biopsien an der Kardia des Magens, direkt am proximalen Ende der Magenfalten, entnommen. Die Infiltration regulatorischer T-Zellen wurde auf Ebene der Genexpression durch qPCR des spezifischen Transkriptionsfaktors FOXP3 sowie immunhistochemisch durch Färbung von FOXP3-exprimierenden T-Zellen analysiert und mittels einer Doppelfärbung von CD45 (LCA, leucocyte common antigen) durch das lymphozytären Gesamtinfiltrats normalisiert. Zusätzlich wurde die mukosale Expression der regulatorischen Zytokine IL-10 und TGF-ß1 gemessen. Wir konnten zeigen, dass sich die mukosale Entzündungsreaktion an der Kardia bei Patienten mit GERD signifikant von der *H. pylori*-induzierten Entzündung des gastroösophagealen Überganges bezüglich der Infiltration regulatorischer T-Zellen unterscheidet. Wir konnten eine mehr als 100-fach verstärkte Genexpression von FOXP3 als spezifischer Transkriptionsfaktor für regulatorische T-Zellen bei einer *H. pylori*-Infektion im Vergleich mit der GERD-assoziierten Entzündung der Mukosa nachweisen. Immunhistochemisch zeigte sich, dass bei Patienten mit GERD sowohl die Gesamtzahl an Mukosa-infiltrierenden regulatorischen T-Zellen als auch ihr proportionaler Anteil am entzündlichen Gesamtinfiltrat in der Mukosa vermindert ist (siehe Abbildung 11). Die funktionelle Bedeutung einer differenziellen regulatorischen T-Zellantwort in der Mukosa der beiden unterschiedlichen Ursachen einer Entzündung am gastroösophagealen

Überganges wurde durch die Analyse der regulatorischen Zytokine IL-10 und TGF-ß1 in der Studie unterstützt. Dabei konnten wir eine verstärkte Expression von TGF-ß1 in der Mukosa von *H. pylori*-infizierten Patienten sowie der positiven Korrelation FOXP3-exprimierender T-Zellen mit der Expression beider regulatorischen Zytokine TGF-ß1 und IL-10 nachweisen.



<u>Abbildung 11:</u> Immunhistochemische Färbung und Analyse regulatorischer T-Zellen in der Mukosa des gastroösophagealen Überganges bei GERD (A) und *H. pylori* -Infektion (B). In der Analyse zeigt sich eine verminderte Anzahl an regulatorischen T-Zellen in der Mukosa von Patienten mit GERD im Vergleich zur *H. pylori* -Infektion (**C**). Der Anteil an regulatorischen T Zellen am entzündlichen Gesamtinfiltrat der Mukosa (% FOXP3/CD45) war bei der GERD-assoziierten Entzündung der Kardia ebenfalls vermindert (**D**) (siehe **<u>Publikation VIII</u>** [124]) .

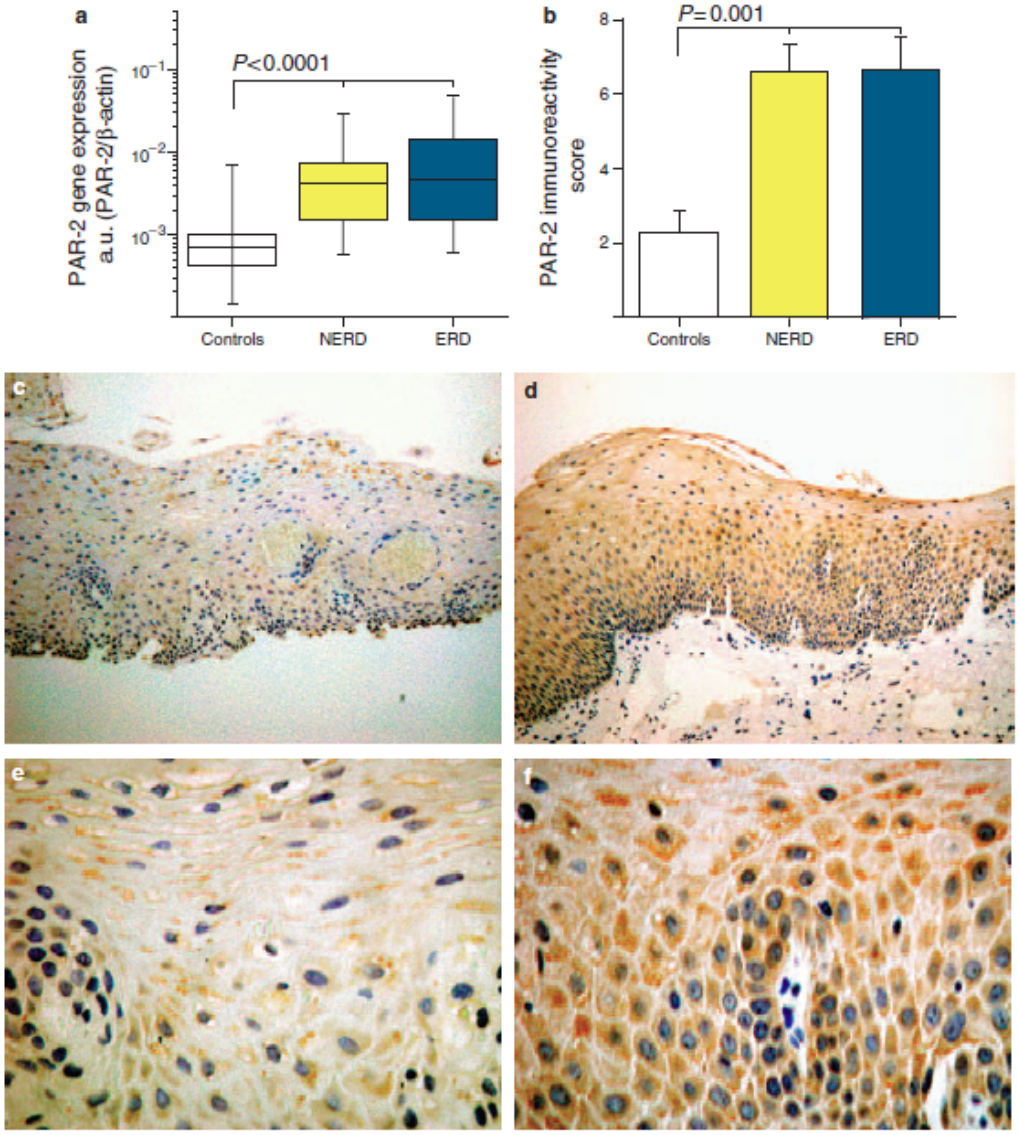
Über die letzten Jahrzehnte wurde zur Erklärung pathophysiologischer Veränderungen der Ösophagusmukosa bei Patienten mit GERD die Hypothese verteidigt, dass die proinflammatorischen Veränderungen der Ösophagusmukosa sekundär als Folge der Infiltration von Immunzellen in die Mukosa zu erklären sind. Diese infiltrieren die Mukosa aufgrund der Verletzung der Schleimhaut durch Säure mit Zerstörung des ösophagealen Epithels.

Die eigenen Arbeiten und Untersuchungen an einem Rattenmodell durch die Arbeitsgruppe um Ronda Souza legen die Initiierung der entzündlichen Veränderungen durch die ösophagealen Keratinozyten durch Sekretion proinflammatorischer Zytokine durch das Epithel nach Exposition mit Mageninhalt nahe [55].

Grundlegende molekulare Mechanismen zur Pathophysiologie und potentielle pathophysiologische Bedeutung des Protease-aktivierten Rezeptors-2 (PAR2) für die Genese der Refluxerkrankung werden im Manuskript zur Publikation IX untersucht und dargestellt [53].
Als zentrale Fragestellung der dargestellten Arbeit wurde die Bedeutung von PAR2 insbesondere für die „Immunpathogenese“ und Initiierung einer proinflammatorischen Zytokinantwort durch die Ösophagusmukosa untersucht.
PAR2 gehört zur Klasse der 7-Transmembran- G Protein-gekoppelten Rezeptoren. Durch proteolytische Spaltung am extrazellulären N-terminalen Ende des Rezeptors kommt es zu einer Konformationsänderung mit Freilegung einer Aminosäure-Sequenz, die als „tethered“ Ligand zur Bindung und Aktivierung des Rezeptors führt. Neben Mastzell-spezifischer Tryptase ist pankreatisches Trypsin als PAR2-aktivierende Proteasen gut charakterisiert [126, 127]. Pankreatisches Trypsin ist bei Patienten mit GERD aufgrund von duodenogastralem Reflux Bestandteil des gastroösophagealen Refluxates und im Lumen des Ösophagus nachweisbar und bei verändertem pH-Wert unter einer Therapie mit PPI proteolytisch aktiv.
Die Ergebnisse anderer Arbeitsgruppen konnten die Bedeutung PAR2-abhängiger Mechanismen für inflammatorische und neuroinflammatorische Veränderungen sowie für die Modulation von Schmerzreizen in unterschiedlichen Tiermodellen belegen [128, 129].
In humanen ösophagealen Epithelzelllinien führt die Aktivierung von PAR2 zu einer verstärkten Expression und Sekretion von IL-8 [88, 130]. Diese proinflammatorischen PAR2-abhängigen Effekte konnten auch für andere Zelllinien und für ausdifferenzierte 3-dimensionalen Zellkulturmodellen (liquid air interface, ALI) *in vitro* belegt werden [131].
In anderen hochrangig publizierten Arbeiten werden die Vermittlung neuroinflammatorischer Effekte und Modulation viszeraler Hypersensitivität durch PAR2-vermittelte Freisetzung von Substanz P (SP) und Calcitonin gene related protein (CGRP) aus freien Endigungen afferenter Nervenfasern sowie Modulation der Reizschwelle von Schmerzrezeptoren wie TRPV1 in der Mukosa beschrieben [132, 133]. In einem Rattenmodell konnte nachgewiesen werden, dass die spezifische Aktivierung von PAR2 durch luminale Proteasen sowohl zu entzündlichen Veränderungen als auch zu einer erhöhten Permeabilität der Dickdarmschleimhaut führt [128, 134-136].

Für die gastroösophageale Refluxerkrankung konnten wir nachweisen, dass PAR2 in der Mukosa von Patienten mit ERD und NERD verstärkt exprimiert wird (siehe Abbildung 12). In dieser Studie wurden prospektiv 123 Patienten untersucht und basierend auf der Montreal-Klassifikation [2] in NERD (n=46) und ERD (n=50) unterschieden. 27 Patienten ohne Reflux-Symptome und ohne PPI Therapie und ohne relevanten Nebenerkrankungen wurden als Kontrollgruppe eingeschlossen. Endoskopisch erfolgte die Entnahme von Biopsien aus dem Ösophagus 3 – 5 cm oberhalb des gastroösophagealen Überganges zur weiteren molekularen Analyse und Auswertung der histopathologischen Veränderungen. Auf diesem Patientenkollektiv basieren auch die Untersuchungen, die unter den Publikationen VI und VII in der Habilitationsschrift dargestellt werden.

Die Lokalisation und Verteilung der PAR2-Rezeptorexpression auf zellulärer Ebene und innerhalb der gesamten Mukosa erfolgte mittels Immunhistochemie. Die verstärkte PAR2 Expression bei Patienten mit GERD zeigte sich in allen epithelialen Schichten der Mukosa. Bezogen auf das Zellkompartiment war eine verstärkte Expression von PAR2 sowohl membran-assoziiert auf der Zelloberfläche als auch im Zytosol der ösophagealen Keratinozyten nachweisbar (siehe Abbildung 12, Panel d+ f).
Nach Rezeptoraktivierung unterliegt PAR2 einer ß-Arrestin und Clathrin-abhängigen Endozytose. In Abhängigkeit unterschiedlicher Faktoren wird PAR2 dann entweder lysosomal degradiert oder nach Re-Synthese des N-terminalen Endes als Rezeptor wieder in die Zellmembran integriert [137, 138]. Der verstärkte zytosolische Nachweis von PAR2 in den ösophagealen Keratinozyten interpretieren wir daher funktionell als Folge einer verstärkten Rezeptoraktivierung mit nachfolgender Endozytose bei Patienten mit GERD.
Wie in den bereits publizierten *in vitro* Versuchen anderer Arbeitsgruppen [88] korreliert die verstärkte PAR2 Expression hoch signifikant mit der Expression von IL-8 und SP in der Mukosa der Patienten mit GERD ($p<0,001$, hier nicht weiter dargestellt).



<u>Abbildung 12</u>: Verstärkte PAR2 Expression in der Mukosa von Patienten mit GERD (aus <u>Publikation IX</u> [53]). Verstärkte PAR2 Expression bei Patienten mit NERD und ERD im Vergleich zu Kontrollen (Panel a + b). Panel d und f zeigen exemplarisch die Mukosa eines Patienten mit NERD im Vergleich mit einem Patienten ohne Symptome (Panel c + e, Kontrolle).
Die PAR2 Expression findet sich in allen Schichten der plattenepithelialen Ösophagusschleimhaut, sowohl Zellmembran-assoziiert als auch verstärkt im Zytosol exprimiert.

Die funktionelle Bedeutung von PAR2 für die Pathogenese entzündlicher Veränderungen in der Mukosa von Patienten mit GERD haben wir weiter in einem plattenepithelialen Zellmodell untersucht. Dafür wurde ein Modell etabliert, in dem die ösophagealen Zelllinien KYSE450 und KYSE150 unter pH neutralen (pH 7,4) und schwach-sauren Bedingungen (pH6, pH5)

kultiviert wurde. Weiter wurde der zusätzliche Effekt einer Exposition mit unterschiedlichen Gallensalzen (Cholsäure, Dihydro-Cholsäure, Deoxy-Cholsäure) untersucht.
Wir konnten zeigen, dass die Kultur unter schwach sauren Bedingungen zu einer bis 20-fach verstärkten Expression von PAR2 in den Zelllinien führt. Die zusätzliche Exposition mit Gallensalzen hingegen hat keinen weiteren Einfluss auf die PAR2 Genexpression.
Die zusätzliche Stimulation des PAR2 Rezeptor erfolgte mittels synthetisch hergestelltem Rezeptoragonisten (SLIGKV-NH2). Dieses Peptid verfügt über die identische Aminosäuresequenz wie das proteolytisch gespaltene N-terminale Ende des Rezeptors. Es bindet anstatt des „tethered ligand“ und führt zu PAR2 Aktivierung ohne proteolytische Konformationsänderung. Durch die zusätzliche Applikation von SLIGKV-NH2 unter den schwach-sauren Kulturbedingungen in unserem Zellmodell konnten wir eine verstärkte IL-8 Sekretion aus den Keratinozyten in den Überstand nachweisen.
Dieses Zellmodell belegt die Bedeutung des Keratinozyten für die Initiierung eines proinflammatorischen Mikromilieus der ösophagealen Mukosa. Die Ergebnisse dieser Arbeit schließen einige Lücken zu den Ergebnissen aus den Tiermodellen und lassen sich sehr gut in die Ergebnisse der Arbeitsgruppe von Ronda Souza integrieren, was in einem Editorial der publizierenden Fachzeitschrift zum Ausdruck gebracht wurde [55, 139].
In der eigenen Arbeitsgruppe konnte Souza et al. die zeitliche Sequenz der entzündlichen Veränderungen der Mukosa in einem Refluxmodell an der Ratte beschreiben. Als erste Veränderungen der Mukosa auf den Reiz eines pathologischen gastroösophagealen Refluxes ist in diesem Modell zunächst ein erhöhtes proinflammatorisches Zytokinmilieu in der Mukosa der Tiere nachweisbar. Eine verstärkte Infiltration von Entzündungszellen und schließlich der Nachweis morphologischer Veränderungen (Ulzerationen, Proliferation, Papillenelongation) treten in der zeitlichen Abfolge erst deutlich später auf.
Die Ergebnisse der eigenen *ex vivo* -Arbeiten in die Daten aus diesem Tiermodell integrierend, konnten wir in zwei hochrangig publizierten Übersichtsarbeiten die pathophysiologischen Zusammenhänge der mukosalen Manifestation und Genese der mukosalen Immunpathogenese der gastroösophagealen Refluxerkrankung zusammenfassend darstellen [54, 99]. Diese ist als Publikation X ebenfalls Bestandteil der eingereichten kumulativen Habilitationsschrift (siehe Abbildung 13).

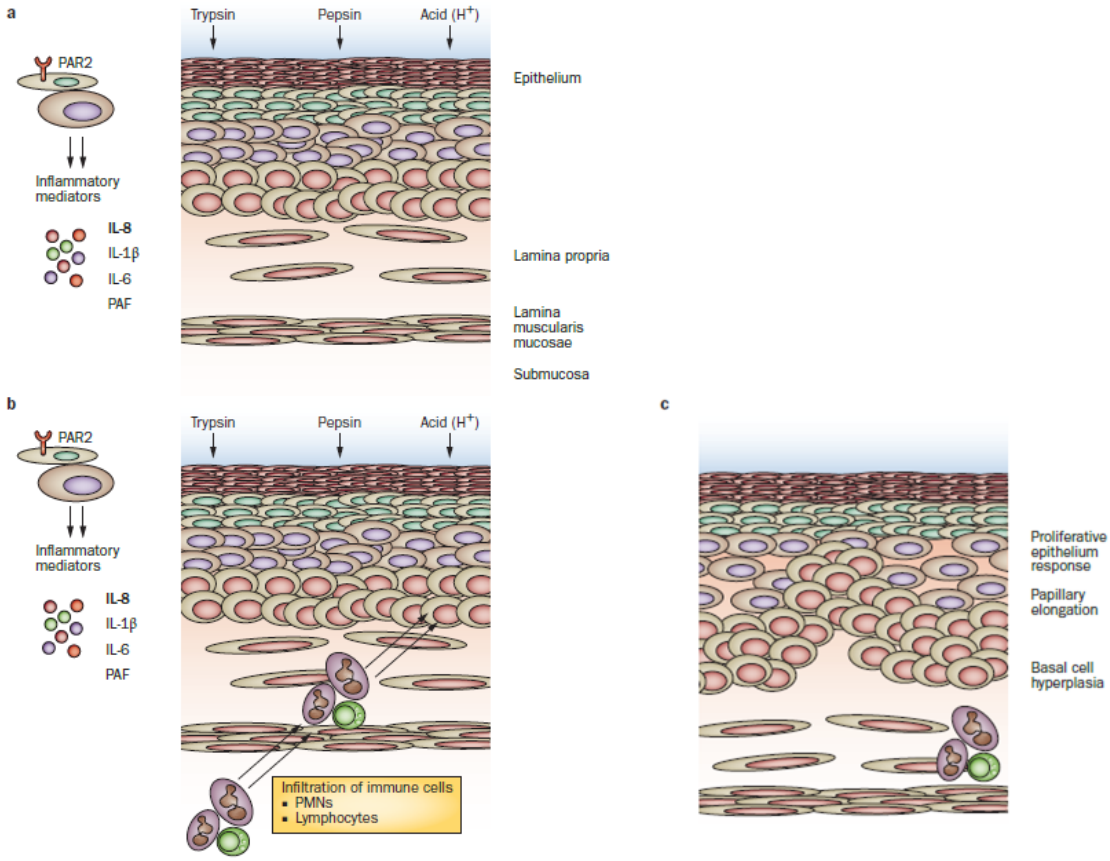


Abbildung 13: Hypothese zur immun-vermittelten Pathogenese der gastroösophagealen Refluxerkrankung (aus Publikation X [54], in Anlehnung an [55]). Initiierung einer proinflammatorischen Immunantwort durch Freisetzung proinflammatorischer Zytokine und Mediatoren aus den Keratinozyten nach Kontakt mit Bestandteilen des Magensaftes und Aktivierung spezifischer Rezeptoren, beispielhaft PAR2 durch Trypsin (a). Chemotaxis und Infiltration von Immunzellen in die basalen Schichten des ösophagealen Plattenepithels mit Zell-vermittelter Zytotoxizität und Ulzeration der Mukosa (b). Reparative und regenerative Veränderungen mit Proliferation und Hyperplasie in der Basalzellschicht, Papillenelongation und erweiterten Interzellularspalten (c).

Neben den bereits aufgeführten proinflammatorischen Zytokinen IL-1ß und IL-8 scheint die Freisetzung von IL-6 und des „Platelet Activating Factor“ (PAF) aus Keratinozyten für die spezifische mukosale Immunreaktion bei NERD und ERD von pathophysiologischer Bedeutung [91, 140].

Im Gegensatz zu Patienten mit NERD und ERD unterscheiden sich Patienten mit einer Barrett-Metaplasie hinsichtlich des prädominanten Zytokinmusters der Schleimhaut. Bereits ältere Arbeiten konnten eine verstärkte Expression von IL-4 nachweisen, die einer T_H2-

gerichteten Immunantwort zugeordnet werden, aber auch der Gehalt an IL-10 ist in der Mukosa von Patienten mit einer Barrett-Metaplasie verstärkt nachweisbar [94, 141].
Neben den epithelialen Zellen sind auch die anderen zellulären Komponenten des Mukosa (mesenchymale Zellen, Endothelium, Zellen neuronalen Ursprungs) an der Immunreaktion der Mukosa beteiligt. Die genauen Mechanismen und Interaktionen sind bisher jedoch nur ungenügend verstanden [54, 142].

5. Zusammenfassung und Ausblick

Die Ergebnisse aus den klinischen Arbeiten unterstreichen nicht nur die Wichtigkeit einer genauen Anamnese der geschilderten Symptome bei Patienten mit vermuteter Refluxerkrankung, sondern machen vielmehr auf die Notwendigkeit einer weiterführenden gastroösophagealen Funktionsdiagnostik aufmerksam. Dies betrifft vor allem Patienten mit fortbestehender Symptomatik unter einer säuresuppressiven Therapie mit PPI und sollte aus meiner persönlichen Sicht bereits initiiert werden, bevor die Dosierung erhöht oder bevor das pharmakologische Präparat gewechselt wird.
Bei adäquater Diagnostik und Selektion der Patienten, bei denen eine GERD nach objektiven Kriterien vorliegt, ist das symptomatische Ansprechen auf eine säuresuppressive Therapie mit PPI gut. Der Ausschluss einer gastroösophagealen Refluxerkrankung und die Differentialdiagnostik sind bei PPI-refraktären Beschwerden insofern von grundlegender Bedeutung, als dass eine medikamentöse Therapie mit PPI beendet wird beziehungsweise im Falle von funktionellem Sodbrennen andere Formen der Behandlung gewählt werden. Auch wenn sich aus der Datenlage in einer aktuellen Meta-Analyse keine allgemeinen Empfehlungen ableiten lassen, kann der medikamentöse Ansatz mit Serotonin-Reuptake Inhibitoren (SSRI) in niedriger Dosierung für ausgewählte Patienten erfolgreich zur Anwendung kommen [143, 144].
In der Differentialdiagnose von GERD und funktionellem Sodbrennen unterstreichen die Ergebnisse der Arbeiten in Zusammenschau mit der aktuellen Literatur den Nutzen von Biopsieentnahmen aus dem Ösophagus. Der histologische Befund der Ösophagusschleimhaut ermöglicht die Abgrenzung zu Erkrankungen wie der eosinophilen Ösophagitis; darüber hinaus liefert er zusätzliche Informationen über Reflux-assoziierte Veränderungen und Integrität der Mukosa.
Über die Analyse der basalen mukosalen Impedanz während der MII-pH können zusätzliche Informationen über die Beschaffenheit und Integrität der Ösophagusschleimhaut erhoben werden und die gastroösophageale Refluxerkrankung von funktionellem Sodbrennen

unterschieden werden [50]. Durch zwei Arbeitsgruppen wurde unabhängig voneinander eine Sonde entwickelt, die es ermöglicht, den Impedanzwert der Mukosa direkt im Rahmen der endoskopischen Untersuchung zu messen [86, 145]. Die Publikationen beider Arbeitsgruppen konnten unsere Ergebnisse bestätigen, dass ein erniedrigtes basales Impedanzniveau mit einer gestörten mukosalen Integrität korreliert und dass dadurch eine Differenzierung gegenüber anderen Erkrankungen der Speiseröhre möglich ist [122, 146]. Die entwickelten Prototypen beider Arbeitsgruppen werden als Sonden durch den Arbeitskanal des Endoskops geschoben und die 2 Impedanzelektroden auf der jeweiligen Sonde werden unter Sicht während der Untersuchung auf der Schleimhaut platziert. Technisch anspruchsvoll und herausfordernd scheint es bis jetzt zu sein, eine stabile Position der Sonde zur Ableitung eines konstanten Signals zu erreichen.
Aus meiner Sicht bietet diese Methodik eine neue Möglichkeit für die tägliche Praxis, die es dem behandelnden Arzt ermöglichen kann, während der Endoskopie eine unmittelbare Aussage über die funktionellen Integrität der Mukosa und zum Vorliegen einer gastroösophagealen Refluxerkrankung zu treffen. Eine sichere Diagnose der NERD wäre so „On-Site“ und in „Real-Time“ durch die Analyse von funktionellen mukosalen Veränderungen möglich. Eine solche Diagnose praktisch „auf Knopfdruck“ könnte – diesen Gedanken weit fortgeführt – dem Patienten die Analyse über 24 Stunden mittels Katheter-geführter Funktionsdiagnostik ersparen [147].

Die Ergebnisse der Arbeiten zur Charakterisierung entzündlicher Veränderungen der Mukosa, mikrostrukturellen Veränderungen und Untersuchungen zur Beteiligung von PAR2 für die Pathogenese der gastroösophagealen Refluxerkrankung bieten Ansatzpunkte, für die Entwicklung neuer medikamentöser Strategien und Ziele.
Obwohl in klinischen Studien eine Wirksamkeit für die sogenannten Refluxinhibitoren mit dem therapeutischen Ansatz einer Reduktion der TLESRs belegt werden konnte [64, 65, 148, 149], wird die Weiterentwicklung dieser Substanzen durch die pharmazeutischen Firmen aufgrund von Nebenwirkungen und unzureichender klinischer Relevanz derzeitig nicht weiter verfolgt.

Interessante molekulare und klinische Daten liegen bezüglich des Capsaicin-sensitiven Rezeptors TRPV1 (transient receptor potential cation channel subfamiliy V member 1) vor. TRPV1 ist ein nicht-selektiver Kationenkanal, der durch Hitze, Capsaicin oder durch Wasserstoffionen bei saurem pH-Wert (zum Beispiel im Refluxat oder im entzündeten Gewebe) aktiviert wird [150]. Die freien Enden afferenter Neuronen in der Mukosa von Patienten mit NERD weisen eine verstärkte Expression von TRPV1 auf [151-153]. Bei diesen Patienten ist funktionell eine erhöhte Sensibilität gegenüber der Perfusion mit Capsaicin

nachweisbar, die symptomatisch das Empfinden von Sodbrennen hervorruft [154]. Die Entwicklung eines TRPV1-Rezeptorantagonisten mit Reduktion der entzündlichen und neuroinflammatorischen Veränderungen und Wiederherstellung der mukosalen Integrität bietet diesbezüglich einen alternativen therapeutischen Ansatz, für den bereits erste humane Studienergebnisse vorliegen [151, 153, 155]. Ein therapeutisches Ansprechen konnte in diesen Studien bisher nur gegenüber der Exposition von Hitze und mechanischen Reizen erreicht werden, nicht jedoch gegenüber der Exposition der Ösophagusmukosa mit sauren Medien [156].

Andere Arbeiten konnten zeigen, dass die Reizschwelle von TRPV1 durch Aktivierung von PAR2 verändert wird und PAR2 so zu Neuroinflammation und Vermittlung von viszeraler Hypersensitivät beiträgt [157]. Neben der Beteiligung PAR2-abhängiger Mechanismen für die Initiierung entzündlicher Veränderungen in der Mukosa, bietet die pharmakologische Interaktion am PAR2-Rezeptor aufgrund seiner pathophysiologischen Bedeutung für die Vermittlung viszeraler Hypersensitivät [134, 135] ein interessantes molekulares Ziel zur Entwicklung alternativer Therapiestrategien. Die Hemmung PAR2-abhängiger Mechanismen in der Mukosa von Patienten mit GERD stellt diesbezüglich ein mögliches molekulares Ziel dar. In einem Refluxmodell an der Ratte konnte durch eine medikamentöse Hemmung des PAR2-Rezeptors eine signifikante Reduktion der entzündlichen Veränderungen in der Speisröhre nachgewiesen werden [158]. Zur Optimierung dieses therapeutischen Ansatzes für den Einsatz beim Menschen wäre eine topische Anwendung eine Möglichkeit, nicht nur entzündliche Veränderungen zu reduzieren, sondern die strukturelle und funktionelle Integrität der Mukosa wieder herzustellen und symptomatisch die Effekte einer viszeralen Hypersensitivät zu therapieren [122, 159, 160].

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7. Publikationsliste der Habilitationsschrift

I. **Helmut Neumann, Klaus Mönkemüller, Arne Kandulski und Peter Malfertheiner. Dyspepsia and IBS symptoms in patients with NERD, ERD and Barrett's esophagus. Dig Dis. 2008; 26(3):243-7.**

II. **Arne Kandulski, Ulrich Peitz, Klaus Mönkemüller, Helmut Neumann, Jochen Weigt und Peter Malfertheiner. GERD assessment including pH metry predicts a high response rate to PPI standard therapy. BMC Gastroenterol. 2013 Jan 16; 13:12.**

III. **Jochen Weigt, Arne Kandulski und Peter Malfertheiner. Nocturnal gastric acid breakthrough is not associated with night-time gastroesophageal reflux in GERD patients. Dig Dis. 2009; 27(1):68-73.**

IV. **Arne Kandulski, Jochen Weigt, Carlos Caro, Doerthe Jechorek, Thomas Wex und Peter Malfertheiner. Esophageal Intraluminal Baseline Impedance Differentiates Gastroesophageal Reflux Disease From Functional Heartburn. Clin Gastroenterol Hepatol. 2014 Dec 9. pii: S1542-3565(14)01740-6.**

V. **Arne Kandulski, Doerthe Jechorek*, Carlos Caro, Jochen Weigt, Thomas Wex, Klaus Mönkemüller und Peter Malfertheiner. Histomorphological differentiation of non-erosive reflux disease and functional heartburn in patients with PPI-refractory heartburn. Aliment Pharmacol Ther. 2013 Sep; 38(6):643-51. (*equally contributed)**

VI. **Klaus Mönkemüller, Thomas Wex, Doerthe Kuester, Lucia Fry, Arne Kandulski, Albert Roessner und Peter Malfertheiner. Role of tight junction proteins in gastroesophageal reflux disease. BMC Gastroenterol. 2012 Sep 20; 12:128.**

VII. **Thomas Wex, Klaus Mönkemüller, Antje Stahr, Doerthe Kuester, Lucia Fry, Simone Völkel, Arne Kandulski, Albert Roessner und Peter Malfertheiner. Gastro-oesophageal reflux disease is associated with up-regulation of desmosomal components in oesophageal mucosa. Histopathology. 2012 Feb; 60(3):405-15.**

VIII. **Arne Kandulski, Thomas Wex, Doerthe Kuester, Klaus Mönkemüller, Ulrich Peitz, Albert Roessner und Peter Malfertheiner. Chronic mucosal inflammation of the gastric cardia in gastroesophageal reflux disease is not regulated by FOXP3-expressing T cells. Dig Dis Sci. 2009 Sep; 54(9):1940-6.**

IX. **Arne Kandulski, Thomas Wex*, Klaus Mönkemüller, Doerthe Kuester, Lucia Fry, Albert Roessner und Peter Malfertheiner. Proteinase-activated receptor-2 in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol. 2010 Sep; 105(9):1934-43. (*equally contributed)**

X. **Arne Kandulski und Peter Malfertheiner. Gastroesophageal reflux disease - from reflux episodes to mucosal inflammation. Nat Rev Gastroenterol Hepatol. 2011 Nov 22; 9(1):15-22.**

8. Erklärungen

Ich erkläre, dass ich die der Medizinischen Fakultät zur Habilitation eingereichte Habilitationsschrift mit dem Titel

„Morphologische, funktionelle und molekulare Charakterisierung der Ösophagusmukosa bei erosiver und nicht-erosiver gastroösophagealer Refluxerkrankung“

in der Klinik für Gastroenterologie, Hepatologie und Infektiologie der Otto-von-Guericke Universität Magdeburg mit Unterstützung durch Prof. Dr. med. habil. Dr. h.c. Peter Malfertheiner ohne sonstige Hilfe durchgeführt und bei der Abfassung keine anderen als die aufgeführten Hilfsmittel verwendet habe.

Bei der Abfassung der Habilitationsschrift sind Rechte Dritter nicht verletzt worden.

Ich habe die Habilitationsschrift bisher an keiner in- oder ausländischen Hochschule/Universität zur Habilitation eingereicht.

Ich übertrage der Medizinischen Fakultät der Otto-von-Guericke Universität das Recht, weitere Kopien meiner Habilitationsschrift herzustellen und zu vertreiben.

Magdeburg, Dr. med. Arne Kandulski

8.1 Erklärungen bezüglich des Eigenanteils an den publizierten Arbeiten zur kumulativen Habilitationsschrift von Herrn Dr. med. Arne Kandulski

I. Helmut Neumann, Klaus Mönkemüller, Arne Kandulski und Peter Malfertheiner

Dyspepsia and IBS symptoms in patients with NERD, ERD and Barrett's esophagus. Dig Dis. 2008; 26(3):243-7.

Der Kandidat war für die Analyse und statistische Auswertung der Fragebögen sowie der Betreuung der Patienten in der gastroenterologischen Spezialsprechstunde der Klinikambulanz zuständig. Die Erstellung des Manuskripts erfolgte in der Arbeitsgruppe unter der Leitung von Prof. Dr. Malfertheiner. Der Kandidat war neben der statistischen Auswertung für die Darstellung, Interpretation und Diskussion der Ergebnisse in der Erstellung des Manuskripts maßgeblich beteiligt.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

II. Arne Kandulski, Ulrich Peitz, Klaus Mönkemüller, Helmut Neumann, Jochen Weigt und Peter Malfertheiner

GERD assessment including pH metry predicts a high response rate to PPI standard therapy. BMC Gastroenterol. 2013 Jan 16; 13:12.

Die Konzeption der Studie erfolgte in der Arbeitsgruppe maßgeblich durch PD Dr. med. Ulrich Peitz. Nach dem Ausscheiden von Herrn PD Dr. med. Ulrich Peitz aus der Klinik für Gastroenterologie übernahm der Kandidat die weitere Koordination der Studie, die Applikation der pH-Metrie Kapseln sowie das Auslesen und Analyse der pH-Metrie Daten. Die Analyse der Studiendaten, statistische Analysen und Interpretation der Ergebnisse lag ebenso in der Verantwortung des Kandidaten wie das konzeptionelle Verfassen des Manuskripts innerhalb der Arbeitsgruppe unter Anleitung und Kooperation mit Prof. Dr. Peter Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

III. **Jochen Weigt, Arne Kandulski und Peter Malfertheiner**

Nocturnal gastric acid breakthrough is not associated with night-time gastroesophageal reflux in GERD patients. Dig Dis. 2009; 27(1):68-73.
Durch den Kandidaten erfolgte die Rekrutierung der Patienten, Durchführung und Analyse der gastroösophagealen Funktionsdiagnostik (MII-pH). Die Interpretation und Diskussion der Ergebnisse sowie der Verfassung des Manuskripts erfolgte innerhalb der Arbeitsgruppe unter maßgeblicher Beteiligung des Kandidaten.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

IV. **Arne Kandulski, Jochen Weigt, Carlos Caro, Doerthe Jechorek, Thomas Wex und Peter Malfertheiner.**

Esophageal Intraluminal Baseline Impedance Differentiates Gastroesophageal Reflux Disease From Functional Heartburn. Clin Gastroenterol Hepatol. 2014 Dec 9. pii: S1542-3565(14)01740-6.
Durch den Kandidaten erfolgte die Konzeption der Studie, Rekrutierung der Patienten, Durchführung und Analyse der der gastroösophagealen Funktionsdiagnostik (MII-pH), Etablierung und Erfassung der basalen Impedanzanalysen sowie der endoskopischen Untersuchungen. Die Interpretation und Diskussion der Ergebnisse erfolgte in Kooperation mit den Kollegen der Arbeitsgruppe, die Verfassung des Manuskripts in Kooperation mit Prof. Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

V. **Arne Kandulski, Doerthe Jechorek*, Carlos Caro, Jochen Weigt, Thomas Wex, Klaus Mönkemüller und Peter Malfertheiner.**

Histomorphological differentiation of non-erosive reflux disease and functional heartburn in patients with PPI-refractory heartburn. Aliment Pharmacol Ther. 2013 Sep; 38(6):643-51.

Durch den Kandidaten erfolgte die Konzeption der Studie, Rekrutierung der Patienten, Durchführung und Analyse der der gastroösophagealen Funktionsdiagnostik (MII-pH) sowie der endoskopischen Untersuchungen.

Die Bewertung der histomorphologischen Veränderungen erfolgte durch Frau Dr. Dörthe Jechorek (*equally contributed). Die Interpretation und Diskussion der Ergebnisse sowie der Verfassung des Manuskripts erfolgte durch den Kandidaten unter Anleitung von Prof. Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

VI. **Klaus Mönkemüller, Thomas Wex, Doerthe Kuester, Lucia Fry, Arne Kandulski, Albert Roessner und Peter Malfertheiner.**

Role of tight junction proteins in gastroesophageal reflux disease. BMC Gastroenterol. 2012 Sep 20; 12:128.

Durch den Kandidaten erfolgte die Analyse der Expressionsdaten, statistische Auswertung und graphische Darstellung der Korrelationsanalysen. In der Interpretation, Diskussion der Ergebnisse und Verfassung des Manuskripts war der Kandidat innerhalb der Arbeitsgruppe maßgeblich beteiligt.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

VII. **Thomas Wex, Klaus Mönkemüller, Antje Stahr, Doerthe Kuester, Lucia Fry, Simone Völkel, Arne Kandulski, Albert Roessner und Peter Malfertheiner.**

Gastro-oesophageal reflux disease is associated with up-regulation of desmosomal components in oesophageal mucosa. Histopathology. 2012 Feb; 60(3):405-15.
Durch den Kandidaten erfolgte die Analyse der Expressionsdaten, statistische Auswertung und graphische Darstellung der Korrelationsanalysen. An der Interpretation und Diskussion der Ergebnisse sowie Verfassung des Manuskripts war der Kandidat innerhalb der Arbeitsgruppe maßgeblich beteiligt.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

VIII. **Arne Kandulski, Thomas Wex, Doerthe Kuester, Klaus Mönkemüller, Ulrich Peitz, Albert Roessner und Peter Malfertheiner.**

Chronic mucosal inflammation of the gastric cardia in gastroesophageal reflux disease is not regulated by FOXP3-expressing T cells. Dig Dis Sci. 2009 Sep; 54(9):1940-6.
Durch den Kandidaten erfolgte die Konzeption der Studie und der experimentellen Versuche sowie im Folgenden die Analyse der Daten, statistische Auswertung und graphische Darstellungen sowie Verfassung des Manuskriptes mit Unterstützung durch Prof. Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

IX. **Arne Kandulski, Thomas Wex*, Klaus Mönkemüller, Doerthe Kuester, Lucia Fry, Albert Roessner und Peter Malfertheiner.**

Proteinase-activated receptor-2 in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol. 2010 Sep; 105(9):1934-43.

Durch den Kandidaten erfolgte die Konzeption der Studie und der experimentellen Versuche sowie im Folgenden die Analyse der Daten, statistische Auswertung und graphische Darstellungen. Die Betreuung der experimentellen Versuche im Funktionslabor der Klinik erfolgte durch Prof. Wex (*equally contributed). Die Verfassung des Manuskriptes erfolgte durch den Kandidaten mit Unterstützung durch Prof. Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

X. **Arne Kandulski und Peter Malfertheiner**

Gastroesophageal reflux disease--from reflux episodes to mucosal inflammation. Nat Rev Gastroenterol Hepatol. 2011 Nov 22; 9(1):15-22.

Durch den Kandidaten erfolgte die Konzeption der Übersichtsarbeit, die Auswahl und Analyse der aktuellen Literaturstellen und Publikationen sowie der Verfassung des Manuskripts in Zusammenarbeit mit Prof. Dr. Malfertheiner.

Dr. med. Arne Kandulski **Prof. Dr. Peter Malfertheiner**

9. Danksagungen

Mein Dank gilt allen, die diese Arbeit durch die Unterstützung, die ich beruflich, wissenschaftlich und privat erfahren habe, möglich gemacht haben.
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Bei Prof. Dr. rer. nat. Thomas Wex möchte ich mich für die private und wissenschaftliche Unterstützung über die Betreuung der Promotionsarbeit hinaus bedanken. Unter seiner Anleitung konnte ich viele der methodischen Grundlagen erlernen, die mir die Erarbeitung vieler wissenschaftlicher Fragestellungen ermöglichte.
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Ihnen und Euch allen vielen Dank.

10. Publikationen

Publikation I

Original Article

Digestive Diseases

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Dyspepsia and IBS Symptoms in Patients with NERD, ERD and Barrett's Esophagus

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Key Words
Dyspepsia · Irritable bowel syndrome · Gastroesophageal reflux disease · Non-erosive reflux disease · Erosive reflux disease · Barrett

Abstract
Introduction: Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are highly prevalent in the general population as does gastroesophageal reflux disease (GERD). Therefore, it is expected that these conditions may frequently overlap. ***Objective:*** We aimed at evaluating the presence of FD and IBS symptoms in patients with erosive (ERD), non-erosive reflux disease (NERD) and Barrett's esophagus (BE). ***Patients and Methods:*** 71 patients presenting at the reflux disease outpatient clinic were prospectively included in this study. 33 patients had NERD, 25 ERD and 13 BE according to the Montreal classification. All patients with ERD and NERD had typical reflux symptoms, as assessed by a validated GERD questionnaire (RDQ). The diagnosis of functional dyspepsia and IBS symptoms was assessed according to the Rome III criteria. ***Results:*** IBS symptoms (bloating, abdominal pain, constipation and diarrhea) were slightly more prevalent in NERD (54.6, 63.6, 21.20, 24.2%, respectively) than in ERD (48.0, 44.0, 12.0, 20.0%, respectively) and in BE (53.9, 23.10, 15,4, 23.1%, respectively), but none of these differences reached statistical significance. NERD patients had more FD symptoms than patients with ERD or BE, but again this difference did not reach statistical significance. ***Conclusion:*** Our data show that IBS and FD are common in the entire spectrum of GERD. The presence of these disorders might explain why many patients with GERD are deemed as treatment failures if they have no complete symptom relief with proton pump inhibitors.

Introduction

Gastroesophageal reflux disease (GERD), irritable bowel syndrome (IBS) and functional dyspepsia (FD) are the most prevalent gastrointestinal (GI) disorders and constitute an enormous health care burden [1]. IBS and FD occur in over 20% of the general population [2]. Therefore, it is expected that these conditions overlap in patients with GERD. Furthermore, it is also possible that IBS and FD are more common in GERD. Indeed, recent data suggest that patients with GERD suffer more commonly from functional GI disorders [3, 14, 15]. In clinical practice, the differentiation of the manifestations of GERD and FD is difficult, in particular in patients presenting with long-lasting symptoms [4]. The current Rome III criteria have attempted to bring clarification for the definition and categorization of these disorders [5]. By using these criteria and well standardized questionnaires an accurate diagnosis can be reached.

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Knowledge of the prevalence of IBS and FD in GERD is very useful, firstly to establish a correct diagnosis, secondly to better categorize these patients, thirdly, to develop therapeutic plans, and lastly, to better understand the therapeutic response. Therefore, a major goal in clinical practice is to avoid unnecessary treatments and thus minimize inconvenience to the patient and further costs. The use of standardized questionnaires may also aid the clinician to determine if the resolution of symptom is mainly due to GERD and if the remaining symptoms, if present at follow-up, are due to coincident functional GI disorders.

The aim of the present study was to investigate the prevalence of functional gastrointestinal symptoms in a consecutive group of patients with different categories of GERD.

Methods

71 patients evaluated at the reflux disease outpatient clinic of the University Hospital of Magdeburg were prospectively included in this study. All patients were taking PPI therapy for typical reflux symptoms and were evaluated with a validated GERD questionnaire (reflux disease questionnaire; RDQ) [6]. The diagnosis of NERD was based on typical reflux symptoms and a negative conventional upper endoscopy using the Montreal classification [7]. Among patients with GERD, 33 had endoscopically non-erosive (NERD) and 25 erosive reflux disease. In 13 patients a BE was histologically confirmed. Inclusion criteria: age 18 to 79, able to provide written inform consent, typical GERD symptoms, BE. Exclusion criteria: patients with abnormal coagulation parameters and thrombocytopenia, esophageal or gastric neoplasia, inflammatory diseases of the gut (Crohn's disease, ulcerative colitis, celiac disease) and/or autoimmune disorders.

Endoscopy was performed under conscious sedation with intravenous midazolam using standard videogastroscopes (Q160, Olympus, Hamburg). Endoscopic characterization of esophagitis was based on the updated Los Angeles classification [8]. The diagnosis of BE was based on Montreal classification [7].

Reflux symptoms were assessed by the RDQ [9]. The RDQ comprises 12 questions in which the frequency, severity of heartburn, acid regurgitation and dyspeptic complaints were assessed and scored on a five-point Likert scale [5]. The questions are structured in 3 sections including (I) heartburn, (II) regurgitation and (III) dyspepsia. The mean of all three sections gives a score ranging from 0 to 5. The specific GERD section is determined by the summary of the scores heartburn and regurgitation. The questionnaire also comprises a section to access the severity of symptoms.

The diagnosis of functional dyspepsia and IBS symptoms were measured according to the Rome III criteria [5]. For this purpose we constructed an additional questionnaire containing the following symptoms: bloating, epigastric pain, constipation, diarrhea at least 3 months, with onset at least 6 months previously with improvement with defecation; *and/or* onset associated with a change in frequency of stool; *and/or* onset associated with a change in form (appearance) of stool as diagnostic criteria for IBS.

FD was defined as the presence of 1 or more dyspepsia symptoms (bothersome fullness, epigastric pain, epigastric burning) that are considered to originate from the gastroduodenal region, in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms [10].

At the time completing the questionnaires all patients were under PPI therapy.

Statistics

Data were entered into Excel worksheets (Microsoft™ Corporation, Seattle, Wash., USA) and analyzed using non-parametric Mann-Whitney U test with a two-sided level of significance. p values of <0.05 were regarded as significant. Box plots were prepared using Microcal™ Origin® Version 7.5 (Microcal™ Software, Inc., Northampton, Mass., USA).

Table 1. Demographics of patients with gastroesophageal reflux disease symptoms

	NERD	ERD	Barrett	p value
Number of patients	33	25	13	
Mean age	55	54	64	ns
Age range	21–74	18–79	26–73	
Percent female	78.8%	28%	30.8%	<0.05

NERD = Non-erosive reflux disease; ERD = erosive reflux disease; ns = not significant.

Results

A total of 71 patients (37 female, 34 male) were included (table 1). Among patients with GERD, 33 and 25 had endoscopically NERD and ERD, respectively and 13 a BE. Significantly more female patients suffered from NERD than male patients ($p < 0.05$).

Irritable Bowel Symptoms in GERD

The prevalence of IBS-like symptoms was 63.6% in NERD and 48% in ERD. Patients with NERD had slightly more IBS symptoms like bloating, abdominal pain, constipation and diarrhea (54.6, 63.6, 21.20, 24.2%, respectively) than those with ERD (48.0, 44.0, 12.0, 20.0%, respectively) or BE (53.9, 23.10, 15.4, 23.1%, respectively) (table 2). Furthermore, NERD patients suffered more frequently from IBS symptoms than ERD or BE (table 3). However, due to the limited number none of these differences reached statistical significance (fig. 1).

Table 2. Irritable bowel syndrome symptoms in erosive reflux disease (ERD), non-erosive reflux disease (NERD) and Barrett's esophagus (BE)

	NERD %	ERD %	BE %	p value		
				NERD vs. ERD	NERD vs. BE	ERD vs. BE
Bloating	54.60	48.00	53.90	ns	ns	ns
Abdominal pain	63.60	44.00	23.10	ns	0.03	ns
Constipation	21.20	12.00	15.40	ns	ns	ns
Diarrhea	24.20	20.00	23.10	ns	ns	ns

ns = Not significant.

Table 3. Frequency of irritable bowel syndrome symptoms in erosive reflux disease (ERD), non-erosive reflux disease (NERD) and Barrett's esophagus (BE)

	NERD %	ERD %	BE %	p value		
				NERD vs. ERD	NERD vs. BE	ERD vs. BE
Less than 3 ×/month	24.20	44.00	53.90	ns	ns	ns
More than 3 ×/month	69.70	56.00	38.50	ns	ns	ns
In the last months	87.90	88.00	53.90	ns	ns	ns
At least 6 months previously	84.90	84.00	46.20	ns	ns	ns

ns = Not significant.

Table 4. Functional dyspepsia symptoms in erosive reflux disease (ERD), non-erosive reflux disease (NERD) and Barrett's esophagus (BE)

	NERD %	ERD %	BE %	p value		
				NERD vs. ERD	NERD vs. BE	ERD vs. BE
Bothersome fullness	45.50	40.00	38.50	ns	ns	ns
Epigastric pain	69.70	60.00	30.80	ns	0.04	ns

ns = Not significant.

Functional Dyspepsia in GERD

Within these groups only one significant difference could be observed between NERD and BE in the subscale of epigastric pain (p = 0.04). Overall it seemed that NERD patients had more functional dyspeptic symptoms like bothersome fullness or epigastric pain (45.5, 69.7%, respectively) than patients with ERD (40.0, 60.0%, respectively) or BE (38.5, 30.8%, respectively), but this difference did not reach statistical significance (fig. 2, table 4).

Discussion

Recent studies have reported on the considerable overlap of different GI symptoms in patients seeking medical attention [11, 12]. The first available report on this relationship indicate that around 30% of IBS patients have daily heartburn and almost 60% have monthly heartburn [13]. Other studies, including a recent Cochrane database analysis, have reported on prevalence rates of IBS in

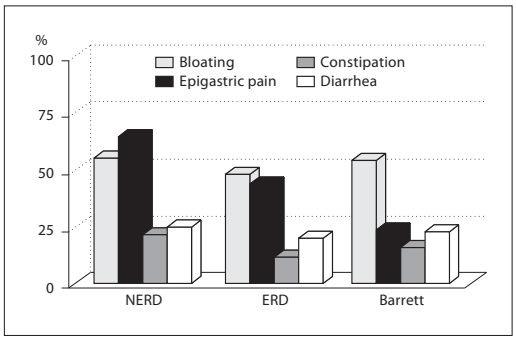

Fig. 1. Percentage of IBS symptoms in non-erosive (NERD), erosive (ERD) and Barrett's esophagus (Barrett). Patients with NERD had slightly more IBS symptoms than those with ERD or BE, but due to the limited number none of these differences reached statistical significance.

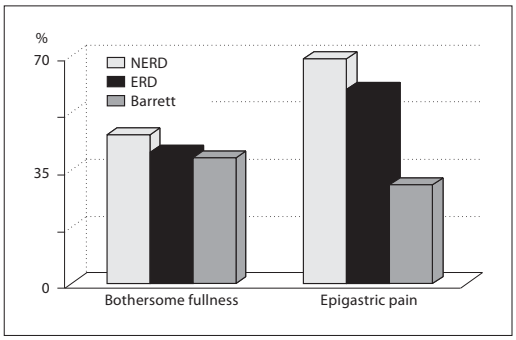

Fig. 2. Percentage of functional dyspepsia symptoms in non-erosive (NERD), erosive (ERD) and Barrett's esophagus (Barrett). There is a significant difference between NERD and BE in subscale of epigastric pain (p = 0.04). Overall, NERD patients had more FD symptoms than patients with ERD or BE.

GERD ranging from 19 to 71% [14–16]. In contrast, the rate of IBS in the non-GERD community was calculated to be only 5% [14]. Therefore, it seems that IBS is relatively common in individuals with GERD and more common than in the general population.

In this study we found that the prevalence of IBS-like symptoms was 53 to 66% in patients with GERD treated at a tertiary referral center. Our study suggests that there is an important overlap between GERD and IBS symptoms. Interestingly, IBS and functional dyspeptic symptoms were almost equally common along the entire spectrum of GERD (NERD, ERD and BE). However, patients with NERD had longer-lasting and more frequent symptoms than ERD and BE. Our data is in contradiction with other studies, where NERD patients are portrayed as having much more IBS symptoms than ERD [3, 16]. However, other studies have also found that IBS is equally common in both NERD and ERD [17]. Recently, Nojkov et al. analyzed in a prospective study of clinically and endoscopically well characterized patients with GERD the influence of IBS and psychological distress on outcomes and quality of life (QOL) following PPI therapy. The authors found that GERD patients, excluding BE, had a level of symptoms comparable to a non-GERD population after 8 weeks of PPI therapy. Furthermore, they found that symptoms and QOL before and after PPI therapy were similar in NERD and ERD. The investigators also found that patients with ERD and NERD had a similar prevalence of IBS symptoms [17].

From the clinical perspective our data and those from Nojkov et al. make sense for two reasons. First, patients with ERD, NERD and BE have all typical GERD symptoms and these symptoms will improve with PPI therapy [17, 18]. However, as it was demonstrated in the large multicenter ProGERD trial [19] and also shown by Nojkov et al., symptom improvement tends to be better in patients with ERD than NERD. Second, many patients who do not 'improve' will then return to the practice or are referred to tertiary centers, such as ours. These patients have persistent symptoms and the symptoms are likely resulting from IBS or FD. PPIs resolve reflux typical symptoms but are generally not useful for these conditions and therefore would not positively influence the patients' symptoms.

Pogromov et al. analyzed psycho-autonomic aspects in patients with GERD and found that patients with NERD were characterized by more pronounced emotional, motivational, and autonomic disorders compared to ERD patients [20]. This might explain why patients with NERD have more severe symptoms than patients with ERD and BE.

In summary, it appears that several mechanisms are responsible for PPI failure, including visceral hyperalgesia, duodenogastroesophageal reflux, psychological comorbidity and functional GI disorders such as FD and IBS [21]. Our data show that IBS and FD symptoms are common in the entire spectrum of GERD. The presence of these disorders might explain why many patients are

deemed as treatment failures. The overlap between both spectra of diseases, GERD and functional GI disorders, has implication for the patients' management and thus, a better definition and categorization should lead to clear strategies for each condition. Furthermore, our data demonstrate the importance of evaluating patients with standardized questionnaires instead of relying only on routine questioning.

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RESEARCH ARTICLE Open Access

GERD assessment including pH metry predicts a high response rate to PPI standard therapy

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Abstract

Background: Inadequate response to proton pump inhibitor (PPI) therapy in patients with gastroesophageal reflux disease (GERD) is reported in up to 40%. Patients with non erosive reflux disease (NERD) have lower response rates compared to patients with erosive reflux disease (ERD); pH metry contributes to GERD diagnosis and is critical for proper diagnosis of NERD.
Aim of the study was to assess the need for doubling esomeprazole standard dose (40 mg) for 4 weeks in PPI naive patients with typical reflux symptoms and diagnosis of GERD based on endoscopy and 48 hours, wireless pH metry.

Methods: All patients underwent upper GI endoscopy. Symptoms were recorded with a structured questionnaire (RDQ) and acid exposure was determined by 48 hours, wireless pH monitoring (BRAVO). In case of abnormal acid exposure, patients received a short term treatment with esomeprazole 40 mg q.d. for 4 weeks. If symptoms persisted, patients underwent a second pH metry on PPI and the dose was increased to 40 mg b.i.d.

Results: 31 consecutive patients with typical reflux symptoms underwent 48 hours pH monitoring. 22 patients (71%) had abnormal acid exposure, 9 patients had normal pH metry (29%). Of the 9 patients with normal pH metry, 2 were found with erosive esophagitis and 7 without endoscopic abnormalities.
24 patients with documented GERD received esomeprazole treatment. 21 patients achieved complete symptom resolution with 40 mg q.d. after 4 weeks (88%). Only 2 patients required doubling the dose of esomeprazole for complete symptom resolution, 1 patient remained with symptoms.

Conclusions: Patients with typical reflux symptoms and abnormal acid exposure have a high response rate to standard dose esomeprazole regardless of whether they have ERD or NERD.

Keywords: GERD, NERD, PPI, Esomeprazole, Treatment, ph metry, Diagnosis, Therapy

Background

GERD is defined as a condition which develops when reflux of gastric contents causes troublesome symptoms and/or mucosal lesions in the distal esophagus [1]. The problems of a symptom-based diagnosis of GERD are demonstrated by Dent and colleagues who found typical symptoms in only 49% of the patients [2] with proven GERD. Nevertheless most guidelines recommend to first administer an empiric trial of proton pump inhibitors (PPIs) for patients presenting with typical GERD-related symptoms without alarm symptoms (dysphagia, weight loss) [3].

Erosive reflux disease (ERD) is diagnosed endoscopically [4,5], however in the absence of erosions, the diagnosis of NERD deserves functional testing. This includes ambulatory pH metry, prolonged pH metry or combined pH and intraluminal impedance measurements to define timing, acid exposure time, reflux characteristics as well as symptom association [3,6,7]. The wireless and prolonged 48 hours capsule pH metry has been demonstrated to exhibit better compliance and patients' satisfaction and better test accuracy for the diagnosis of GERD due to the prolonged measurement and frequent day-to-day variations in the reflux characteristics of

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GERD patients [8,9]. Normal acid exposure to the distal esophagus or missing association between reflux episodes and patients' symptoms are defined as functional heartburn according to ROME III criteria [10].

Adequate acid inhibition with PPI is the current standard therapy for GERD [11,12]. The efficacy in healing reflux esophagitis is very high for PPI with a number needed to treat of 1.7 (95% CI 1.5-2.1) [13]. Furthermore, PPIs are effective for the symptomatic response in GERD [14] but their efficacy differs between the subgroups of ERD and NERD with a larger proportion of non-responders in NERD even when standard dose has been increased to a twice daily dosage [15,16]. We believe that this is most likely due to an incorrect diagnosis of NERD.

Our study was designed to test whether, and in which proportion of patients, PPI standard dose is effective in achieving complete symptom relief if GERD (ERD and NERD) is properly diagnosed by either abnormal endoscopic findings or abnormal acid exposure using 48 hours pH metry. A secondary aim was to determine the proportion of patients that need the escalation of esomeprazole dosage to 40 mg b.i.d for complete symptom relief.

Methods

The study was approved by the institutional ethics committee at the Otto-von-Guericke University and the German "Bundesinstitut für Arzneimittel und Medizinprodukte" (BfArM), funded by Astra Zeneca, Wedel, Germany (Protocol No. GS0205; Eudract No. 2005-000761-19; Title: Control of Symptoms and Acid Reflux by Esomeprazole in Patients with GERD) and conducted according to the ethical guidelines of the declaration of Helsinki.

Patients' population

Patients presenting at the outpatients department of the Department of Gastroenterology, Hepatology and Infectious Diseases with GERD associated symptoms were evaluated. Only patients without prior PPI medication were included in the study (PPI naïve). After given their written informed consent patients were included in the screening (for demographic details see Table 1).

Objectives and study design

The primary objective was to determine the proportion of patients that achieve complete symptom relief with esomeprazole 40 mg q.d. or b.i.d. Complete symptom relief was defined as absence of reflux symptoms during seven days, as assessed by the self-administered Reflux Disease Questionnaire (RDQ) and a diary. Secondary, the response rates for symptomatic response for proper diagnosed NERD were assessed and related to ERD.

A further objective was to assess the relation between gastrointestinal symptom pattern and 48 hour acid reflux profile during esomeprazole treatment in patients with incomplete symptom relief.

The study was designed as an open, mono-centric treatment study with measurement of symptoms and pH monitoring before and during therapy with esomeprazole. The diagnosis of GERD was confirmed by 48 hours BRAVO pH monitoring and/or erosions during upper GI endoscopy.

In case of abnormal findings in BRAVO pH monitoring, patients entered a short term treatment (I) with esomeprazole 40 mg q.d. for 4 weeks. During acid suppressive therapy, symptom severity was again assessed by RDQ and a symptom diary. Complete symptom relief was defined as no GERD symptoms during the last 7 days as documented in the diary and in the RDQ questionnaire.

In case of persisting symptoms, the patients underwent a second diagnostic EGD and functional testing, followed by escalating dosage (II) with esomeprazole

Table 1 Demographic data pH data and endoscopic results for patients before therapy with esomeprazole at baseline assessment

	Screening	pH negative	symptom relief 40 mg q.d.	symptom relief 40 mg b.i.d.	persistence
	n = 31	n = 9	n = 19	n = 2	n = 1
Gender (male/female)	12/19	0/9	9/10	2/0	1/0
Age (mean±SD)	52.4±17.1 years	47.5±3.5 years	52.5± 2.8 years	23.7± 5.1 years	66 years
Endoscopy					
NERD		7 (no erosions)	7		
ERD Los Angeles A		2	4	1	1
ERD Los Angeles B			5	1	
ERD Los Angeles C			1		
Barrett's Esophagus			2		
DeMeester score ±SD	_	5.5 ± 1.8	26.9 ± 3.9	20.9 ± 15.7	30.3
- DeMeester [day 1]	_	5.9 ± 2.9	28.2 ± 4.8	21.6 ± 19.8	23.2
- DeMeester [day 2]	_	4.9 ± 2.4	25.6 ± 3.9	10.7 ± 3.5	34.8

40 mg b.i.d. for another 4 weeks. The symptom relief was evaluated under escalating dosage as described before (Figure 1).

The study medication was to be ingested 30 minutes before breakfast (I) and dinner (I, II) with 100 ml of table water.

Esophagogastroduodenoscopy (EGD)

After an overnight fast, all patients underwent EGD under intravenous conscious sedation (midazolam 2–5 mg) using a standard videogastroscope (GIF Q160, Olympus Optical Europe, Hamburg, Germany). Endoscopic esophageal landmarks were defined as the gastroesophageal junction with the beginning of the gastric folds and the Z-line as the squamocolumnar junction. Erosive esophagitis was characterized according to Los Angeles classification [4].

NERD was defined as normal appearing GEJ and abnormal acid exposure during 48 hours pH metry.

Wireless 48 hours BRAVO™ pH monitoring

Ambulatory pH monitoring was performed over 48 hours using the wireless BRAVO capsule pH monitoring device (Medtronic, Minneapolis/GIVEN). Capsules were calibrated at pH 1.0 and 7.0 by submersion in buffer solutions (Medtronic/GIVEN) according to the product information. During EGD the gastroesophageal junction (GEJ) was visualized and the capsule was attached at the esophageal mucosa at 6 cm above GEJ with vacuum suction of 700 mmHg for 2 minutes. The correct placement of the capsule was confirmed endoscopically. The pH data was transmitted by the capsule to a recording device with 433 Hz and a sampling interval of 6 seconds. The patients were asked to carry or keep the recording device within a maximum distance of 100 cm maximum from their bodies. The patients were instructed to follow their normal daily activities and diet. During the period of 48 hours, meal time, sleep disturbances, supine and upright positions were marked in a patients' diary.

After 48 hours patients returned to hospital and the data were downloaded from the recording device. The recordings were completed by entering the diary information manually and analyzed based on the manufacture's software (POLYGRAM NET™ Version 14.1.1322.287).

Total numbers of acid episodes, acid exposure time (AET, pH < 4) and DeMeester score were analyzed for day 1 and day 2 separately as well as for 48 hours in total. Acid exposure time ≥ 4.2% and/or a DeMeester score ≥ 13.9 were considered abnormal [6].

Evaluation of symptoms by validated reflux disease questionnaire (RDQ) and patient's diary

Complete symptom relief was defined as absence of GERD-related symptoms during the last 7 days as documented by RDQ (<5 points) and a symptom-assessing diary. The self-administered patient's diary documented the severity of symptoms on 7 days before and under treatment with esomeprazole. The diary graded heartburn objectively on a 5 point Likert-scale for each day as used in the EXPO study [17].

The RDQ was designed to grade different reflux symptoms during the last seven days. The RDQ is a self-

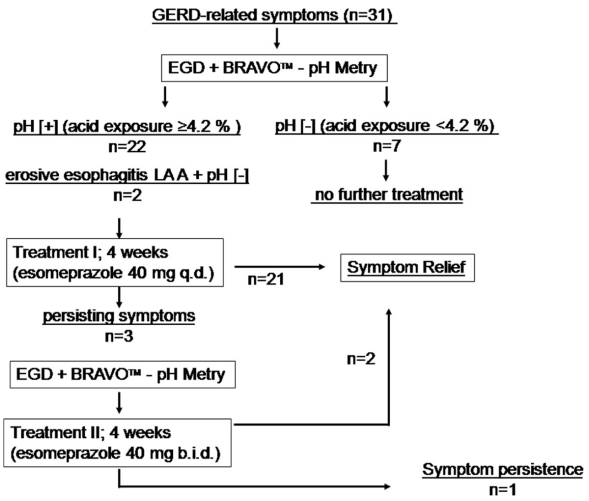

Figure 1 Study design and disposition of patients.

administered questionnaire in which subjects are asked to report the frequency and severity of their upper gastrointestinal symptoms. There are three subscales that evaluate regurgitation, heartburn, and dyspepsia. Response options were also scaled as Likert-type with scores ranging from 0 to 5 for frequency and severity. Each subject's score was calculated as the mean of item responses with higher scores indicating more severe or frequent symptoms [18,19].

Statistical analysis

According to the results of the EXPO study, where 91.1% of patients experienced had complete symptom relief after four week treatment with esomeprazole 40 mg orally the sample size was calculated. With focus on complete symptom relief, the hypothesis H0 complete symptom relief < =68% vs. H1 complete symptom relief > 85% was tested by a one-sided binomial test, which will have a power of 80% (type-I-error 5%). 40 patients were calculated as the requested sample size.

All data entered into a database using the Microcal Origin™ 5.0 program package (Northhampton, MA, USA) and SPSS© 12.0. Data is expressed as mean and 95%-CI (confidence intervals), if not stated otherwise. For statistical analysis (pre- and post-treatment) parametric *T*-test was used. All test were applied two-sided with a level of significance of $P < 0.05$.

Results

Patients' characteristics

A total of 40 patients with predominantly female gender were included in the screening phase of the study (mean age 52 years; range: 18–79 years) (Table 1).

31 patients met the inclusion criteria with either endoscopic findings of erosive reflux disease (n = 2), abnormal pH metry (NERD n = 7) or both (n = 15). In 7 patients with normal appearing gastroesophageal junction (all female), GERD was excluded by normal results in BRAVO pH monitoring (Table 2).

There was a withdrawal of 9 patients for different reasons: 5 patients did not complete neither questionnaire nor diary; one patient stopped the study medication because of an newly appeared exanthema; 3 patients presented with technical problems in BRAVO capsule testing (1 patient suffered from severe chest pain that required endoscopic removal of the capsule; 2 patients documented an early drop off the capsule).

Control of symptoms and acid reflux by standard dose and doubled standard dose of esomeprazole

Finally, 24 patients entered the treatment phase. Endoscopic diagnosis revealed 7 patients with non-erosive reflux disease (NERD), 15 patients with erosive reflux disease (ERD) and 2 patients with newly diagnosed short segment Barrett's esophagus without dysplasia (Tables 1, 2).

After 4 weeks of treatment with esomeprazole 40 mg q.d., 21 (88%) patients achieved complete symptom relief. 2 patients achieved symptom relief after escalating dosage of esomeprazole to 40 mg b.i.d. (8%), but only 1 patient presented with persisting symptoms even after escalating dosage. In this patient, pH metry revealed an even unchanged cluster of pH metry during esomeprazole treatment (Figure 1).

No differences were obtained between patients with NERD and ERD (Figure 2) and no substantial differences were found between day 1 and day 2 of pH analysis (Table 1).

Relief of symptoms documented RDQ and patient's diary

Patients with complete relief of symptoms according to RDQ and diary are shown in Figure 3. The calculated RDQ means pre- and post PPI therapy differed 6.3–times in total (19.1 [14.07 – 24.02] vs. 3.2 [0.51 – 5.4]; $p < 0.0001$), 8.2-times for heartburn (6.5 [4.5 – 8.4] vs. 0.8 [0.19 – 1.78]; $p < 0.0001$), 5.1-times for regurgitation (7.2 [4.7 – 9.63] vs. 1.4 [0.08 – 2.75]; $p < 0.0001$) and 4.2-times for dyspepsia (5.4 [3.36 – 7.47] vs. 1.3 [0.17 – 2.3]; $p < 0.0001$). Similar to the RDQ, the diaries were evaluated for the patients included and documented a 3.9-fold reduction of the mean value (17.2 [13.9 – 20.49] vs. 4.4 [3.3 – 5.1]; $p < 0.0001$; Figure 4).

Discussion

The main finding of this study is that patients with typical reflux symptoms and abnormal acid exposure have a high response rate to standard esomeprazole regardless of whether they have ERD or NERD. Two thirds (22/31) of patients with typical GERD-related symptoms had an abnormal acid exposure in esophageal BRAVO pH metry. Including 2 patients with erosive changes but

Table 2 Diagnosis and response to esomperazole 40 mg q.d. for 4 weeks

diagnosis	n	response to esomeprazole 40 mg q.d.
abnormal pH metry only (NERD)	7	100%
ERD and abnormal pH metry [pH +]	15	80%
ERD and normal pH metry [pH -]	2	100%
normal endoscopy and normal ph metry [pH-]	7	no therapy

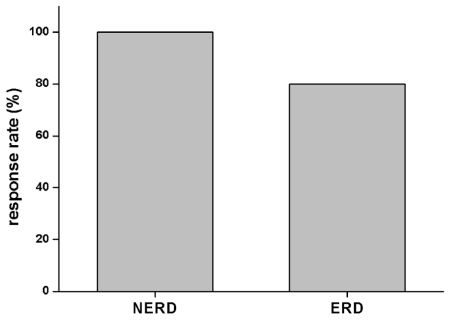

Figure 2 Therapeutic response to esomeprazole 40 mg o.d. in patients with NERD and ERD.

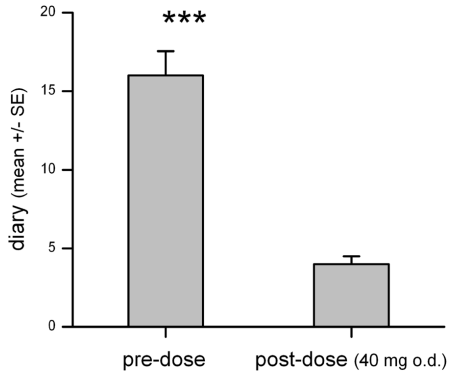

Figure 4 Mean severity of symptoms at screening and after 4 weeks of treatment with esomeprazole 40 mg q.d. according to patients' diary.

normal pH metry, 24 patients were eligible for PPI treatment in our study.

88% of this well selected patient group achieved complete symptom relief on esomeprazole standard dose for 4 weeks. Symptomatic response was similarly obtained in patients with ERD and NERD (Table 2; Figure 2). The claim that patients with NERD would have a worse response to PPI is therefore most likely due to the inclusion of patients without abnormal gastroesophageal reflux in previous studies. Misdiagnosis of GERD – NERD in particular – might also explain reasonably the high PPI failure rate in previously published data. Weijenborg and colleagues systematically reviewed previous outcome studies and found only 2 studies defining NERD by both negative endoscopy and a positive pH-test. In contrast to poor response rates in empirical treated or endoscopy-negative patients, the pooled estimate rate of complete relief of heartburn after 4 weeks of for those accurately diagnosed NERD was 0.73 (95% CI 0.69-0.77) and comparable to patients with ERD [20]. This clinical data indicates to careful asses the diagnosis of NERD and differentiate especially from functional heartburn to predict a therapeutic success of current PPI therapy.

We excluded patients with normal acid exposure as there is no rationale for PPI treatment. This category of patients is likely to account for the frequent reports with up to 30-40% PPI failure to standard dose [15,21]. In

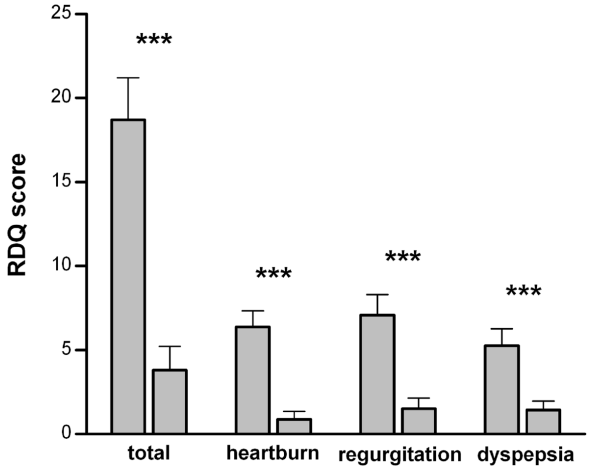

Figure 3 Mean heartburn, regurgitation, dyspepsia and total RDQ scores at screening and after 4 weeks of treatment.

routine practice in Germany, the response rate to PPI is 60% [22]. For patients not responding to PPI in presence of typical symptoms, functional testing is performed to test the initial diagnosis and to further investigate for conditions that might explain PPI refractoriness. Among them, persistent acid or non-acid reflux episodes have been reported to be responsible for incomplete symptom relief [6,23-26].

In a further subset of patients, reflux symptoms may be unrelated to reflux episodes at all and related to a functional syndrome (functional heartburn) [27]. Although unable to determine the proportion of "non acidic" reflux episodes by BRAVO pH metry, our study reemphasizes the importance of the patients' interview and interpretation of symptoms to distinguish between acid-related symptoms and functional disorders that often overlap and requires different medical treatment [27,28].

For patients not responding to PPI, pH metry should be considered to confirm the diagnosis of abnormal gastroesophageal reflux. Mechanisms involved in symptom generation or perpetration are either hypersensitivity to visceral stimuli or weakly acidic reflux episodes, a fast hepatic metabolism of PPIs [29] or duodenogastroesophageal reflux (DGER) [15,30]. Intestinal proteases in the refluxate and interaction with epithelial protease-activated receptors are also involved in the pathogenesis of mucosal inflammation in GERD pathogenesis [31,32].

The shortcomings of the study are the missing control group and the small sample size. This was mainly due to the inclusion criteria of PPI naive patients in a referral centre. As calculated before, the recruitment was finalized after having screened 40 patients.

In spite of the small sample size, the results indicate daily clinical practice. Nevertheless, our study has the true advantage of having included truly PPI-naive patients, a fact that is very hard in routine clinical practice, as most physicians administer PPI very quickly based on current guidelines. However, this "aggressive" approach might need to be rethought, as we believe that many patients receiving PPI do not suffer from NERD or ERD, and thus being over treated. Thus, a careful initial assessment of symptoms combined with functional testing may identify the patients who respond well to PPI therapy. This fact needs to be reconsidered in the interpretation of many clinical trials concerning response to PPI therapy, especially in NERD [20].

Conclusion

PPI naïve patients with characteristic GERD-related symptoms and abnormal findings in pH metry had an excellent response to standard dose esomeprazole. Due to the small sample size of our study it cannot be concluded, but patients with NERD diagnosed with pH metry and endoscopy did not differ in their response rates to esomeprazole in comparison with ERD. This corresponds to the systematic review cited above and responds to the studies investigating the PPI test, and documented symptom relief in up to 90% in case of pathological acid exposure [33,34].

29% of the patients in our study suffered from typical GERD-related symptoms but had no abnormal acid exposure in 48 hours pH metry, predominantly with unsuspicious results in EGD (no erosions). This may partly explain the high proportion of PPI non-responsiveness in the literature, since the patients may all have been grouped as NERD [21].

For non-responders with abnormal 48 hours pH metry, in clinical practice it may be appropriate to escalate PPI to double standard dose before embarking in functional testing (MII-pH) and seek for other mechanisms in GERD pathogenesis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PM, UP and KM designed the study. AK, HN and JW enrolled the majority of patients. AK and JW provided pH metry analyses and clinical data. The manuscript was drafted by AK, UP and KM and reviewed for important intellectual content by JW, HN and PM. All authors read and approved the final manuscript.

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Nocturnal Gastric Acid Breakthrough Is Not Associated with Night-Time Gastroesophageal Reflux in GERD Patients

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Key Words
Nocturnal acid breakthrough · Gastroesophageal reflux disease · Impedance · pH-metry · Proton pump inhibitors

Abstract
Background: Nocturnal acid breakthrough (NAB) is defined as gastric pH below 4 over 60 consecutive minutes at night-time in subjects who take proton pump inhibitors twice daily. The link between NAB and gastroesophageal reflux (GER) episodes has not been investigated using combined multichannel intraluminal impedance and pH-metry (MII-pH). ***Aims and Methods:*** The aim was to investigate the relationship between NAB and GER by means of MII and gastroesophageal pH-metry. We reanalyzed MII-pH recordings obtained in patients on twice-daily proton pump inhibitors. ***Results:*** Overall 15 eligible recordings were reanalyzed in detail (7 males, 8 females; age 59 ± 10 years, range 36–68 years). NAB was detected in 7/15 (46%) recordings with one NAB in 4 subjects and two 2 NABs in 3 subjects. In 6 of the cases with NAB, reflux symptoms were reported at night, but in no case did these occur in association with NAB. Patients with NAB reported significantly more typical than atypical symptoms (77 times vs. 47 times), whereas patients without NAB reported fewer typical than atypical symptoms (48 times vs. 60; p = 0.011). Over the total 24-hour period, acid reflux was more frequent (9 ± 11 times) in patients with NAB than in patients without NAB (1 ± 0.5 times; p = 0.04). Weakly acid reflux also occurred more frequently (15 ± 9 times) in subjects with NAB than in subjects without NAB (6 ± 4 times; p = 0.02). Weakly alkaline reflux occurred in equal frequencies in subjects with and without NAB (2 ± 4, 0 ± 0.4, respectively). Esophageal acid exposure in the upright position was not different between subjects with NAB and subjects without NAB, but subjects with NAB presented a higher recumbent esophageal acid exposure than subjects without NAB (2.0 ± 2.4 vs. 0%, respectively). ***Conclusion:*** Esophageal acid exposure is not increased during NAB episodes. However, over a period of 24 h, patients with NAB presented with increased gastroesophageal reflux. Although NAB and GER are not strongly associated, symptoms of patients with and without NAB are different.

Introduction

Nocturnal acid breakthrough (NAB) is defined as a decrease in gastric pH below 4 over 60 consecutive minutes at night-time in subjects who take proton pump inhibitors (PPI) twice daily (bid) [1]. Peghini et al. [1] were the first to describe the phenomenon of NAB, which can be detected in up to 70% of the patients who are taking PPI bid. NAB occurs independently of the type of PPI [2] and is detectable in healthy subjects as well as in patients with gastroesophageal reflux disease (GERD) [2]. The impact of NAB on gastroesophageal reflux (GER) is not well characterized and is a subject of contentious debates.

[1] This article is part of the doctoral thesis of Franziska Büsch.

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NAB was suggested to play a critical role in GERD [2, 3], and several pharmacological attempts have been made to overcome this phenomenon with different regimens and doses of PPI [4] as well as with the additional administration of H2 receptor antagonists [2, 5]. Recently, NAB has been described to be an isolated gastric phenomenon and to be not associated to GER [6].

Combined multichannel intraluminal impedance and pH-metry (MII-pH) allows to identify all types of GER by detecting retrograde bolus movement [7, 8]. Using a MII-pH catheter with two pH electrodes, acidity of the stomach and the esophagus can be measured simultaneously. As MII-pH detects GER episodes independent of pH value, it is the best available instrument to characterize the link between NAB and GER and is therefore better than pH-metry alone.

Previous studies demonstrated that the total number of reflux episodes as defined by a change in impedance is similar with and without PPI medication. Only the acidity of the refluxate is altered by PPI [9]. Therefore, MII-pH allows to detect GER in patients also while they remain on PPI therapy.

The aim of this retrospective analysis was to investigate the relationship between gastric NAB and gastroesophageal reflux and reflux symptoms by means of MII-pH combined with intragastric pH-metry.

Methods

From our database of 93 MII-pH recordings in clinical routine, 15 patients were identified taking PPI bid during the examination and these subjects were further analyzed retrospectively. In all of them, important confounding factors were excluded. Exclusion criteria were defined (table 1). The data were analyzed using the BioView Analysis® software from Sandhill Scientific, Inc. All records were also analyzed manually with special attention to gastric pH, esophageal pH and all kinds of retrograde bolus movements to obtain the highest detection rate of reflux episodes. Meal periods were excluded from the analysis [for further details, see also 10]. A pH above 7 defined a reflux episode as weakly alkaline. A pH between 4 and 7 was defined as weakly acidic and a pH below 4 was defined as acidic. Medical files of all patients were browsed for the results of endoscopy.

Before MII-pH monitoring, we performed esophageal manometry in each patient to rule out motility disorders and to label the distance between the lower esophageal sphincter (LES) and the nostril for defining the depth of insertion of the MII-pH probe. Immediately after the manometry, the impedance catheter (ZAN 62C01E, Sandhill Scientific®) was inserted. The pH electrode was placed 5 cm above and 10 cm below the LES and the impedance channels were located at 3, 5, 7, 9, 15 and 17 cm above the LES (fig. 1). Data were sampled and stored in a portable recorder (Sleuth, Sandhill Sientific®). The patients were advised on the use of the recorder and instructed to keep a detailed diary report in addition to the electronic data sheet recording documenting body position, meal intake, medication and symptoms. The MII-pH recording lasted over a period of 24 h. Reflux episodes were defined by occurrence of retrograde bolus movement. Figure 2 shows an example of a reflux episode of a patient of this study. Symptoms were divided into typical (heartburn and regurgitation) and atypical symptoms according to the Montreal classification [11].

Statistical analysis of patient groups was performed using the Student's t test for analysis of reflux parameters and the χ^2 test for analysis of symptom distribution. A p value below 0.5 was considered to express statistical significance.

Table 1. Exclusion criteria and their rationales

Exclusion criterion	Rationale
Artifacts in MII-pH recording	Recording not reliable
Lack of gastric acid suppression during night-time	Other causes than NAB of acid recovery during night-time
Meals during night-time	Meals influence gastric pH directly and lead to increased postprandial reflux
Gastric surgery	Parietal cell mass is reduced, gastrin-secreting cells are reduced, gastric emptying is affected
Barrett's metaplasia longer than 3 cm	Alters detection of reflux episodes with MII-pH

Results

Patient Characteristics

The recordings of 15 subjects (7 male, 8 female; age 59 ± 10 years, 36–68 years) were reanalyzed for gastric pH profile and GER patterns with special attention to reflux during NAB episodes. Eight patients had nonerosive GERD, 3 patients showed erosions during endoscopy (Los Angeles grade A/B) and 4 patients had Barrett's esophagus. 13 patients were on esomeprazole 40 mg bid, 1 patient on omeprazole 40 mg bid and 1 patient on pantoprazole 20 mg bid.

Analysis of the MII-pH Recordings

NAB was detected in 7 patients (46%). NAB occurred as a solitary phenomenon in 4 out of these 7 subjects (57%), two episodes of NAB occurred in 3 patients (43%). Mean duration of NAB was 130 min, ranging from 62 to

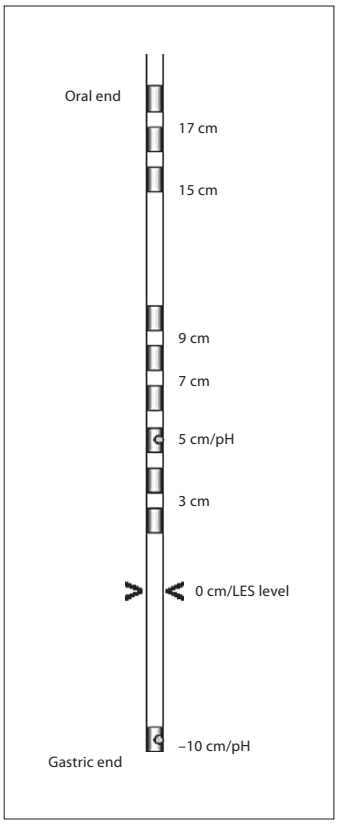

Fig. 1. Sketch of a six-channel impedance probe with dual-channel pH-metry electrodes. The probe is positioned with the pH-metry electrodes 5 cm above and 10 cm below the level of the LES.

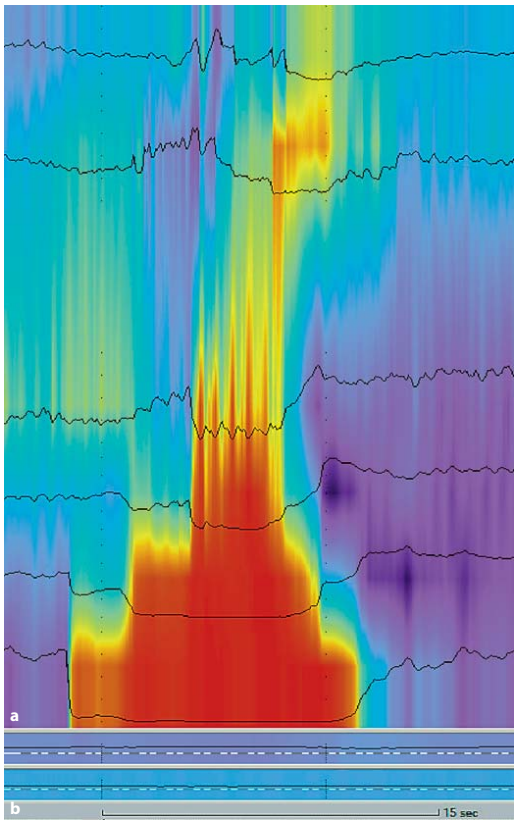

Fig. 2. Example of an MII-pH tracing demonstrating an episode of nonacid reflux. **a** Six impedance channels (black lines) with an overlay of colored expression of change in percentage of baseline impedance. High impedance/baseline impedance is expressed in blue and violet color. Low impedance/fall in impedance is expressed as red and yellow color. **b** Esophageal pH (upper part) and gastric pH (lower part).

389 min. Additional gastric acid recovery periods of more than 20 min duration but shorter than 60 min were detected in 3 subjects (42%). Patient characteristics are demonstrated in table 2.

Analysis revealed acid reflux in 2 recordings and weakly acid reflux in 2 recordings during NAB episodes. Weakly alkaline reflux was absent during all recorded NAB episodes. Subjects presenting with NAB were compared with those without NAB during the recording according to the overall (24-hour) reflux pattern.

Acid reflux episodes were found 9 ± 11 times in subjects with NAB and only 1 ± 0.5 times in subjects without NAB ($p = 0.04$). Weakly acid reflux episodes occurred 15 ± 9 times in patients with NAB and 6 ± 4 times in patients without NAB ($p = 0.02$). The number of weakly alkaline reflux episodes was not different in subjects with and without NAB (2 ± 4, 0 ± 0.4, respectively). Esophageal acid exposure (pH only reflux episodes) in the upright position was not different in subjects with NAB and subjects without NAB. Nevertheless, subjects with NAB

Table 2. Patient characteristics and demographics

NAB	Sex	Age, years	Duration of 1st NAB, min	Duration of 2nd NAB, min	Duration of 3rd NAB, min	Duration of 4th NAB, min	Johnson-DeMeester score	Endoscopy finding
NAB negative	M	46					0.9	NERD
	F	42					0.9	BM 1 cm
	F	44					12.0	NERD
	F	50					1.8	NERD
	F	47					0.9	LA-A
	M	36					2.1	LA-B
	M	64					8.2	BM 0.5 cm
	F	41					0.9	NERD
NAB positive	F	57	390				0.9	NERD
	F	38	60	67			11.3	BM 1 cm
	F	50	66	132			21.0	NERD
	M	68	74	85	30*	29*	6.3	NERD
	M	53	79				28.8	NERD
	M	36	265	62			19.5	BM 1 cm
	F	61	81	34*	24*		12.4	LA-B

Patients are grouped according to NAB. The Johnson-DeMeester score is higher in the NAB group, although the subjects do not present increased GER during the NAB episodes. Asterisks point to values not matching the common definition of NAB. LA = Los Angeles grade of erosive esophagitis; BM = Barrett's metaplasia given with the maximal length of segment.

Table 3. pH-MII analysis over 24 h according to the presence of NAB

	NAB+ (46%)	NAB– (54%)	p value
Acid reflux episodes	9 ± 11	1 ± 0.5	0.04
Weakly acidic episodes	15 ± 9	6 ± 4	0.02
Acid exposure recumbent	2.0 ± 2.4%	0%	0.04
DeMeester score	14.3 ± 9.5	3.5 ± 4.2	0.01

Values are presented as means ± standard deviation. Reflux episodes were more frequent in the patient group with NAB over the whole 24-hour period.

presented a higher recumbent esophageal acid exposure as compared to subjects without NAB (2.0 ± 2.4 vs. 0%, respectively; p = 0.04; for detailed information, see table 3).

Symptom Analysis

Symptoms during night-time were reported by 4 patients (27%) with NAB, but none of these symptoms occurred during a NAB episode or showed association with any kind of reflux. 3 of the 4 patients experienced only one type of symptom, the other patient reported two different symptoms. In 3 of the 4 patients, the symptoms that occurred at night were also reported at daytime. One patient had no symptoms at daytime. Only 2 patients without NAB reported night-time symptoms.

Patients with NAB significantly more frequently reported typical than atypical symptoms (77 times vs. 47 times), whereas patients without NAB reported fewer typical than atypical symptoms (48 times vs. 60; p = 0.011). The average amount of reported symptoms was not statistically different in patients with NAB (18.4 ± 14.1) and patients without NAB (15.1 ± 15.8).

Discussion

Gastric NAB was not directly associated with esophageal acid reflux in our study. NAB was detected in 46% of patients on PPI bid, similar to previously reported findings [1]. About 70% of GERD patients, 80% of patients with Barrett's esophagus and 67% of normal controls presented NAB in a study by Katz et al. [3]. More severe forms of GERD showed significantly increased esophageal acid exposure during NAB. Up to 33% of GERD patients and 50% of patients with Barrett's esophagus had increased esophageal acid exposure. Normal controls had

increased esophageal acid exposure in only 8% [3]. The prevalence of night-time reflux was rare in our study and similar in patients with and without NAB. A widespread belief is that GER is more frequent in the recumbent or the supine position. But recent studies indicate the opposite and show that in most cases GER is more frequent in the upright position [12–16]. This was observed in our study as well.

Patients with NAB have more overall reflux episodes compared to patients without NAB, but GER episodes are rarely seen during NAB episodes. A possible explanation for the association between NAB and gastroesophageal reflux may be the presence of additional short episodes of gastric acid recovery that do not fit the definition of NAB but may still contribute to GER.

In our study, NAB showed no correlation with either acid, weakly acid or weakly alkaline gastroesophageal reflux. In addition, the nocturnal GERD symptoms were not associated with NAB or reflux episodes during NAB. Similar findings were reported by Nzekao and Murray [17], who found that only in the minority of cases (36%), symptoms occurred in direct association with NAB. Although there is no association between NAB and nocturnal GER, night-time symptoms that lead to an arousal were more frequent in patients with NAB and absent in patients without NAB. The increased overall GER based on the 24-hour analysis leads to more night-time symptoms in patients with NAB, while the direct association between GER and night-time symptoms is low. Interestingly, the experience of typical and atypical symptoms shows association to NAB. Patients with NAB more often experienced typical than atypical symptoms, whereas patients without NAB reported more atypical symptoms. This demonstrates a link between reflux symptoms and NAB in patients on PPI treatment even if GER cannot be directly observed.

An interesting observation of the study of Katz et al. [3] is that one patient investigated twice had increased esophageal reflux during NAB only in one recording. GER does not regularly occur during NAB.

In our study, patients with NAB did not have increased acid reflux during NAB but had more total esophageal reflux over a period of 24 h (table 3). MII-pH measurement reveals that the esophageal exposure to reflux is significantly lower than the estimated total acid exposure if common pH-metry alone is performed [18]. Previous studies that reported increased esophageal acid reflux during NAB and during the rest of the night accompanied by decreased acid clearance during night-time were performed using standard pH-metry [2–6, 19]. This is a methodological limitation of previous studies. To our best knowledge, our study is the first that investigates NAB in correlation to reflux by means of combined multichannel intraluminal impedance and pH-metry.

Another limitation of isolated pH-metry is related to sampling errors of pH measurements in the stomach. A single pH electrode in the stomach is not able to measure the complete gastric pH environment. Apart from this, pH-metry results do not reflect the amount of gastric acid secretion but only the H+ activity [20]. Several studies have shown a compartimentation of the stomach with areas that markedly differ in pH values at the same time [21].

In contrast to the investigations by Peghini et al. [2] and Katz et al. [3], Ours et al. [6] found no correlation between NAB and GER, concluding that gastric acidity and GER are independent phenomena. Recent data demonstrate the lack of association between the severity of GERD and nocturnal symptoms and gastric acidity [19].

The limitation of our study is its retrospective nature. This is compensated by the fact that only well-characterized recordings were analyzed in detail in a well-characterized population. Thus, the obtained data allow some new insights into the complexity of NAB and GERD.

In summary, gastric NAB is not associated with gastroesophageal reflux but presents as a risk factor for nocturnal gastroesophageal reflux episodes. Short episodes of gastric acid recovery that do not fit the criteria of NAB may play a role in nocturnal gastroesophageal reflux. The clinical impact of NAB needs to be further evaluated.

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Publikation V

AP&T Alimentary Pharmacology and Therapeutics

Histomorphological differentiation of non-erosive reflux disease and functional heartburn in patients with PPI-refractory heartburn

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SUMMARY

Background
Proton pump inhibitor (PPI)-refractory heartburn may be due to persistent gastro-oesophageal reflux, oesophageal hypersensitivity or functional heartburn (FH). The differentiation between non-erosive reflux disease (NERD) and FH may be very difficult. However, this differentiation is important for appropriate therapeutic management. Dilated intercellular spaces (DIS), papillary elongation (PE) and basal cell hyperplasia (BCH) can be all assessed by light microscopy. Whether these mucosal abnormalities allow the differentiation of NERD from FH in PPI-refractory patients is uncertain.

Aim
To assess histopathological findings by light microscopy in patients with refractory heartburn to differentiate NERD from FH.

Methods
Sixty-two patients with PPI-refractory symptoms underwent EGD and MII-pH after pausing PPI medication for 2 weeks before investigation. Twenty-five subjects without upper gastrointestinal symptoms were included as controls. Symptom assessment was based on the reflux disease questionnaire (RDQ). Biopsies were taken 3–5 cm above the gastro-oesophageal junction. DIS, PE, BCH and infiltration of immune cells were evaluated and a sum score was calculated.

Results
Based on endoscopy and MII-pH, GERD was diagnosed in 43 patients (NERD: 20; ERD: 23) and FH in 19 patients. There was no difference in symptoms between the groups. Each individual histopathological item was different between the groups ($P < 0.0001$). Between NERD and FH, the most significant difference was found for DIS and the histopathological sum score ($P < 0.001$).

Conclusions
These findings suggest that oesophageal biopsies are useful to differentiate NERD from FH. Increased DIS and a histological sum score are the most significant histopathological abnormalities in NERD as compared with FH.

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INTRODUCTION

Up to 40% of patients with gastro-oesophageal reflux disease (GERD) have no adequate improvement of reflux symptoms with a standard dose of proton pump inhibitors (PPIs).[1] Neither typical symptoms (i.e. heartburn or regurgitation) nor a short course of PPI (PPI test) allows establishing the diagnosis of GERD with certainty.[2–4]

Erosions detected during endoscopy lead to the diagnosis of erosive reflux disease (ERD). However, patients with reflux symptoms most often do not have oesophageal mucosal abnormalities at endoscopy. If they do not respond to PPI, it is uncertain whether they have non-erosive reflux symptoms (NERD) or functional heartburn (FH). 24 h-pH-metry or combined pH-impedance monitoring (MII-pH) is helpful to differentiate between the two conditions. In case of negative upper gastrointestinal endoscopy and a normal reflux monitoring without association with symptoms, the diagnosis is more likely FH.[5, 6] The distinction between NERD and FH is clinically relevant as the therapeutic approach is different between the two conditions.

Several studies have addressed the question whether oesophageal mucosal changes such as dilated intercellular spaces (DIS) are helpful in the characterisation of GERD and diagnosis of NERD.[7–10] DIS have been induced in animal perfusion studies by acid solutions and bile acids [11] and are recognised as common but distinct morphological findings in patients with NERD.[12, 13] So far, the functional role of DIS is suggested to be related to symptom generation, as oesophageal contents may reach deeper mucosal layers and intramucosal nerve endings.[14]

While DIS were originally investigated by transmission electron microcopy (TEM),[12, 13, 15] subsequently, DIS were also reported to be detectable by standard light microscopy (LM).[8, 10, 16] DIS measured by TEM allow distinguishing NERD from FH in PPI-refractory patients.[17]

The aim of our study was to investigate whether standard LM with GI pathological examination will allow the differential diagnosis of FH and NERD.

METHODS

Study subjects and study protocol

This is a prospective study enrolling consecutive patients with PPI-refractory heartburn who were evaluated at our out-patient department. None of the patients had EGD or functional diagnostics before, but all had heartburn as typical GERD symptom based on Montreal classification.[18] PPI-refractoriness was defined as <50% symptom improvement and a medical history of PPI double standard dose for at least 6 weeks in the past.

Previous upper GI surgery, alarm symptoms, gastric or duodenal ulcer disease, Barrett's oesophagus or oesophageal motility disorders were exclusion criteria.

The study protocol was performed according to the declaration of Helsinki and approved by the local ethical committee. Eligible patients (>18 years) were included after having given their informed consent.

All patients were asked to taper their antisecretory drugs and stop for at least 2 weeks before endoscopy and MII-pH. The exact period during which patients were actually off PPI therapy beyond the required period was not recorded systematically, but we did not experience differences between patients with NERD and FH as all patients had adhered closely to the required period of 2 weeks.

Symptoms were recorded using the validated reflux disease questionnaire in German translation.[19] Then, patients underwent EGD and MII-pH monitoring at the same day.

Based on the endoscopic results and MII-pH, patients were divided into three groups:

(i) When erosions were clearly visible during endoscopy according to Los Angeles classification[20];
(ii) NERD, in case of negative EGD but abnormal MII-pH including oesophageal hypersensitivity (HE);
(iii) FH, with a negative EGD and negative MII-pH without symptom association.

Additionally, 25 patients without any GERD-related symptoms and upper GI pathologies and without acid suppressive medication were included as controls.

Upper GI endoscopy and oesophageal biopsies

After an overnight fast, all patients underwent EGD under intravenous conscious sedation using midazolam (Dormicum V® 5 mg/mL; Roche Deutschland Holding GmbH, Penzberg, Germany) and/or 1% propofol (Propofol-Lipuro® 10 mg/mL; Braun Melsungen AG, Melsungen, Germany) with a standard videogastroscope (GIFQ180, Olympus Optical Europe, Hamburg, Germany).

Endoscopic oesophageal landmarks were defined as the gastro-oesophageal junction (GEJ) with the beginning of the gastric folds and the Z-line as the squamocolumnar junction and diaphragmatic pinch. In the distal oesophagus, 2 oesophageal biopsies were taken 3–5 cm above the GEJ not including visible changes (no erosions) and immediately transferred to 4% neutral-buffered formalin for later embedding in paraffin.

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Combined 24-h impedance pH monitoring (MII-pH)

After recovery from endoscopy, the MII-pH catheter (Sandhill Scientific, Highland Ranch, CO, USA) was inserted and located with oesophageal pH electrodes 5 cm above the gastro-oesophageal junction (LES) according to endoscopy or stationary manometry. Oesophageal impedance electrodes were located at 3, 5, 7, 9, 15 and 17 cm above the LES.

Patients were asked to take three meals and beverages at fixed times and not to lie down during day time. Event markers were set for meal times, body posture and the occurrence of specific symptoms. Manual analysis of the tracings was performed by two experienced operators (AK, CC), independently. In case of disagreement, the case was discussed with a third experienced investigator (JW).

Reflux episodes were defined as drop of >50% from baseline impedance moving from distal to the proximal extend. They were characterised as liquid + mixed or gas and by the pH of the refluxate.

Acid exposure time (AET) was defined abnormal when pH <4 was measured more than 4.2% over 24 h. Symptom association probability (SAP) and symptom index (SI) were assessed as previously described [21, 22] for acid, weakly acidic and weakly alkaline reflux events.

NERD was diagnosed if no erosions were visible endoscopically, but AET was increased (>4.2%). Patients with normal AET but positive SI or SAP had hypersensitive oesophagus (HE) and were considered in the diagnosis of NERD according to Rome III criteria.[6] A normal endoscopic appearance of the gastro-oesophageal junction in combination with normal AET without any symptom association (negative SI and SAP) was considered as functional heartburn (FH).

Histopathological evaluation

Oesophageal tissue specimens were embedded in paraffin and submitted for histopathological examinations with haematoxylin and eosin and periodic-acid shift staining. The degree of basal cell hyperplasia, presence of papillary elongation and dilated intercellular spaces was assessed [10, 23, 24] and semi-quantitatively scored as either 0 (absent), 1 (mild), 2 (moderate) or 3 (severe) as described previously.[25] Furthermore, inflammation was scored by the density of granulocytes, eosinophiles and intraepithelial lymphocytes (see Figure 1).

A histopathological sum score was calculated by adding the individual scores of each specific histological feature (DIS + BCH + PE + inflammation). The expert

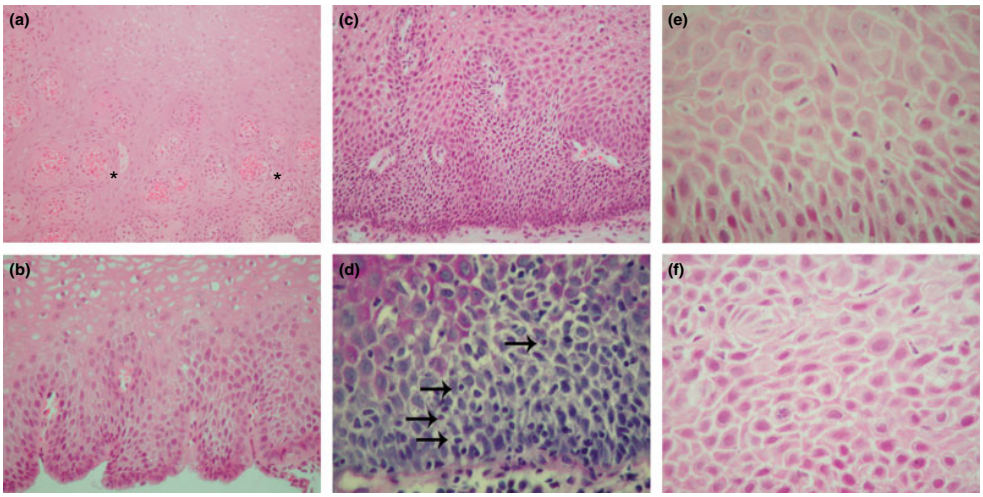

Figure 1 | Histopathological Evaluation. Exemplary display of papillary elongation (a + b), basal cell hyperplasia (c), inflammatory infiltration oesophageal mucosa (d) and dilated intercellular spaces (e + f). (*) in panel a shows elongated and dilated intrapapillary vessels with typical hyperaemia. Arrows mark infiltrating granulocytes and intramucosal lymphocytes (microscope: Zeiss Axioskop 50; magnification ×200, ×400; camera: Nikon coolpix 990.

pathologist (DJ) was blinded to endoscopic data and results from MII-pH.

Data collection and statistical analysis

All collected data were entered in an Excel sheet (Microsoft Corporation Redmont, Redmont, WA, USA) and statistically analysed using SPSS 12.0 or GraphPad Prism (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard error (S.E.) or 95% CI (confidence intervals), if not stated otherwise. One-way ANOVA was applied for comparisons among the groups (controls, FH, NERD and ERD). If significant differences were identified, Bonferroni's analysis for multiple testing for *post hoc* analysis was performed to calculate differences between the histopathological items. Correlation analysis was performed by Pearson's correlation test. All tests were applied two-sided with a level of significance of $P < 0.05$.

We performed a receiver-operated characteristic (ROC analysis) for the sum scores between NERD and FH to calculate a cut-off value. Sensitivity, specificity and predictive values for the discrimination between NERD and FH were calculated with a cut-off ≥5 using chi-squared test.

RESULTS

Demographic data and patients' symptoms

Sixty-two patients with refractory heartburn were included. Additionally, twenty-five patients without any symptoms of GERD, without PPI therapy and unsuspicious gastro-oesophageal junction were included as GERD-negative controls (Table 1).

There was no difference for age, but a predominant female gender in controls, NERD and FH. In ERD, male gender was significantly increased.

No differences were found for symptoms defined by the different items of the RDQ. Symptom severity was equal between ERD, NERD and FH evaluating the RDQ scores for dyspepsia for heartburn or regurgitation (Figure 2a–c).

Histological evaluation

Each histopathological feature differed significantly between the groups (Figure 3, $P < 0.0001$). In *post hoc* analysis, the distinction between NERD and FH was statistically significant different for BCH, DIS and the inflammatory score ($P < 0.05$–0.001), with highest discrimination for DIS. FH showed similar scores for each

Table 1 | Demographic data

	Controls $n = 25$	FH $n = 19$	NERD $n = 20$	ERD $n = 23$
Gender (male/female)	8/17	3/16	8/12	15/8
Age (years) (mean ± S.E.; range)	54.8 ± 3.4 23–79 years	51.0 ± 4.1 23–77 years	59.1 ± 2.05 43–72 years	51.7 ± 3.3 24–78 years

FH, functional heartburn; NERD, non-erosive reflux disease; ERD, erosive reflux disease.

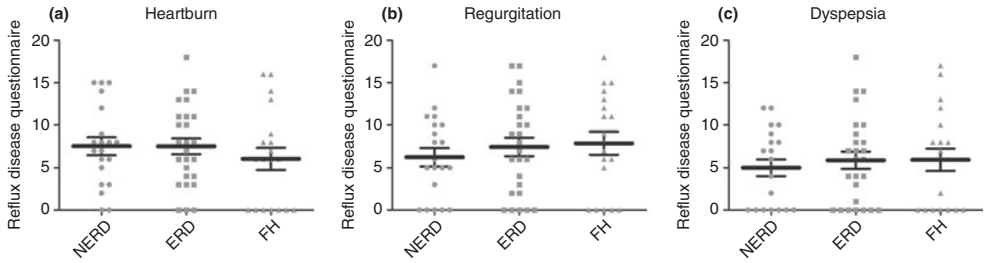

Figure 2 | Symptom characteristics according to the reflux disease questionnaire. Panels a + b display the reflux items for heartburn (a) and regurgitation (b). Panel b shows the dyspepsia score. For all items, no significant differences were obtained between the diagnoses.

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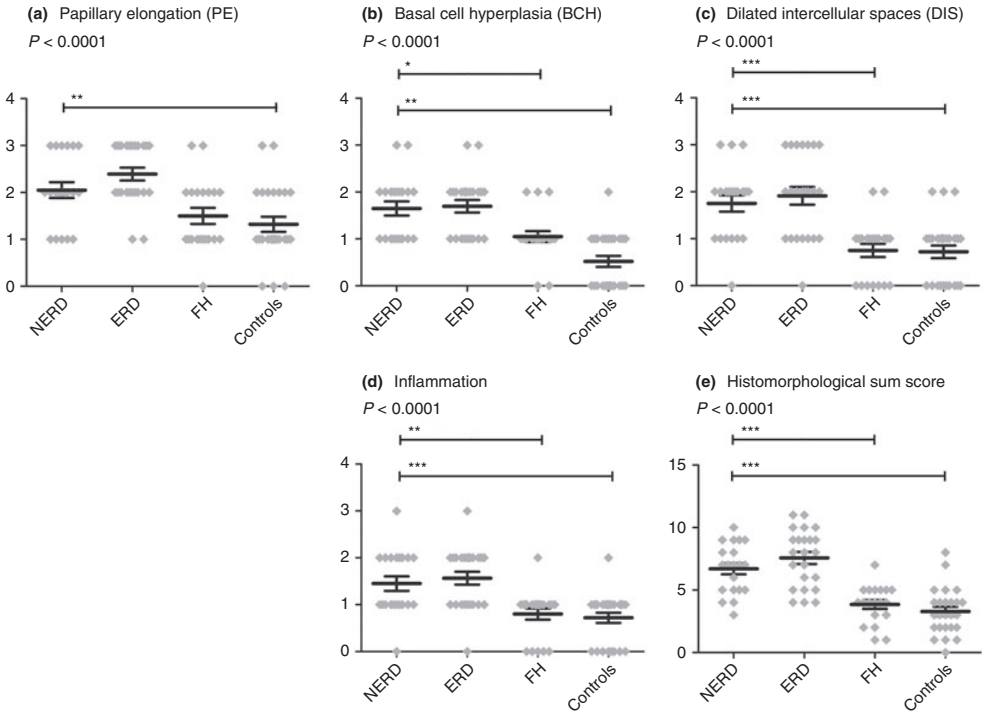

Figure 3 | Histopathological analysis and differentiation. Each histopathological feature is displayed as single values including bars representing mean ± S.E. One-way ANOVA revealed significant differences ($P < 0.0001$) for each item. *Post hoc* analysis calculated most distinct differences between NERD and FH for DIS, inflammation and histomorphological sum score [$P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$(***)]. For additional information, see Table 2.

item as asymptomatic controls. Mean values for each histopathological item and statistical results are shown in Table 2.

As histopathological evaluation revealed also minor changes in semi-quantitative scoring of controls, we decided to calculate a histomorphological sum score. The sum score adds the single values of each individual histopathological feature.

By using this sum score, we were able to differentiate patients with FH from patients with NERD with high statistical significance ($P < 0.0001$; Figure 3e). Again, the values of the histopathological sum score for FH were comparable to those of controls.

The subgroup of NERD includes also patients with oesophageal hypersensitivity (EH) ($n = 10$). These patients had normal oesophageal acid exposure, but positive symptom association. Figure 4 shows the histomorphological findings of patients with NERD separately from EH compared to patients with FH and controls. DIS and the sum score (Figure 4a,b) distinguish EH from FH and controls with statistical significance. For PE as well as BCH, the differences in EH compared with FH were not significant.

For histopathological discrimination between NERD and FH, we performed a receiver-operated model with an area under the ROC curve of 0.83 (95% CI 0.70–0.96, $P = 0.0005$). Using a cut-off value of ≥5 for the discrimination of NERD and FH, sensitivity was 0.85 (95% CI 0.62–0.97) and specificity was 0.64 (95% CI 0.40–0.84), with a positive predictive value of 0.71 (95% CI 0.49–0.87) and negative predictive value 0.8 (95% CI 0.52–0.96) (Table 3).

Assuming a relationship of histomorphological changes and reflux parameters, a weak correlation of DIS was found for AET (r: 0.29, $P = 0.04$), reflux (acid) episodes in pH-metry (r: 0.32, $P = 0.02$) and gas acid

Table 2 | Histopathological characteristics in relation to diagnosis

	Controls	FH	NERD	ERD	*P*-value ANOVA *Bonferroni's*
Papillary elongation	1.32 ± 0.16	1.5 ± 0.17	2.05 ± 0.17	2.39 ± 0.14	$P < 0.0001$
NERD vs. ERD					N.S.
NERD vs. FH					N.S.
NERD vs. controls					$P < 0.01$
ERD vs. FH					$P < 0.01$
ERD vs. controls					$P < 0.001$
FH vs. controls					N.S.
Basal cell hyperplasia	0.52 ± 0.12	1.05 ± 0.11	1.65 ± 0.15	1.7 ± 0.13	$P < 0.0001$
NERD vs. ERD					N.S.
NERD vs. FH					$P < 0.05$
NERD vs. controls					$P < 0.001$
ERD vs. FH					$P < 0.01$
ERD vs. controls					$P < 0.001$
FH vs. controls					N.S.
Dilated intercellular spaces	0.72 ± 0.14	0.75 ± 0.14	1.75 ± 0.18	1.91 ± 0.19	$P < 0.0001$
NERD vs. ERD					N.S.
NERD vs. FH					$P < 0.001$
NERD vs. controls					$P < 0.001$
ERD vs. FH					$P < 0.001$
ERD vs. controls					$P < 0.001$
FH vs. controls					N.S.
Inflammation	0.72 ± 0.11	0.80 ± 0.12	1.45 ± 0.15	1.57 ± 0.14	$P < 0.0001$
NERD vs. ERD					N.S.
NERD vs. FH					$P < 0.01$
NERD vs. controls					$P < 0.001$
ERD vs. FH					$P < 0.001$
ERD vs. controls					$P < 0.001$
FH vs. controls					N.S.
Sum score	3.28 ± 0.37	3.84 ± 0.35	6.7 ± 0.45	7.57 ± 0.37	$P < 0.0001$
NERD vs. ERD					N.S.
NERD vs. FH					$P < 0.001$
NERD vs. controls					$P < 0.001$
ERD vs. FH					$P < 0.001$
ERD vs. controls					$P < 0.001$
FH vs. controls					N.S.

The table shows the evaluation of each histomorphological feature as mean ± standard error (S.E.). Statistical analysis was performed using One-way ANOVA for comparisons among the groups and Bonferroni's analysis for multiple testing as *post hoc* analysis.

FH, functional heartburn; NERD, non-erosive reflux disease; ERD, erosive reflux disease; N.S., not significant.

episodes in MII-pH (r: 0.44, $P = 0.004$). The correlation analysis for histopathological sum score did not reveal any other association than with gas acid episodes (r: 0.46, $P = 0.003$).

DISCUSSION

Recent data from a multicentric study demonstrated the limited value of the PPI test and of questionnaires to identify patients with GERD diagnosed by wireless capsule pH-metry.[3] Noncorrectly diagnosed NERD may explain the low success rate of PPI therapy in achieving complete symptom relief.[26] Therefore, a clear distinction between NERD from FH may help predict the therapeutic success of PPI therapy.

The aim of our study was to differentiate NERD from FH by standard light microscopy. We found significant histomorphological abnormalities of the oesophageal mucosa in patients with NERD as well as ERD, but not in FH and controls. By light microscopy, we were able to distinguish patients with FH from patients with NERD in PPI-refractory heartburn. Unlike electron microscopy, light microscopy permits describing additional morpho-

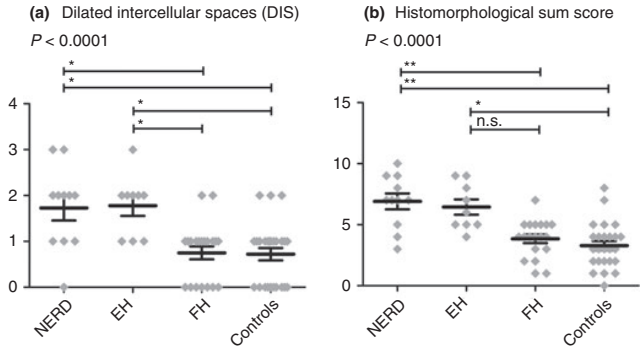

Figure 4 | Histopathological analysis of NERD, EH and FH. EH and NERD were analysed separately in comparison with FH. Each histopathological feature is displayed as a single value including a bar representing mean ± S.E.

Table 3 | Discrimination between NERD and FH by using the histomorphological sum score (cut-off value ≥5)

Histomorphological sum score (cut-off: ≥5)		95% CI
Sensitivity	85%	0.62–0.97
Specificity	63%	0.38–0.84
Positive predictive value (PPV)	71%	0.49–0.87
Negative predictive value (NPV)	80%	0.52–0.96

According to ROC analysis, sensitivity and specificity of the histomorphological sum score for the discrimination of NERD and FH were calculated with a cut-off value of ≥5 (chi-squared test, $P = 0.002$).

logical changes to DIS. However, the diagnostic utility of LM in daily practice is limited and controversially discussed.[16]

The prevalence of DIS in patients with documented GERD in our study was comparable to results from a study using TEM.[17] Because we detected mild changes with low scoring for DIS in GERD-negative controls, we included additional morphological changes such as BCH, PE and inflammatory changes to calculate a sum score. This indeed allowed better discrimination between NERD and FH. The accuracy in distinguishing NERD from FH with a cut-off value ≥5 was good (Table 3) and comparable to the results recently published by Savarino *et al.*[27] Thus, our results reinforce the statement that oesophageal biopsies may provide additional value in the differential diagnosis of endoscopy negative patients with heartburn.

In our study, only one experienced and dedicated GI pathologist evaluated histopathological changes. However, the interobserver value between two expert GI pathologists for microscopic oesophagitis in GERD has been reported to be excellent in two previous studies ($k = 0.88$).[27, 10]

In our study, DIS were associated with acid reflux parameters by means of acid exposure time, number of acidic reflux episodes as well as acidic gas reflux episodes. In several studies, DIS was induced by perfusion of acid and other components such as bile acids.[11, 15] In these studies, the presence of DIS seems not to be directly involved in symptom perception, but it is still a marker for oesophageal damage and impaired mucosal integrity due to abnormal gastro-oesophageal reflux.[14] Mechanisms of pain generation within oesophageal mucosa need further elucidation. Theses mechanisms involve mucosal inflammation,[28, 29] in particular neuroinflammation.[30, 31] Certainly, there are components of the refluxate other than acid that interact with mucosal receptors such as proteinase-activated receptors.[32–34]

In our patients, we did not collect information about the effect of antisecretory therapy on oesophageal histomorphology. Calabrese *et al.* report about complete recovery of DIS assessed by TEM after PPI therapy in patients with GERD.[35] In the LOTUS trial, DIS and other histomorphological changes analysed by LM showed recovery after antireflux surgery as well as medical treatment with PPI.[36]

In conclusion, our study provides further indications that histopathological assessment of DIS, BCH and PE

including a simple histological sum score contributes to the distinction between NERD and FH. In line with previous studies from animal models and humans, DIS were found to be associated with acid reflux parameters in MII-pH analysis. Thus, our results reinforce that oesophageal biopsies may provide additional value in the differential diagnosis of patients with PPI-refractory symptoms.

AUTHORSHIP

Guarantor of the article: None.

Author contributions: AK: study design, endoscopy, tissue and data collection, MII-pH, drafting and review of the manuscript. DJ: tissue processing, histological analysis, drafting and review of the manuscript. CC: MII-pH analysis review of the manuscript. JW: endoscopy; MII-pH. TW: data collection, statistics and discussion of the data, important intellectual input, review of the manuscript. KM: endoscopy, important intellectual impact, review of the manuscript. PM: study design, review of the manuscript. All authors approved the final version of the manuscript.

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Publikation IV

Clinical Gastroenterology and Hepatology 2015;■:■–■

Esophageal Intraluminal Baseline Impedance Differentiates Gastroesophageal Reflux Disease From Functional Heartburn

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Q5 **BACKGROUND & AIMS:** **Mucosal integrity can be assessed in patients with gastroesophageal reflux disease (GERD) by measuring intraluminal baseline impedance (BI). However, it is not clear whether BI is abnormal in patients with functional heartburn (FH), or can be used to distinguish them from patients with GERD. We compared differences in BI between patients with FH vs GERD.**

METHODS: **We performed a prospective study of 52 patients (16 men; mean age, 55 y; range, 23–78 y) seen at a tertiary university hospital from February 2009 through December 2012. Thirty-five patients had GERD (19 had nonerosive reflux disease [NERD], 16 had erosive reflux disease [ERD]) and 17 had FH. All patients discontinued proton pump inhibitor therapy and then underwent esophagogastroduodenoscopy and multichannel intraluminal impedance and pH monitoring. BI was assessed at 3, 5, 7, 9, 15, and 17 cm proximal to the lower esophageal sphincter in recumbent patients. Biopsy specimens were taken from 3 cm above the gastroesophageal junction; histology analysis was performed to identify and semiquantitatively score (scale, 0–3) dilated intercellular spaces.**

RESULTS: **Baseline impedance in the distal esophagus was significantly lower in patients with NERD or erosive reflux disease (ERD) than FH ($P = .0006$). At a cut-off value of less than 2100 Ω, BI measurements identified patients with GERD with 78% sensitivity and 71% specificity, with positive and negative predictive values of 75%. Also in the proximal esophagus, reduced levels of BI levels were found only in patients with ERD. There were negative correlations between level of BI and acid exposure time ($r = -0.45$; $P = .0008$), number of acidic reflux episodes ($r = -0.45$; $P = .001$), and proximal extent ($r = -0.40$; $P = .004$). Biopsy specimens from patients with NERD or ERD had significant increases in dilation of intercellular spaces, compared with those from patients with FH; there was an inverse association between dilated intercellular spaces and BI in the distal esophagus ($r = -0.28$; $P = .06$).**

CONCLUSIONS: **Measurement of BI in the lower esophagus can differentiate patients with ERD or NERD from patients with FH (78% sensitivity and 71% specificity), and therefore should be considered as a diagnostic tool for patients with proton pump inhibitor–refractory reflux. Low levels of BI are associated with increased exposure to acid and dilation of intercellular spaces, indicating that BI is a marker of mucosal integrity.**

Q6 *Keywords:* MII-pH; Esophageal Mucosa; Acid-Suppressive Therapy; Diagnosis.

Q7 Q8 Q9 The esophageal squamous epithelium is a tight protective barrier against luminal components. Disruption of this epithelial defense is a common phenomenon in gastroesophageal reflux disease (GERD), even in the absence of lesions visible at endoscopy (nonerosive reflux disease [NERD]). Microscopic alterations and dilation of cell–cell contacts usually are found in GERD and are associated with impaired mucosal integrity.[1–3] In addition, in NERD, altered microscopic architecture with dilated intercellular spaces has been

Abbreviations used in this paper: AET, acid exposure time; BI, baseline impedance; CI, confidence interval; DIS, dilated intercellular spaces; ERD, erosive reflux disease; FH, functional heartburn; GERD, gastroesophageal reflux disease; LES, lower esophageal sphincter; MII-pH, impedance pH monitoring; NERD, nonerosive reflux disease; PPI, proton pump inhibitor; SAP, symptom association probability; SI, symptom index.

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linked to impaired transepithelial permeability in several functional studies.[4] Besides the measurements of transepithelial electrical resistance and permeability in Ussing chambers, impaired mucosal integrity has been associated with in vivo assessment of esophageal baseline impedance (BI).[5,6] In a rabbit model, perfusion with acidified solution was found to reduce BI levels, which persisted even beyond the end of perfusion. Ex vivo, BI levels correlated with transepithelial electrical resistance in Ussing chambers and with dilated intercellular spaces.[5] Patients with GERD have lower BI impedance levels compared with asymptomatic controls as well as with symptomatic patients with normal acid exposure of the distal esophagus. Furthermore, these changes have normalized with increasing BI levels after acid-suppressive therapy.[6]

In clinical practice, NERD is the most frequently diagnosed entity of GERD but poses a diagnostic challenge to conditions that are not GERD related (ie, functional heartburn [FH]).[7] In case of a normal pH-impedance analysis without symptom association, the diagnosis most likely is FH with no or only a weak response to acid-suppressive therapy.[8] Several studies have addressed the assessment of morphologic changes in esophageal mucosa and were able to distinguish NERD from FH by using transmission electron microscopy as well as standard histopathologic evaluation.[9–11]

To date, functional investigations to assess intramucosal BI levels showing impaired mucosal integrity with respect to histomorphologic alterations have not been performed to differentiate FH from GERD. The aim of our study was to assess BI levels in patients with FH and to differentiate them from GERD. We further aimed to evaluate histomorphologic alterations such as dilated intercellular spaces (DIS) to correlate with BI levels as a parameter of mucosal electrical conductivity and integrity.

Methods

Study Subjects and Study Protocol

Fifty-two consecutive patients (16 men, 36 women; age, 55 y; [23–78]) were referred to our outpatient department and functional gastrointestinal laboratory and investigated for typical reflux symptoms (heartburn and acid regurgitation). In this prospective study we enrolled 17 patients with FH (age, 53.8 y; [23–78 y]). By definition, these patients suffered from proton pump inhibitor (PPI)-refractory heartburn with less than 50% symptom improvement and a past medical history of a PPI double standard dose for at least 6 weeks. Diagnostic criteria for FH were a normal endoscopic appearance of the gastroesophageal junction in combination with normal acid exposure time without any symptom association (negative symptom index [SI] and symptom association probability [SAP]) (see later). In addition, 16 patients with erosive reflux disease (ERD) (age, 53.8 y; [23–78 y]) and 19 patients with NERD (age, 64.9 y; [56–72 y]), including patients with esophageal hypersensitivity, were investigated.

All patients were interviewed and clinically characterized before planning further diagnostic steps. The patients were asked to taper and stop potential acid-suppressive medication for at least 3 weeks before endoscopy and impedance pH monitoring (MII-pH) to minimize effects of potential acid hypersecretion on BI levels and histology. Symptoms were recorded using the validated reflux disease questionnaire translated into German,[19] and all patients were scheduled to be investigated endoscopically and by MII-pH monitoring on the same day.

The study protocol was performed according to the Declaration of Helsinki and approved by the local ethical committee. Eligible patients (>18 y) were included after providing informed consent. None of the patients had an esophagogastroduodenoscopy or functional diagnostics previously, but all patients had heartburn as a typical GERD symptom based on the Montreal classification.[12] Previous upper gastrointestinal surgery, alarm symptoms, gastric or duodenal ulcer disease, Barrett's esophagus, or esophageal motility disorders were considered exclusion criteria.

Upper Gastrointestinal Endoscopy and Esophageal Biopsy Specimens

After an overnight fast, all patients underwent an esophagogastroduodenoscopy under intravenous conscious sedation using midazolam (Dormicum V 5 mg/mL; Roche Deutschland Holding GmbH, Penzberg, Germany) and/or 1% propofol (Propofol-Lipuro 10 mg/mL; Braun Melsungen AG, Melsungen, Germany) with a standard videogastroscope (GIFQ180; Olympus Optical Europe, Hamburg, Germany).

Endoscopic esophageal landmarks were defined as the gastroesophageal junction, with the beginning of the gastric folds and the Z-line as the squamocolumnar junction and diaphragmatic pinch. In the distal esophagus, 2 esophageal biopsy specimens were taken from 3 to 5 cm above the gastroesophageal junction, not including visible changes (no erosions), and immediately transferred to 4% neutral-buffered formalin for later embedding in paraffin.

Combined 24-Hour Impedance pH Monitoring and Assessment of Intraluminal Baseline Impedance Levels

After endoscopy, the MII-pH catheter (Sandhill Scientific, Highland Ranch, CO) was inserted and located with esophageal pH electrodes 5 cm above the gastroesophageal junction (lower esophageal sphincter [LES]). Manometry was not performed in all patients to localize the LES. In a subset of patients, localization of the LES and placement of the MII-pH catheter was performed

Table 1. Comparison of Symptoms and 24-Hour MII-pH in Patients With FH and Patients With GERD

	ERD	NERD	FH	*P* value
RDQ heartburn, mean ± SE	1.8 ± 0.4	2.0 ± 0.3	1.5 ± 0.3	NS
RDQ regurgitation, mean ± SE	2.1 ± 0.4	1.7 ± 0.3	1.9 ± 0.3	NS
RDQ dyspepsia, mean ± SE	1.6 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	NS
AET, %	6.1 ± 1.8	5.1 ± 1.0	0.8 ± 0.2	.008
Reflux episodes (pH)	37.1 ± 7.0	34.9 ± 6.9	10.9 ± 2.6	.002
Esophageal acid percentage time (MII)	1.5 ± 0.3	1.7 ± 0.3.0	0.7 ± 0.2	.02
Acidic reflux events (MII)	37.5 ± 5.8	37.3 ± 5.4	16.4 ± 5.2	.01
Proximal acidic reflux events (MII)	25.3 ± 4.6	21.2 ± 3.4	8.0 ± 2.2	.004

RDQ, reflux disease questionnaire.

after endoscopy by using a transversion factor of 4 cm,[13] noting that there is a difference of 0.9 cm between the endoscopically determined squamocolumnar junction and manometrically detected LES.[14]

Esophageal impedance electrodes were located at 3, 5, 7, 9, 15, and 17 cm above the LES. Patients were asked to eat 3 meals and beverages at fixed times and not to lie down in the supine position during the day. Event markers were set for meal times, body posture, and the occurrence of specific symptoms. Manual analysis of the tracings was performed by 2 experienced operators (A.K. and C.C.) independently. In case of disagreement, the case was discussed with a third experienced investigator (J.W.).

Reflux episodes were defined as a decrease of more than 50% from baseline impedance moving from the distal to the proximal extend. They were characterized as liquid, mixed, or gas, and by the pH of the refluxate. Acid exposure time (AET) was defined as abnormal when a pH less than 4 was measured for more than 4.2% of the time over 24 hours. SAP and SI were assessed as previously described for acid, weakly acidic, and weakly alkaline reflux events.[15,16] NERD was diagnosed if no erosions were visible endoscopically but AET was increased (>4.2%). Patients with a normal AET and a positive SI or SAP have a hypersensitive esophagus and were considered to have a diagnosis of NERD according to the Rome III criteria.[6] A normal endoscopic appearance of the gastroesophageal junction in combination with a normal AET without any symptom association (negative SI and SAP) was considered FH.

BI levels were assessed at 3, 5, 7, 9, 15, and 17 cm above the lower esophageal sphincter. Swallows and reflux-induced changes were excluded. In the recumbent position at night, we identified a period with a stable and constant BI signal without any severe changes or interference over time. During these periods the BI levels were analyzed for at least 30 minutes.

Histopathologic Evaluation

Esophageal tissue specimens were embedded in paraffin and submitted for histopathologic examination with H&E and periodic acid–Schiff staining. The degree of basal cell hyperplasia, presence of papillary elongation, and dilated intercellular spaces was assessed[10,23,24] and semiquantitatively scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), as described previously.[10,17] The expert pathologist (D.J.) was blinded to endoscopic data and results from MII-pH.

Data Collection and Statistical Analysis

All collected data were entered in an Excel sheet (Microsoft Corporation, Redmond, WA) and statistically analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Data are expressed as mean ± SE or 95% confidence intervals (CIs), if not stated otherwise. One-way analysis of variance was applied for comparisons among the groups (FH, NERD, and ERD). If significant differences were identified, Bonferroni analysis for multiple testing for post hoc analysis was performed to calculate differences between the histopathologic items. Correlation analysis was performed using the Pearson correlation test. All tests were 2-sided with a *P* level less than .05 indicating significance.

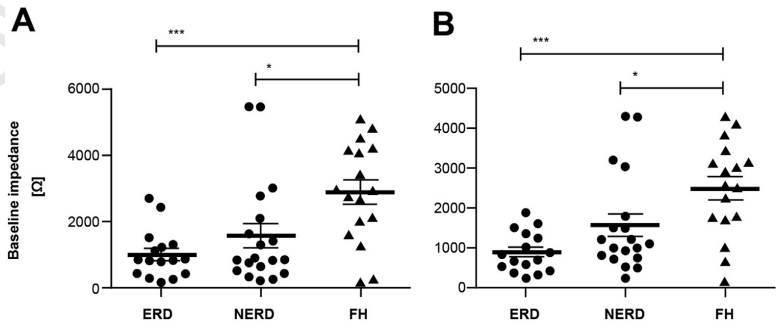

Figure 1. Distal BI levels at (*A*) 3 cm and (*B*) 5 cm above the LES in patients with ERD, NERD, and FH. *< .01; ***< .0001.

Table 2. Comparison of BI Levels in Patients With FH, NERD, and ERD at 3, 5, 15, and 17 cm Above the LES

	ERD	NERD	FH	*P* value
Baseline impedance 3 cm Ω, mean ± SE	994.0 ± 182.2	1558 ± 362.3	2884 ± 364.8	.0006
Baseline impedance 5 cm Ω, mean ± SE	880.1 ± 124.7	1555 ± 281.4	2489 ± 288.2	.0002
Baseline impedance 15 cm Ω, mean ± SE	1307 ± 231.4	2514 ± 260.8	2649 ± 187.5	.0003
Baseline impedance 17 cm Ω, mean ± SE	2412 ± 352.8	3482 ± 325.8	3879 ± 410.8	.0003

We performed a receiver operating characteristic analysis for distal baseline impedance at 3 cm above the LES. Sensitivity, specificity, and predictive values for the discrimination between NERD and FH were calculated with a cut-off value of less than 2100 Ω using the Fisher exact *t* test.

Results

Twenty-Four–Hour Impedance pH Monitoring and Patient Characteristics

According to the reflux disease questionnaire, there were no differences in symptom severity between the groups for either heartburn, regurgitation, or dyspepsia.

Results from MII-pH monitoring are shown in Table 1, which summarizes the most important parameters in the distinction between ERD, NERD, and FH. Within NERD patients, 10 patients had no pathologic AET but a positive symptom association (esophageal hypersensitivity).

Intraluminal Baseline Impedance Analysis in the Distal and Proximal Esophagus

BI levels in the distal esophagus at 3 and 5 cm above the LES differed significantly between FH and GERD (Figure 1). At 3 cm, BI levels of ERD (994.0 ± 182.2 Ω) and NERD (1558 ± 362.3 Ω) were significantly lower than in patients with FH (2884 ± 364.8 Ω) ($P = .0006$). Similar measurements were obtained at 5 cm above the LES (Table 2), but not for the more proximal impedance electrodes of the catheter.

In the proximal esophagus at 15 and 17 cm above the LES, BI levels of only patients with ERD were significantly lower ($P = .02$–$.0003$). No differences in BI levels were obtained between NERD and FH (Table 2).

Correlation analyses of BI levels in the distal esophagus with functional parameters from MII-pH measurements showed significant associations of low BI levels with parameters of acidic reflux. Exemplary displayed for BI at 3 cm, we found a negative correlation with AET (r = -0.45; $P = .008$) and esophageal acid percentage time defined by MII (r = -0.45; $P = .001$), with the numbers of acidic reflux episodes (r = -0.45; $P = .001$) and numbers of proximal reflux episodes (r = -0.4; $P = .003$) (Figure 2).

For discrimination between NERD and FH, we performed a receiver-operated model with an area under the receiver operating characteristic curve of 0.73 ± 0.09 (95% CI, 0.55–0.91; $P = .01$). With a cut-off value of less than 2100 Ω for discriminating NERD from FH, sensitivity was 0.79 (95% CI, 0.54–0.94) and specificity was 0.71 (95% CI, 0.44–0.90); with a positive negative predictive value of 0.75 (95% CI, 0.51–0.91) as well as a negative predictive value 0.75 (95% CI, 0.48–0.93) (Table 3).

Histopathologic Evaluation of Dilated Intercellular Spaces Correlating With Baseline Impedance

Histologically, patients with FH had less dilated intercellular spaces than NERD and ERD (Figure 3*A*).

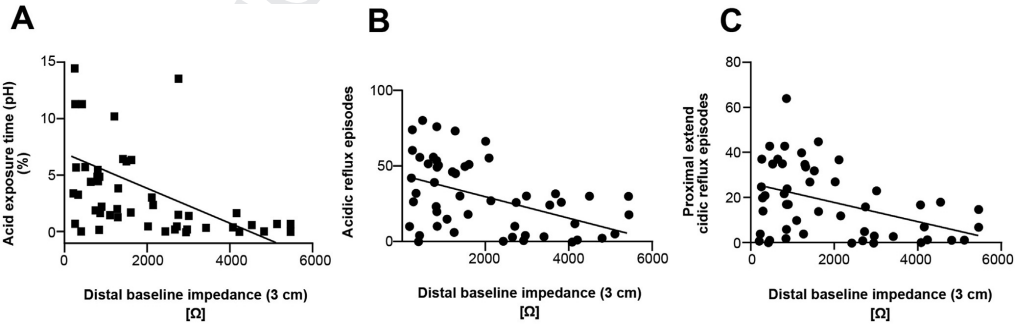

Figure 2. Negative association of distal baseline impedance levels at 3 cm (Ω) and (*A*) acid exposure time (r = -0.45; $P = .008$), (*B*) acidic reflux episodes (r = -0.45; $P = .001$), and (*C*) numbers of proximal reflux episodes (r = -0.4; $P = .003$).

Table 3. Discrimination of Patients With NERD From FH by Assessment of Distal Baseline Impedance Levels at 3 cm Above LES

Distal baseline impedance (3 cm above LES), cut-off < 2100 Ω		95% CI
Sensitivity	78 %	0.54–0.94
Specificity	71%	0.44–0.90
Positive predictive value (PPV)	75%	0.51–0.91
Negative predictive value (NPV)	75%	0.48–0.93

NOTE. The sensitivity and specificity for the discrimination between NERD from FH was calculated with a cut-off value of less than 2100 Ω according to receiver operating characteristic curve analysis (Fisher exact *t* test, $P = .006$).

Furthermore, low BI levels correlated with DIS evaluated with standard light microscopy that marginally missed statistical significance (r = -0.28; $P = .06$) (Figure 3*B*).

Discussion

Assessment of BI levels allows us to distinguish patients with FH from patients with GERD. Our findings confirm previous observations of low BI levels in patients with GERD including NERD,[6,18,19] but there are very few publications regarding basal impedance in FH. Vaezi et al developed an impedance probe that determines mucosal baseline impedance by direct contact of the probe with the mucosa during endoscopy. They found higher impedance levels in patients with a normal endoscopic appearance and normal acid exposure time during pH-metry.[20] Placed under endoscopic guidance, direct mucosal impedance is likely to be comparable with measurement of BI levels as conducted in our study. However, the distance between the electrodes and the size of the electrical field certainly has an impact on the measurement. The size of the electrical field is dependent on the distance between the electrodes. With a larger distance between impedance electrodes on the conventional MII-pH catheter, the electrical field of BI measurements might be enlarged and therefore often is speculated to measure impedance signals that are influenced by deeper layers of the esophageal wall. However, based on structural components of the mucosa (ie, tight junctions) forming a tight barrier for electrodes, the mucosal contribution to BI levels probably is most important for the impedance signal. Both methods have their specific limitations (ie, localization, pressure, and time of contact), but direct comparisons of both methods have not been performed.

Recently, Martinucci et al[21] described higher BI levels in patients with characteristics of FH as well. Impaired BI levels in the distal esophagus have been associated with parameters of acidic reflux in MII-pH in our study. Again, this was similarly reported by other groups for patients with GERD.[6,22–24] These publications clearly showed a more significant decrease of BI levels associated with more moderate to severe reflux episodes and also with more severe forms of esophagitis shown by the direct impedance technique.[20]

Similar to the BI levels in our study, Woodland et al[22] described comparable low BI levels in the distal esophagus of patients with NERD (1669 ± 182 Ω), but not with FH (2384 ± 211 Ω). In addition, Woodland et al[22] described slower impedance recovery rates in patients with NERD when compared with FH.

Although our study cohort included a rather small sample size, we calculated a cut-off level of less than 2100 Ω for differentiation between NERD and FH with a sensitivity and specificity of 78% and 71%, respectively. Including all patients with GERD (NERD as well as ERD), the sensitivity and specificity were 83% and 71%, respectively (data not shown). Therefore, analysis of the BI level should be considered a useful additional parameter during MII-pH analysis in the differential diagnosis of NERD and FH.

Morphologically, DIS was associated with ERD as well as NERD, but not with FH. Direct assessment of impaired mucosal integrity includes measurement of increased paracellular permeability, which also is associated with the presence of DIS,[3,25] as well as impaired mucosal integrity, which was linked functionally to reduced BI levels and was induced by acidic perfusion in a rabbit model and in healthy volunteers.[5] PPI therapy increases baseline impedance levels as well as microstructural changes of dilated intercellular spaces, a further

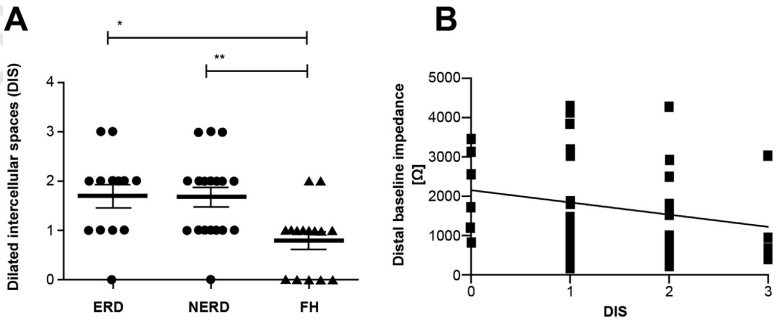

Figure 3. Assessment of (*A*) DIS by light microscopy, and a (*B*) negative association with baseline impedance levels (r = -0.28; $P = .06$). *$P < .01$; **$P < .001$.

indication for acidic-induced abnormalities.[6] Our study also shows the differences of BI levels in the proximal esophagus. This was significant for patients with ERD when compared with FH. Structural changes of the esophageal mucosa also can be induced by infusion with acidic solutions even in the proximal esophagus.[5,26,27] Because measurements were assessed at 15 and 17 cm above the LES these abnormalities were unlikely to be associated with mucosal erosions.

Recently, in patients with GERD, low baseline impedance values were described to be associated with dilated intercellular spaces by light microscopy and with the expression of claudin-1 and occludin.[28] Both proteins are involved in the structural integrity of tight junctions and were found to be increased in patients with GERD.[17,29]

What actually causes changes of BI levels needs to be investigated further. Besides structural changes with increased paracellular conductivity, inflammatory changes also need to be considered. We did not find an association with histopathologic assessment of inflammatory cell infiltration, although inflammation-related mucosal edema needs to be discussed as a possible explanation for altered baseline impedance as well. We further showed a significant association of low BI with parameters of acidic reflux and with dilated intercellular spaces in light microscopy.

Impaired mucosal integrity assessed by BI levels is a characteristic finding in patients with GERD, but not in patients with FH. These results provide us with a greater insight into the pathophysiological differences between the entities. Although the sole coincidence of reflux-associated mucosal abnormalities needs to be considered, induced paracellular conductivity in the presence of DIS to date is the most likely explanation for low BI levels in patients with GERD.

In conclusion, we found that patients with FH can be distinguished from patients with GERD, in particular NERD, based on baseline impedance levels. Based on the test parameters calculated in our study, the measurement of BI as a single parameter does not allow us to adequately differentiate patients with FH from patients with GERD. The increasing number of PPI-refractory patients has lead to growing medicoeconomic efforts and costs, and thus accurate differential diagnosis is crucial. Measurement of BI levels is therefore a complementary tool in addition to endoscopy, conventional reflux monitoring, and esophageal biopsies. In difficult cases all methods should be considered for an accurate diagnosis to guide further adequate therapeutic management.

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Conflicts of interest
The authors disclose no conflicts.

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RESEARCH ARTICLE Open Access

Role of tight junction proteins in gastroesophageal reflux disease

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Abstract

Background: Gastroesophageal reflux disease (GERD) is associated with impaired epithelial barrier function that is regulated by cell-cell contacts. The aim of the study was to investigate the expression pattern of selected components involved in the formation of tight junctions in relation to GERD.

Methods: Eighty-four patients with GERD-related symptoms with endoscopic signs (erosive: n = 47) or without them (non-erosive: n = 37) as well as 26 patients lacking GERD-specific symptoms as controls were included. Endoscopic and histological characterization of esophagitis was performed according to the Los Angeles and adapted Ismeil-Beigi criteria, respectively. Mucosal biopsies from distal esophagus were taken for analysis by histopathology, immunohistochemistry and quantitative reverse-transcription polymerase chain reaction (RT-PCR) of five genes encoding tight junction components [Occludin, Claudin-1, -2, Zona occludens (ZO-1, -2)].

Results: Histopathology confirmed GERD-specific alterations as dilated intercellular spaces in the esophageal mucosa of patients with GERD compared to controls ($P < 0.05$). Claudin-1 and −2 were 2- to 6-fold upregulation on transcript ($P < 0.01$) and in part on protein level ($P < 0.015$) in GERD, while subgroup analysis of revealed this upregulation for ERD only. In both erosive and non-erosive reflux disease, expression levels of Occludin and ZO-1,-2 were not significantly affected. Notably, the induced expression of both claudins did not correlate with histopathological parameters (basal cell hyperplasia, dilated intercellular spaces) in patients with GERD.

Conclusions: Taken together, the missing correlation between the expression of tight junction-related components and histomorphological GERD-specific alterations does not support a major role of the five proteins studied in the pathogenesis of GERD.

Keywords: Gastroesophageal reflux disease, Tight junction, Claudins, Esophagitis, Inflammation

Background

Gastroesophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders in the world [1,2]. Based on endoscopic findings GERD is differentiated in erosive (erosive reflux disease or ERD), non-erosive reflux disease (NERD) and Barrett's esophagus (BE) [3,4]. ERD is characterized by endoscopic visible breaks of esophageal mucosa integrity and classified according to various endoscopic classifications, most recently the Los Angeles classification [5,6]. However, two thirds of patients with typical GERD symptoms do not exhibit visible mucosal changes in conventional esophagogastroduodenoscopy (EGD) and are thus diagnosed as having NERD [6,7]. Although histology is not used in clinical practice for GERD diagnosis, frequent histological changes as basal cell hyperplasia, elongation of the papilla, inflammatory infiltrates and dilatation of the intercellular spaces are observed in the distal esophagus of patients with both ERD and NERD [8-11]. Dilations of the intercellular spaces (ICS) are characteristic changes of the esophageal mucosa of patients with ERD and NERD. ICS were described by various others using electron microscopy and are even characterized by light microscopy. This feature is being more widely proposed as an additional morphological feature of acid-

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induced damage to the squamous epithelium [10,12-14]. The widened ICS are supposed to permit the diffusion of molecules to the lamina propria where sensory nerve endings are located [15]. Therefore, ICS dilation even in the absence of endoscopically visible mucosal damage may explain the occurrence of symptoms in patients with NERD [16,17]. Furthermore, recent studies have provided evidence that the impaired barrier function of esophageal mucosa is a "hallmark" of GERD [18-20]. The integrity of epithelial surfaces is based on various cell-cell contacts that provide the structural basis for barrier function by regulating the diffusion of molecules and sorting of transmembrane proteins to apical and basolateral surfaces. Tight junctions, adherens junction and desmosomes are the three major structural units mediating barrier and sorting function [21,22]. Their structural composition, general functions, and pathophysiological relevance have been reviewed extensively by others [21,23,24]. In line with the current concept in GERD, the role of molecules contributing to cell-cell contacts in esophageal mucosa in relation to GERD has been investigated in animal and human studies recently. Notably, the majority of studies were focused on the role of tight junction molecules (e.g. Claudin-2, -3, -4, -7 and −18) in Barrett's metaplasia and carcinogenesis towards esophageal adenocarcinoma [25-30]. In regard to the other 2 endoscopic entities (ERD, NERD), distinct alterations in the expression and or localization were described for Claudins 3 and 4 in GERD-related animal and *in vitro* models [31-33]. Rat model revealed decreased expression of Claudin-3 and no change of Claudin-1 and 4 [31,32], while an *in vitro* model of esophageal-like squamous cells demonstrated a prominent role of Claudin-4 [33].

Here, we studied the expression patterns of five tight-junction related molecules (Occludin, Claudin-1, -2 and Zonula occludens-1-, 2) in the esophageal mucosa of a prospective cohort of patients with GERD as well as reflux-negative individuals. Gene expression was assessed both on transcriptional and protein level, and changes were studied in context to histopathological alterations associated with GERD.

Methods

Study design and patients' characteristics

Between 2005 and 2007, a cohort of patients with GERD and individuals lacking any symptom or endoscopic sign of GERD as GERD-negative controls were enrolled [34]. Patients with typical GERD-related symptoms based on Montreal classification [4] and patients without any reflux-related clinical symptoms undergoing EGD for screening or non-reflux dyspepsia (GERD-negative controls with a reflux disease questionnaire, RDQ score of 0) were invited to participate. All the patients underwent a detailed history and physical examination. The demographic data and endoscopic findings of the study population are presented in Table 1A and Table 1B. Written informed consent was obtained from all patients before endoscopy, after the endoscopist had explained the procedure to the patient in detail and answered all questions. The study was approved by the ethical committee of our institution and conducted according to the ethical guidelines of the declaration of Helsinki as revised in 1989.

Functional investigations such as 24 hour-pH-metry or MII-pH analysis were performed in individual cases only, and could not be included as separate parameter. The assignment of NERD was additionally based on the responsiveness to PPI therapy that was subsequently assessed.

Inclusion criteria

Female or male, age 18 to 80, able to provide written informed consent. Patients with typical reflux symptoms had to present symptoms at least three times a week. Typical reflux symptoms were defined as heartburn and regurgitation, as evaluated by the RDQ score. Patients with other types of reflux symptoms were not included in this study.

Exclusion criteria

Upper gastrointestinal pathology (e.g. peptic ulcers, cancers, polyps, and Barrett's mucosa), systemic inflammatory, neoplastic or malabsorptive diseases (e.g. Crohn's disease, ulcerative colitis, vasculitis, celiac disease), and acute medical conditions such as pneumonia, stroke, coronary ischemia and acute renal failure. Patients with known abnormal coagulation parameters and thrombocytopenia at the time of the procedure (i.e. INR > 1.2, platelet count < 80,000) were also excluded. None of the patients had taken antibiotics, or bismuth compounds or any H2-blockers or proton-pump inhibitors (PPI) in the last 2 weeks before entering the study. It is notable that the majority of patients enrolled had various anti-secretory medications in their past, and does not present GERD-naïve patients. Each patient was assigned a coded number. Histopathological assessment was done by pathologist (DK) blinded to clinical data.

Endoscopy and histopathology

The patients underwent the procedure after an overnight fast. The endoscopy was performed under conscious sedation with intravenous midazolam using a video-gastroscope (Q160, Olympus, Hamburg). Endoscopic characterization of esophagitis was performed according to the "Los Angeles classification" [35] describing the following endoscopic landmarks: gastroesophageal junction (GEJ), Z-line, beginning of the gastric folds and

Table 1 Patient groups analyzed by quantitative RT-PCR and immunohistochemistry

Quantitative RT-PCR	**Controls (n = 26)**	**NERD (n = 37)**	**ERD (n = 47)**
Sex (male/female)	6/20	6/31	31/16[#]
Age (mean, sd, range)	52.3 ± 17.6	47.0 ± 14.1	47.5 ± 15.4
	(20–79)	(18–72)	(20–79)
H. pylori-status (positive)	5/21	7/30	12/35
	(23.1 %)	(22 %)	(29.2 %)
Immunohistochemistry	**Controls (n = 12)**	**NERD (n = 13)**	**ERD (n = 16)**
Sex (male/female)	2/10	4/9	10/6[#]
Age (mean, sd, range)	46.2 ± 19.1	48.9 ± 9.5	48.6 ± 14.1
	(20–75)	(35–64)	(29–72)
H. pylori-status (positive)	4/8	2/11	7/9
	(33.3 %)	(15.4 %)	(43.8 %)

diaphragmatic pinch. The GEJ was defined as the beginning of the gastric folds, whereas the Z-line was defined as the squamocolumnar junction. The cardia was defined as the mucosa lying immediately below the GEJ.

In the distal esophagus, 3 biopsies were taken 2 cm above the squamous-columnar junction at the 3 o'clock position. In case of erosions, specimens were taken 2 cm above the tip of the erosion. One biopsy was snap-frozen in liquid nitrogen for molecular analysis. The two other biopsies were immediately fixed in 4 % neutral-buffered formalin and submitted for histopathological examinations using hematoxilin and eosin, modified Giemsa and PAS stain. In analogy to the Sydney classification for gastritis, the density of intraepithelial neutrophils/eosinophils and lymphocytes were scored to evaluation active and chronic inflammation. Furthermore, degree of basal cell hyperplasia, presence of papillary elongation and dilated intercellular spaces were semiquantitatively scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) as described previously [34]. Notably, several subgroups of the study cohort were published in regard to inflammatory mediators (e.g. cytokines, Protease-activated receptor 2) [34,36], molecules related to barrier functions [37,38], desmosomal proteins [39] and histopathological alterations [34].

Extraction of RNA and quantitative reverse transcription - polymerase chain reaction (RT-PCR) analysis of tight junction-related genes

Extraction of total RNA and cDNA synthesis were performed by the "two-step" protocol as described previously [40]. Transcript levels of Occludin, Claudin-1, -2, Zonula occludens-1, -2, and β-Actin were determined by quantitative real-time RT-PCR using an iCycler (BioRad, Munich, Germany) and the QuantiTect™ SYBR Green kit (Qiagen) using primers and standard conditions described in Table 2. Initial template mRNA amounts for all genes were calculated using iCycler software (Ct-values) and serial dilutions of plasmid DNA standard containing the corresponding PCR-fragments. Calculating template concentrations based on the Ct method and standard dilutions allowed an individual assessment of different efficiency for each PCR assay that were between 0.95 and 0.99. Gene-specific levels were normalized to the corresponding ß-actin level of the sample. Final results are expressed as arbitrary units (a.u.) and represent ratios between investigated gene and ß-Actin transcript amounts. All together, gene expression levels are identical to those calculated by the 2-ΔΔ Ct-method [41], but they are additionally adjusted to the assay-specific efficiency. Due to the primer design (usage of intron-spanning regions), amplification of genomic DNA was excluded. All amplification products were checked for their correct size by agarose gel electrophoresis. Therefore, gene expression levels (a.u.) illustrate the mRNA pool of the individual gene studied.

Immunohistochemical analysis of tight junctional components

Immunohistochemistry was performed using the avidin-biotin complex immunostaining method and the automated immunohistochemistry slide staining system by Ventana NexES (Ventana Medical System, Strasbourg, France) as described previously [36]. Details for antigen retrieval and primary antibodies are illustrated in Table 2. Dilutions of primary antibodies were determined using appropriate positive and negative controls. For negative controls, primary antibody was replaced by irrelevant rabbit IgG that did not reveal specific signals (data not shown). Immunoreactivity was assessed in 5 representative high power fields (Zeiss Axioskop 50) of each sample by one blinded pathologist (DK). For semiquantitative assessment an adaptation of a score system originally described by Remmele et al. was applied [42]. Briefly, staining intensity ([SI], 1 = weak, 2 = moderate, 3 = strong) and the percentage of positive cells ([PPC], 1 = <10%,

Table 2 Characteristics of primers, RT-PCR protocol and antibodies

	Primer sequence, length of fragment, annealing temperature	Antibody, Company, Antigen retrieval, final dilution
Occludin	fw: GGCCATTGCCATTGTACTGGG rv: GGAACCGGCGTGGATTTATAGG315 bp; 58°C	polyclonal rabbit anti-occludin antibody No. 71–1500 (Invitrogen, Carlsbad, CA, USA), Protease-retrieval, Final dilution: 1:50
Claudin-1	fw: ATGGTGGTTGGCATCCTCCTG rv: GGCCTTGGTGTTGGGTAAGAGG344 bp, 58°C	polyclonal rabbit anti-Claudin-1 antibody No. 51–9000, clone JAY.8 (Invitrogen, Carlsbad, CA, USA), EDTA-retrieval, Final dilution: 1:50
Claudin-2	fw: TCTCTTGGCCTCCAACTTGTGGG rv: GCACTGGATGTCACCATCATGGC259 bp, 60°C	polyclonal rabbit anti-Claudin-2 antibody No. 51–6100 (Invitrogen, Carlsbad, CA, USA), EDTA-retrieval, Final dilution: 1:50
ZO-1	fw: TCTGATCATTCCAGGCACTCGC rv: CCACATCTGGTTGCCAACTTGG225 bp, 58°C	polyclonal rabbit anti-ZO-1 antibody No. 61–7300, (Invitrogen, Carlsbad, CA, USA, Protease retrieval, Final dilution: 1:30
ZO-2	fw: AGAGGACACGCCGAGCAGATTG rv: TCCCGACATCATTGCCACCAG272 bp, 60°C	polyclonal rabbit anti-ZO-2 antibody No. 71–1400, (Invitrogen, Carlsbad, CA, USA, EDTA retrieval, Final dilution: 1:150
β-Actin	fw: CATGCCATCCTGCGTCTGGACC rv: ACATGGTGGTGCCGCCAGACAG400 bp, 60°C	not performed
Standard protocol	95°C: 15 min; (94°C: 30s, 58°-60°C: 30s, 72°C: 30s) 40 cycles; 72°C: 5 min	

mab: monoclonal antibody, fw: forward, rv: reverse.

2 = 10-50%, 3 = 51–80%, 4= > 80%) were scored semi-quantitatively, resulting in an immunoreactive score [IRS = SI x PC] between 0 and 12. Furthermore, a score for membranous staining (0 = none, 1 = weak, 2 = moderate, 3 = strong/complete) was added resulting in a possible maximum of 15 points for each sample.

Statistical analysis

Data are expressed as absolute number, relative proportion, median + range or mean ± standard deviation (SD) if not stated otherwise. Since the majority of data sets revealed skewed distribution, non-parametric Kruskal-Wallis test were applied for all comparisons made among the three groups (controls, NERD and ERD). If significant differences were identified ($P < 0.05$), post hoc analyses for pairwise comparisons between groups were performed using Mann–Whitney *U* test for gene expression analysis and immunohistochemistry. Age and histopathological parameters were analyzed by ANOVA and *T* test; frequencies by chi-square test. Non-parametric correlation analysis was performed by Spearman's rank correlation test to investigate potential association between gene expression levels and histomorphological changes. Correlation analyses were performed in explorative manner only; adjustment for multiple comparisons was not performed. All tests were applied two-sided with a level of significance of $P < 0.05$.

Results

Patients and GERD-specific histomorphological changes

The three groups as well as the subgroups (randomly selected for immunohistochemistry) did not differ with respect to age and *H. pylori* status (Table 1). Histomorphological alterations are shown in Table 3. Activity and chronicity scores in esophageal mucosa were slightly higher in patients with NERD or ERD *vs.* controls without reaching significance. Basal cell hyperplasia, dilated intercellular spaces and elongation of papilla were significantly increased in both endoscopic entities (Table 3).

Upregulation of tight junction-related proteins in esophageal mucosa in context to the presence of gastroesophageal reflux disease

As exemplarily demonstrated in figure 1, Claudin-1 transcript and protein levels in esophageal mucosa were significantly increased in patients with ERD, while a weaker increase was noted in NERD compared to controls. Corresponding data for the other four genes (Claudin-2, ZO-1, ZO-2; Occludin) including those of Claudin-1 are summarized in tables 4A and 4B. Claudin-2 had a similar expression pattern as Claudin-1, and both ZO-1 and ZO-2 showed a tendency to higher transcript levels in ERD and NERD (*P*-values <0.07, Table 4A). In addition to the upregulation in context to controls, both transcript levels and immunohistochemical scores of Claudin-1 were significantly higher in patients with ERD compared to those with NERD (Figure 1).

In general, higher transcript levels were accompanied by higher immunohistochemical scores for most proteins. In addition to these quantitative changes in gene expression, different patterns of protein distribution within the cell compartment and within different mucosal layers were noted (Figure 2). In controls, the expression of tight junction-related proteins was mainly observed in the basal epithelial layers and in a cytoplasmatic pattern. In GERD, expansion of protein expression to the suprabasal und spinous epithelial layers was observed. Furthermore, expression of Claudin-1, Claudin-2 and ZO-1 was partly membrane-associated with a stronger intensity in GERD compared to controls.

Table 3 Histopathological parameters

	Controls	NERD	ERD	P-value One way ANOVA
Activity	0 ± 0	0.23 ± 0.54	0.22 ± 0.41	n.s.
Chronicity	0.72 ± 0.54	0.97 ± 0.51	1.05 ± 0.67	n.s.
Basal cell hyperplasia [BSH]	0.52 ± 0.59	1.11 ± 0.63	1.42 ± 0.84	<0.001
Papillary elongation [PE]	1.32 ± 0.80	1.71 ± 0.86	2.07 ± 0.85	<0.001
Dilated intercellular spaces [ICS]	0.72 ± 0.68	1.49 ± 1.01	2.10 ± 0.13	<0.001

Parameters were scored semiquantitatively as described in "Patients and Methods". Data are presented as mean ± sd.

Increased gene expression of tight junction-related molecules (transcript level) does not correlate with histomorphological changes in esophageal mucosa

In order to study potential correlations between gene expression levels (transcript level) and the degree of histopathological alterations, all three groups were analyzed together in the first step. As exemplarily illustrated in figure 3, gene expression levels of Claudin-1 and Claudin-2 marginally correlated with the degree of basal cell hyperplasia, but not with dilated intercellular spaces and length of papilla (data not shown). Since most analyses were negative, these data are summarized in Table 5A for all five genes. Since basal cell hyperplasia revealed some even weak correlations in the complete study cohort, these correlation analyses were performed again for all three groups individually and for patients with GERD (NERD + ERD) combined. Only 3 out of 20 subanalyses revealed marginally significant correlations (without adjustment for multiple comparisons), two of those were identified in controls (data summarized in Table 5B).

In addition to the correlation based on transcript levels (Figure 3, Table 5), correlation analysis between protein expression levels (immunohistochemical scores, Table 4B) and histopathological alterations (Table 3) was performed. Here, only one significant correlation (between Claudin-1 and activity of inflammation, $r = 0.51$, $P < 0.01$) was identified (data not shown).

Discussion

In this study, we demonstrated (I) distinct expression patterns of five genes encoding for proteins involved in the formation of tight junctions in esophageal mucosa. In particular Claudin-1 in ERD and to lesser extent Claudin-2 was expressed at higher levels in patients with GERD. In contrast, ZO-1, ZO-2, and Occludin were not affected by the presence of GERD. (II) In general, altered gene expression of Claudin-1/-2 did not correlate with the degree of histomorphological changes in the esophageal mucosa of patients with GERD.

Tight junctions are composed of transmembrane proteins such as Occludin, 24 Claudins, several junctional adhesion molecules (JAMs) with different isoforms, E-Cadherin as well as cytosolic binding partners [43,44]. The selection of the five genes studied was based on functional aspects. Occludin is critical for the formation of tight junctions in most tissues [45]. Claudin-1 is one of the numerous Claudins that seals intercellular space leading to higher barrier function [46], while Claudin-2 is the only pore-forming member of this family resulting in increased permeability [47]. Zonula occludens (ZO)-1 and-2 are cytosolic partners of tight junctions in most epithelial surfaces [48,49]. The selected genes present important components of the tight junctional complex, and were considered to allow assessment about alterations of tight junctions in relation to GERD. A comprehensive analysis concerning the general expression pattern of other junctional proteins was not performed.

Recently, several studies demonstrated characteristic histopathological alterations in esophageal mucosa of patients with GERD and a proinflammatory response including the activation of related pathways such as NFκB, PAR-2, ROS and iNOS [50,51]. Several *in vitro* and animal studies have provided evidence that incubation of esophageal mucosa or squamous cell lines either with acidified media with/without bile acids or proinflammatory cytokines can provoke changes in transepithelial electric resistance and increased transepithelial permeability [52-55]. Notably, several studies demonstrated a cytokine-mediated change of tight junction-related molecules in various cell models. For instance, IL-6 markedly induces Claudin-2 expression *via* MEK and PI3K signaling leading to increased tight junction permeability [56]. In a rabbit model of GERD, elevated IL-6 expression correlated with induction of several tight junction-related proteins (Claudin-1, Occludin, JAM-1, ZO-1) [57] and altered the motogenic activity of smooth muscle cells [58].

All together, there is sufficient data showing that the exposure of mixed gastric or gastroduodenal refluxate causes altered esophageal epithelial barrier function, inflammation and cellular damage, although the timely order of these processes is a matter of debate [20]. As today, it is well accepted that impaired epithelial barrier function of the distal esophagus presents a major pathophysiological process in GERD.

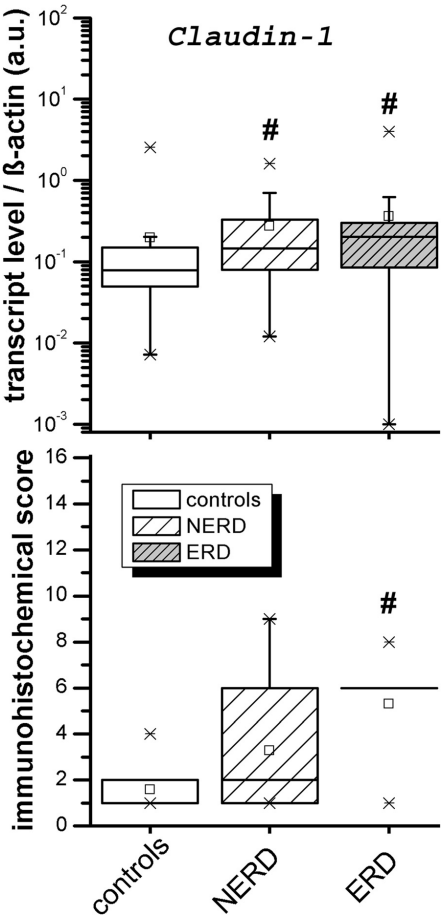

Figure 1 Expression of Claudin-1 in the esophageal mucosa of patients with NERD and ERD. The upper panel presents data of RT-PCR analysis; the lower panel illustrates immunohistochemical scores. Data are shown as boxplots illustrating 25, 75 percentile, median and 5–95 range. Note that due to skewed data distribution Claudin-1 (ERD) is presented as line only. Significant differences compared to controls are marked by a star (#); further details are presented in Tables 4A, and B.

This study shows an upregulation of tight junction-related proteins in relation to ERD and NERD in mucosal samples. In particular, Claudin-1 and Claudin-2, though mediating opposite functionally effects, were induced, while cytoplasmic adapters and Occludin were rather unchanged in relation to controls. The higher expression of Claudin-1 (both on transcript and protein level) was the only significant difference identified between patients with ERD and NERD. The fact that all other identified changes were similar between NERD and ERD supports the concept of similar pathophysiological mechanisms between both diseases. Unexpectedly, these changes did not correlate with histomorphological alterations, in particular with dilated ICS in esophageal mucosa. This finding is in contrast to the recently identified correlation between histopathological alterations, in particular basal cell hyperplasia, and elevated gene expression of desmosomal proteins [39]. In this study, few borderline correlations were found for basal cell hyperplasia and some genes only, but notably these findings were mostly restricted to reflux-negative controls, whereas patients with GERD did not reveal significant correlations between histopathological alterations and transcript levels of the five genes. Since correlation analyses were performed in an explorative way (without adjustment for multiple comparison), the few significant correlations (with borderline significance) do not support a general role of these findings for the pathophysiology of GERD. Taken into consideration this limitation and the fact that that the overall majority of our comparisons (17 out of 20) revealed no correlations, we conclude that our data do not give evidence for an association between the gene expression of the five genes studied and the histopathological changes in our study groups. It is well known that extent of basal cell hyperplasia reflects proliferative status of esophageal mucosa [9]. Since the identified correlations between gene expression levels and basal cell hyperplasia were mostly restricted to controls, it is unlikely that elevated Claudin-1 levels in ERD reflect tissue repair in context to mucosal damage caused by refluxate in these patients. Since we and others demonstrated more severe histomorphological alterations in ERD than NERD, the overall consistent changes of the 5 genes and their corresponding proteins in both diseases seem to be of limited relevance to the mucosal integrity and function. Furthermore, it is notable that some of the stainings revealed not the typical membrane-restricted expression pattern as demonstrated for these tight junction-related molecules im most gastrointestinal tissues [59,60]. However, cytoplasmic or diffuse membranous expression patterns have been identified for Claudin-2 [60] and ZO-1 [61] in human gastrointestinal tissue and for Claudin-1 in esophageal mucosa of rat [62]. Occludin staining pattern or expression in esophageal mucosa differs frequently also from those identified in gastric or intestinal mucosa [60,63]. Overall, the subcellular distribution of the 5 tight junction-related proteins seems to differ partially from those identified in columnar-lined epithelium. However, the study was

Table 4 Expression of tight junction-related components in esophageal mucosa in patients with GERD

Panel ATranscript level	**Gene expression/ß-actin (a.u.) median (range)**	**Change *vs.*controls (x-fold)**	***P*-values (* Kruskal-Wallis; posthoc: Man Whitney *U* test)**
Occludin			*0.098**
controls	0.041 (0.0086 - 0.38)		
NERD	0.060 (0.0052 - 0.57)	1.46	n.a.
ERD	0.035 (0.0026 - 1.09)	0.85	n.a.
Claudin-1			***0.0097****
controls	0.078 (0.0072 - 2.54)		
NERD	0.15 (0.012 - 1.6)	1.92	**0.016**
ERD	0.20 (0–4.1)	2.56	**0.0032**
Claudin-2			***0.0027****
controls	0.000038 (0–0.003)		
NERD	0.0002 (0–0.019)	5.26	**0.0041**
ERD	0.000083 (0–0.021)	2.18	0.11
ZO-1			*0.069**
controls	0.0060 (0.0012 - 0.073)		
NERD	0.0081 (0–0.067)	1.35	n.a.
ERD	0.0077 (0.0015 - 0.21)	1.28	n.a.
ZO-2			*0.061**
controls	0.011 (0.0022 - 0.038)		
NERD	0.019 (0.002 - 0.27)	1.72	n.a.
ERD	0.021 (0.0019 - 0.59)	1.9	n.a.
Panel BProtein level	**IHC scoremedian (range)**	**Change (x-fold)** *vs.* controls	***P*-values (* Kruskal-Wallis; posthoc: Man Whitney *U* test)**
Occludin			***0.02****
controls	3 (1 – 9)		-
NERD	6 (1 – 15)	2.0	***0.026***
ERD	8 (1 – 12)	2.7	***0.012***
Claudin-1			***0.014****
controls	1 (1 – 4)		-
NERD	2 (1 – 9)	2.0	***0.14***
ERD	6 (1 – 8)	6.0	***0.0004***
Claudin-2			***0.0057****
controls	4 (1 – 9)		-
NERD	6 (1 – 12)	1.5	0.28
ERD	9 (4 –15)	2.25	***0.0025***
ZO-1			*0.62**
controls	2 (0 – 6)		-
NERD	4 (1 –12)	2.0	n.a.
ERD	3 (0 – 3)	1.5	n.a.
ZO-2			*0.58**
controls	4 (0 – 10)		-
NERD	4 (0 – 8)	1.0	n.a.
ERD	1 (0 – 8)	0.25	n.a.

Transcript levels are shown in relation to controls (Panel A). Immunohistochemical scores are illustrated similarly (Panel B). Statistical analyses for both datasets were done first by non-parametric Kruskal-Wallis (*P-value italic style*); if significant post-hoc analysis was done by Mann Whitney *U* test. Significant changes are demonstrated by bold letters. n.a.: not applicable.

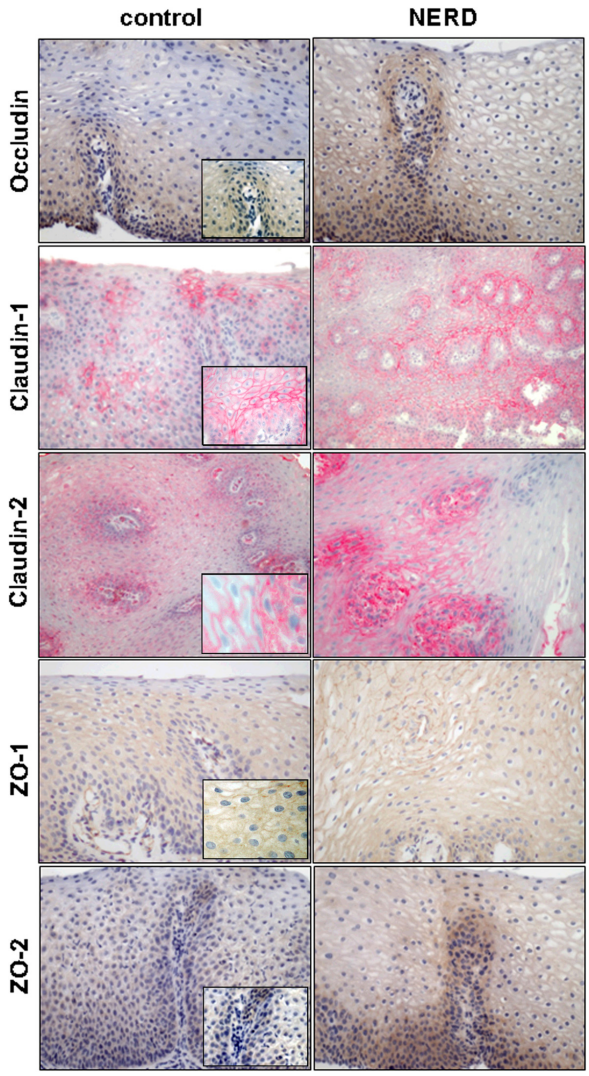

Figure 2 Immunohistochemical stainings of tight junction-related proteins in esophageal mucosa. Occludin, Claudin-1, -2 and ZO-1,-2 are displayed by brown or red staining, respectively. Panels illustrate representative staining for controls and samples obtained from patients with NERD. Immunohistochemical staining was observed in the esophageal squamous epithelium mainly at the basal and suprabasal zone. Claudin-1/2 and ZO-1 showed partly a membranous staining. (Zeiss Axioskop 50; camera: Nikon coolpix 990).

not aimed to analyze the subcellular distribution pattern of the molecules in esophageal mucosa on the subcellular level. The presence of appropriate negative and positive control stainings in other tissues, and the good concordance between expression data on transcript and protein level in general provide further indirect evidence for the specificity of immunohistochemical stainings.

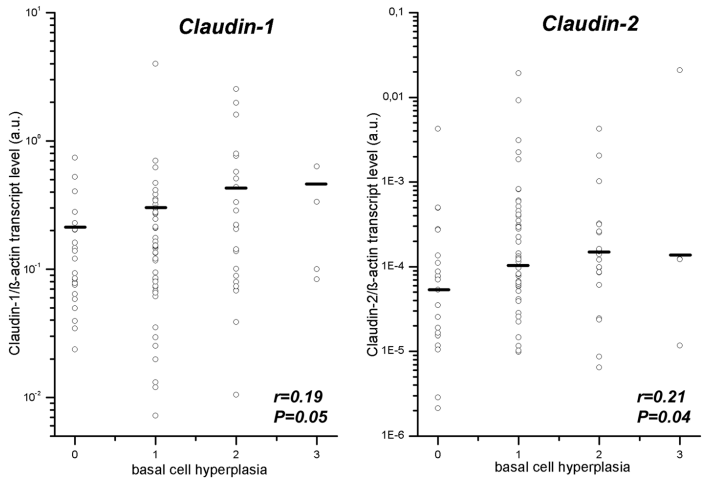

Figure 3 Correlation of Claudin-1 and Claudin-2 with histomorphological changes in esophageal mucosa. Panels illustrate correlations between transcript levels and histomorphological alterations as indicated. Data are shown as open dot plots; medians are presented filled dot. Non-parametric correlation analysis was performed by Spearman's rank correlation test; *P values* are presented in figure. Detailed data of other correlations are presented in Tables 5A, and 5B.

Based on the descriptive study design, it remains open whether the altered gene expression levels of Claudin-1 and −2 contribute to GERD pathophysiology or merely are markers for the existing disease. Furthermore it is notable that the majority of patients received GERD medications (PPI, H2RA) in the past before entering study. Even a stop of at least 2 weeks was mandatory to enter the study, we can not exclude that the effects of long-term therapy in the past or the changes induced by the 2-week stop of medication (e.g. acid rebound) [64]

Table 5 Correlation between GERD-specific histopathological alterations and gene expression level of tight junction-related genes (transcript level)

Panel A: All samples	***Occludin***	***Claudin-1***	***Claudin-2***	***ZO-1***	***ZO-2***
Activity	n.s.	r = 0.23	n.s.	n.s.	n.s.
		P = 0.07			
Chronicity	n.s.	n.s.	n.s.	n.s.	n.s.
Basal cell hyperplasia	n.s.	r = 0.19	r = 0.21	r = 0.22	n.s.
		P = 0.05	***P* = 0.04**	***P* = 0.03**	
Elongation of papilla	n.s.	n.s.	n.s.	n.s.	n.s.
Dilated intercellular space	n.s.	n.s.	n.s.	n.s.	n.s.
Panel B: Basal cell hyperplasia	***Occludin***	***Claudin-1***	***Claudin-2***	***ZO-1***	***ZO-2***
Controls	r = 0.47	n.s.	n.s.	r = 0.36	r = 0.42
	***P* = 0.02**			*P* = 0.08	***P* = 0.04**
NERD	r = 0.36	n.s.	n.s.	n.s.	n.s.
	***P* = 0.03**				
ERD	n.s.	n.s.	n.s.	n.s.	n.s.
GERD (ERD + NERD)	n.s.	n.s.	n.s.	n.s.	n.s.

Panel A: The histopathological scores (Table 3) were correlated with gene expression levels (transcript levels, Table 4) for each gene individually in the combined study cohort (n = 110). Data (r-, and P-values) represent potential correlations between these parameters (n.s. = not significant). Panel B: Since basal cell hyperplasia demonstrated significant correlations in global analysis (Panel A), this parameter was further analyzed by correlating expression values for all five genes with basal cell hyperplasia within each group and for patients with GERD individually as identified in table.

could have affect the expression of the five genes studied. Another limitation is the assessment of protein expression by an immunohistochemical score that can be done semiquantitatively at best. Besides this methodological aspect, posttranscriptional regulatory mechanisms can lead to different findings between gene expression analysis performed on transcript and protein levels. But as mentioned above, overall we observed a good concordance between both levels even not all significant findings were confirmed by both methodologies. Since we studied five selected components of tight junction complexes in GERD only, general conclusions can not be made. Assessment of other tight junction related molecules (e.g. Claudins, JAMs, Tricellulin) [44,46,65] in regard to GERD needs to be performed.

Conclusions

In summary, this study demonstrates a partial upregulation of tight junction-related components, in particular Claudin-1, in relation to GERD. Since identified molecular changes do not correlate with histomorphological alterations in general, a major role of Claudin-1 as of the other four tight junction-related proteins in the pathogenesis of GERD can not be concluded from our study.

Abbreviations

BE: Barrett's esophagus; ERD: Erosive reflux disease; NERD: Nonerosive reflux disease; GEJ: Gastroesophageal junction; GERD: Gastroesophageal reflux disease; H2RA: Histamine-receptor antagonist; H. pylori: Helicobacter pylori; ICS: Intercellular spaces; INR: International normalized ratio; iNOS: Inducible nitro oxygen synthetase; JAM: Junctional adhesion molecule; NFκB: Nuclear factor kappa B; PAR-2: Protease activated receptor-2; PPI: Proton pump inhibitor; RDQ: Reflux disease questionnaire; ROS: Reactive oxygen species; RT-PCR: Reverse transcription polymerase chain reaction; ZO: Zona occludens.

Competing interests

The authors declare that they have no competing interest concerning the content of this article.

Authors' contributions

KM, PM and TW designed the study. KM, LCF enrolled the majority of patients. AK provided clinical data. TW coordinated and performed laboratory work. DK and AR provided histopathological and immunohistochemical data. TW and SK performed statistical analysis. The manuscript was drafted by TW, AK, DK, and reviewed for important intellectual content by KM and PM. All authors read and approved the final manuscript.

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Gastro-oesophageal reflux disease is associated with up-regulation of desmosomal components in oesophageal mucosa

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Gastro-oesophageal reflux disease is associated with up-regulation of desmosomal components in oesophageal mucosa

Aims: Gastro-oesophageal reflux disease (GERD) is associated with impaired epithelial barrier function. This study was aimed at investigating the role of desmosomal proteins in relation to GERD.
Methods and results: Ninety-five patients with GERD-related symptoms (erosive, $n = 51$; non-erosive, $n = 44$) and 27 patients lacking those symptoms were included. Endoscopic and histological characterization of oesophagitis was performed according to the Los Angeles and Ismeil–Beigi criteria, respectively. Multiple biopsies were taken from the oesophageal mucosa of each patient. Gene expression analysis of plakoglobin, desmoglein-1, desmoglein-2 and desmoglein-3 was performed by quantitative real time (RT)-polymerase chain reaction and immunohistochemistry in the oesophageal mucosa. Routine histology revealed specific GERD-related alterations, such as dilatation of intercellular spaces (DIS), basal cell hyperplasia (BCH), and elongation of the papillae, in the oesophageal mucosa of patients with GERD, as compared with controls (all parameters: $P < 0.05$). All four genes and corresponding proteins were found to be up-regulated by between 1.7 and 8.1-fold (transcript level, $P < 0.05$; protein level, $P < 0.05$). Induced gene expression levels of plakoglobin, desmoglein-1 and desmoglein-2 correlated significantly with DIS and BCH.
Conclusions: Taken together, the uniform up-regulation of desmosomal genes/proteins in the oesophageal mucosa of patients with GERD supports the concept of architectural and molecular changes in the desmosomal compartment in the pathogenesis of GERD.

Keywords: desmogleins, desmosomes, gastro-oesophageal reflux disease, GERD, inflammation, oesophagitis, oesophagus, plakoglobin

Abbreviations: BCH, basal cell hyperplasia; DIS, dilatation of intercellular spaces; ERD, erosive reflux disease; GERD, gastro-oesophageal reflux disease; GEJ, gastro-oesophageal junction; IL, interleukin; NERD, non-erosive reflux disease; PCR, polymerase chain reaction; PP, percentage of positive cells; PPI, proton-pump inhibitor; ROS, reactive oxygen species; SI, staining intensity

Introduction

Gastro-oesophageal reflux disease (GERD) affects 20–44% of the Western population, and comprises three different endoscopic entities: non-erosive reflux disease (NERD), erosive reflux disease (ERD), and Barrett's oesophagus (BO).[1] According to the Montreal classification, GERD is defined as a condition that develops

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when reflux of stomach contents causes troublesome symptoms and/or mucosal lesions in the distal oesophagus.[2] Several pathophysiological mechanisms have been shown to contribute to the pathogenesis of GERD. Besides the increased frequency of transient lower oesophageal relaxation as the underlying mechanism for reflux episodes, impaired oesophageal clearance, decreased epithelial resistance and hypersensitivity to luminal contents are further mechanisms involved in the pathogenesis of GERD.[3,4] The recurrent exposure to acidic and non-acidic refluxate containing gastric acid contents, pepsin, bile acids and neutral peptidases (chymotrypsin and trypsin) leads to the acute and chronic inflammation that is typically associated with GERD.[5,6]

Specific histopathological alterations of the oesophageal mucosa in GERD were categorized for the first time by Ismail-Beigi in 1970,[7] and later confirmed by other groups.[8,9] Today, elongation of the papillae, basal cell hyperplasia (BCH) and dilatation of intercellular spaces (DIS) are accepted histological phenomena that are present in both ERD and NERD.[10–13] Pathophysiologically, DIS is regarded as a morphological correlate of impaired oesophageal barrier function, allowing toxic components of the refluxate to penetrate through the oesophageal mucosa into the deeper layers of the oesophagus, so initiating oesophagitis and also perception of reflux-related symptoms.[14,15] In line with this concept, dysregulation of tight junction components functionally linked to the regulation of epithelial permeability has been described in animal models and in one study evaluating patients with BO.[16–19]

Desmosomes are intercellular adhesive organelles, mostly in the epidermis, that were first characterized decades ago.[20] Together with tight and adherence junctions, desmosomes form a third cell–cell contact at the basolateral membrane in epithelial cell layers.[21–24] The molecular structure is complex and involves 'desmosomal cadherins' localized in the intercellular space, linker proteins and intracellular components that connect this desmosomal complex with the cytoskeleton of the epithelial cells.[24,25] The impairment of desmosomal components has been causally linked to several human diseases of skin and heart, such as pemphigus (desmoglein-3), skin fragility syndrome (plakophilin), lethal acantholytic epidermolysis bullosa (desmoplakin), and arrhythmogenic right ventricular dysplasia (desmoglein-2).[26–30] On the basis of the pathophysiological role of impaired barrier function in GERD and various pathologies caused by the dysregulation of junctional components, we conjectured that there would be an alteration of desmosomal components in relation to GERD. To prove this hypothesis, we analysed the gene expression pattern of plakoglobin, desmoglein-1, desmoglein-2 and desmoglein-3 in the oesophageal mucosa of patients with ERD and NERD.

Materials and methods

STUDY DESIGN

The study was approved by the local ethics committee and government authorities, and was conducted according to the ethical guidelines of the declaration of Helsinki (revised in 2000). All patients provided written informed consent; a detailed interview for history and GERD-related symptoms and physical examination were performed. The study included 122 randomly selected patients from a large prospective cohort (n = 210) assembled with the aim of characterizing GERD (recently published in detail[31]). Briefly, patients with typical reflux symptoms had to suffer symptoms at least three times a week. Typical reflux symptoms were defined as heartburn and regurgitation. Patients with other types of reflux symptom were not included in this study. Additional exclusion criteria included: upper gastrointestinal pathology (e.g. peptic ulcers, cancers, polyps, and Barrett's mucosa), systemic inflammatory, neoplastic and malabsorptive diseases, acute medical conditions, and abnormal coagulation parameters. None of the patients had taken antibiotics, bismuth compounds or any H2-blockers or proton-pump inhibitors (PPIs) in the last 2 weeks before entering the study. Patients were stratified by the endoscopic appearance of GERD: NERD (n = 44), ERD (n = 51), and patients without any clinical symptoms or endoscopic signs for GERD as controls (n = 27). Patients without GERD (controls) underwent oesophagogastroduodenoscopy (EGD) for screening purposes or non-reflux dyspepsia. ERD was classified according to the Los Angeles classification, comprising LA-A (n = 21), LA-B (n = 24), LA-C (n = 4), and LA-D (n = 2). For detailed demographic data, see Table 1A. The patient groups were not matched for age and gender. As pH-metry and/or combined multichannel impedance analysis was performed in only a few patients, these data were not statistically analysed. Note that all patients diagnosed with NERD had typical reflux-related symptoms and responded to PPI therapy. Further details of study design, inclusion criteria and exclusion criteria were published recently.[31,32]

For immunohistochemical analysis of desmosomal proteins, 12, 17 and 14 patients from the control, NERD and ERD groups, respectively, were randomly selected from the 122 included in the qRT-PCR) analysis. The details of both study populations (qRT-

Table 1. Demographic data and *H. pylori* status of patients stratified for the presence of gastro-oesophageal reflux disease and corresponding endoscopic findings [non-erosive reflux disease (NERD)/erosive reflux disease. (ERD)] (A) The study population used for quantitative RT-polymerase chain reaction (PCR) and histopathology. (B) Immunohistochemistry of a subset of the patients in (A)

	Control *n* = 12	NERD *n* = 17	ERD *n* = 14
(A)			
Gender (male/female)	6/21	8/36	32/19*
Age (years), mean ± SD (range)	50.0 ± 17.5 (20–79)	47.0 ± 13.8 (18–72)	48.0 ± 15.0 (20–79)
H. pylori status, no. (%)	6/21 (22.2)	8/36 (18.2)	16/35 (31.4)
	Control *n* = 27	NERD *n* = 44	ERD *n* = 51
(B)			
Gender (male/female)	4/8	7/10	8/6
Age (years), mean ± SD (range)	54.8 ± 14.4* (21–76)	47.2 ± 13.4 (26–72)	49.4 ± 18.2 (20–78)
H. pylori status, no. (%)	0/12 (0)	0/17 (0)	2/12 (14.3)

Data are presented as mean ± standard deviation (SD), range, and relative proportion. Comparisons were performed using the χ^2-test or ANOVA.

*Significant differences ($P < 0.05$). Note that not all biopsies from each location were available or applicable for molecular and immunohistochemical analysis. The reasons for this were missing biopsies and low amounts or quality of either extracted RNA or protein from mucosal biopsies, and limited material for immunohistochemistry. Minimal numbers for independent samples in RT-PCR analysis were 23, 29 and 36, and those for immunohistochemical analysis were 10, 14 and 12, for controls, NERD, and ERD, respectively.

PCR and immunohistochemistry) are summarized in Table 1.

ENDOSCOPY

The patients underwent the procedure after an overnight fast. The endoscopy was performed under conscious sedation with intravenous midazolam, using a videogastroscope (Q160; Olympus, Hamburg, Germany). Endoscopic characterization of oesophagitis was performed according to the Los Angeles classification: grade A, one or more mucosal breaks confined to the mucosal folds, each no longer than 5 mm; grade B, at least one mucosal break more than 5 mm in length, confined to the mucosal folds but not continuous between the tops of two mucosal folds; grade C, at least one mucosal break continuous between the tops of the two or more mucosal folds but not circumferential; and grade D, circumferential mucosal break. During endoscopy, care was taken to document the following landmarks: gastro-oesophageal junction (GEJ), Z-line, beginning of the gastric folds, and diaphragmatic pinch. The GEJ was defined as the beginning of the gastric folds, and the Z-line was defined as the squamocolumnar junction. The cardia was defined as the mucosa lying immediately below the GEJ. In the distal oesophagus, three biopsies were taken 2 cm above the squamocolumnar junction at the 3 o'clock position. In case of erosions, specimens were taken 2 cm above the tip of the erosion or mucosal break. One biopsy was separately snap-frozen in liquid nitrogen and subsequently stored in 0.5 ml of TRIZOL reagent (Life Technologies, Grand Island, NY, USA) at −80°C for molecular analysis. The two other biopsies were used for histopathology and immunohistochemical analysis, and fixed in 4% neutral-buffered formalin.

ROUTINE HISTOPATHOLOGICAL TECHNIQUES

Gastric and oesophageal tissue specimens were immediately fixed in buffered formalin and submitted for histopathological examination performed with haematoxylin and eosin, modified Giemsa and periodic acid–Schiff stains. Acute and chronic inflammation were scored by the densities of intraepithelial neutrophils/eosinophils and lymphocytes, respectively. Also, the degree of BCH, the presence of papillary elongation and DIS were assessed. [7,11,12] *Helicobacter pylori* infection was assessed by histological

examination (Sydney classification) and the rapid urease test (HUT; Astra, Wedel, Germany). All histological parameters were semiquantitatively scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), as described previously.[32]

EXTRACTION OF RNA AND QUANTITATIVE RT-PCR ANALYSIS OF DESMOSOMAL GENE EXPRESSION

Total RNA from oesophageal biopsies was isolated with a two-step protocol including TRIZOL extraction and the RNeasy kit (Qiagen, Hilden, Germany), and 500 ng of total RNA was then transcribed in a final volume of 40 µl with a standard protocol, as described previously.[33] Transcript levels of the genes encoding plakoglobin, desmoglein-1, desmoglein-2, desmoglein-3 and β-actin (as a housekeeping gene) were determined by quantitative RT-PCR with an iCycler (BioRad, Munich, Germany) and the QuantiTect SYBR Green kit (Qiagen), using standard conditions. Primer sequences and further details are summarized in Table 2. Initial template mRNA amounts were calculated with iCycler software and serial dilutions of plasmid DNA standard containing the corresponding PCR fragments. The final results are expressed as artificial units, and represent ratios between the investigated gene and β-actin transcript amounts. Owing to the primer design (use of intron-spanning regions), amplification of genomic DNA was excluded.

IMMUNOHISTOCHEMICAL ANALYSIS OF DESMOSOMAL COMPONENTS

Immunohistochemistry was performed with the avidin–biotin complex immunostaining method using the automated Ventana NexES immunohistochemistry slide staining system (Ventana Medical Systems, Strasbourg, France). Three-micrometre-thick, formalin-fixed, paraffin-embedded serial sections were deparaffinized and dehydrated. For antigen retrieval, pretreatment was performed by microwave heating in 1 mM sodium citrate buffer (30 min, 600 W, pH 6.0). Incubation with primary antibodies (for details see Table 2) was conducted at 37°C for 32 min and followed by washing with phosphate-buffered saline. Positive immunohistochemical reactions were revealed with either the iVIEW DAB Detection Kit (desmoglein-1, desmoglein-2, and desmoglein-3) or the UltraVIEW Universal Alkaline Phosphatase Red Detection Kit (plakoglobin) (both Ventana, Tuscon, AZ, USA) as chromogen substrate. Specimens were counterstained with haematoxylin and mounted with DEPEX. For negative controls, primary antibody was replaced by irrelevant polyclonal rabbit serum that did not reveal specific signals (data not shown). Immunoreactivity was assessed in five representative high-power fields (Zeiss Axioskop 50; Carl Zeiss GmbH, Jena, Germany) for each sample, using a semiquantitative score: staining intensity (SI) was classified as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong); the percentage of positive cells (PP) was classified as 0 (no

Table 2. Characteristics of primers, RT-PCR protocol and antibodies

	Primer sequence, length of fragment, annealing temperature	Antibody, company, antigen retrieval, final dilution
Plakoglobin	Forward: 5′-aag gac gac atc acg gag cct g-3′ Reverse: 5′-gat caa gcc gat ggt tgc ctt g-3′ 174 bp, 60°C	Monoclonal anti-plakoglobin antibody No. 138500, clone PG11E4 (Zymed Lab.oratories, Carlsbad, CA, USA), EDTA retrieval, 1:150
Desmoglein-1	Forward: 5′-tcc gaa ggc aga aac gtg aat g-3′ Reverse: 5′-ggc cca ttg agt tca gag ctc g-3′ 273 bp, 58°C	Monoclonal anti-desmoglein-1 antibody No. 326000, clone 27B2 (Zymed Laboratories), EDTA retrieval, 1:10
Desmoglein-2	Forward: 5′-cct ggc acc ata gag atg ctg c-3′ Reverse: 5′-tcc cac ctt cca tcc atc tcg-3′ 215 bp, 57°C	Monoclonal anti-desmoglein-2 antibody No. 326100, clone 6D8 (Zymed Laboratories), EDTA retrieval, 1:10
Desmoglein-3	Forward: 5′-tac gta tgc cag agg cac agc g-3′ Reverse: 5′-ctc cgc aca ggc aaa tgc ttt c-3′ 292 bp, 58°C	Monoclonal anti-desmoglein-3 antibody No. 326300, clone 5G11 (Zymed Laboratories), citrate retrieval, 1:40
β-Actin	Forward: 5′-cat gcc atc ctg cgt ctg gac c-3′ Reverse: 5′-aca tgg tgg tgc cgc cag aca g-3′ 400 bp, 60°C	Not performed

The standard conditions for the real-time PCR were as follows: intial cycle: 95°C for 15 min, repetitive 40 cycles each: 94°C for 30 s, XX°C for 30 s, 72°C for 30 s), final cycle: 72°C for 5 min (annealing temperature XX is given in the table).

positive cells), 1 (<10%), 2 (10–50%), 3 (51–80%), or 4 (>80%). For each slide, the immunohistochemical score was calculated as SI × PP, with a possible maximum score of 12. Appropriate positive and negative controls (isotype controls) were subjected to immunohistochemical procedures in parallel to validate specific staining (data not shown).

STATISTICAL ANALYSIS

All data were entered into a database with the MICROCAL ORIGIN 8.0 program package (MicroCal, Northampton, MA, USA) and SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as raw, median, mean ± standard error, or 95% confidence intervals, if not stated otherwise. Non-parametric Kruskal–Wallis tests and Dunn's multiple comparisons were applied when comparisons were made among the three groups (controls, NERD, and ERD). If significant differences were identified in global tests ($P < 0.05$), *post-hoc* analyses for pairwise comparisons between groups were performed with the Mann–Whitney *U*-test for gene expression analysis. Histopathological parameters were analysed by one-way ANOVA as a global test for multiple testing; and least significant difference (LSD) as *post-hoc* analysis for pairwise comparisons if global

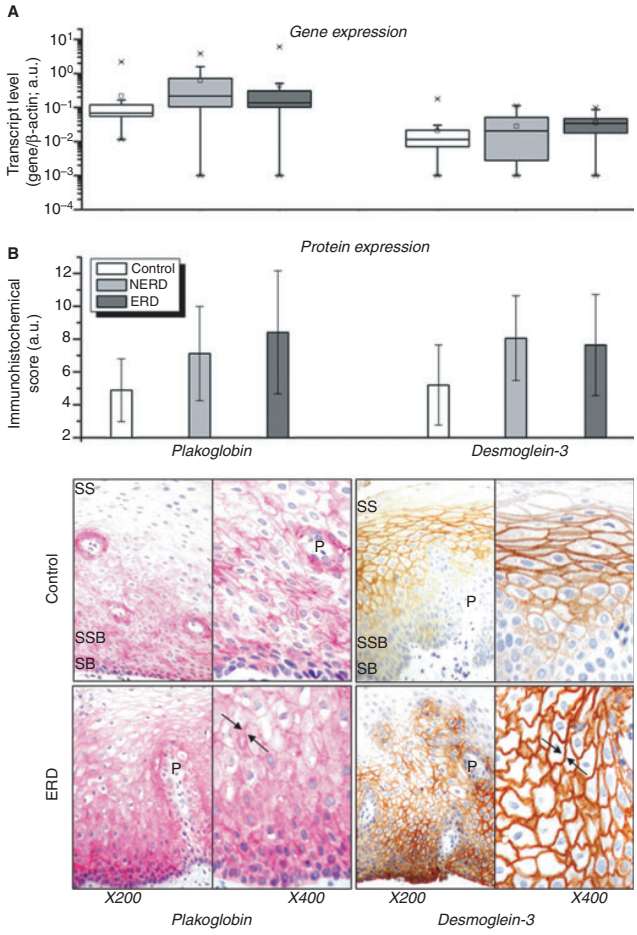

Figure 1. Up-regulation of plakoglobin and desmoglein-3 in the oesophageal mucosa of patients with non-erosive reflux disease (NERD) and erosive reflux disease (ERD). **A**, The upper panel presents the data from real-time polymerase chain reaction analysis; data are presented as boxplots illustrating 25th and 75th percentiles, median, and 5–95 range. The lower panel illustrates immunohistochemical scores of plakoglobin and desmoglein-3; data are presented as means ± standard deviations (SDs). Significant differences as compared with controls are marked by an asterisk; further details are presented in Tables 3 and 4. **B**, Immunohistochemical staining of plakoglobin and desmoglein-3 is shown. Layers of stratified epithelium and papillae are marked in controls: SB, stratum basale; SSB, stratum suprabasal; SS, stratum spinosum; P, papillae. The arrow points to expression of desmoglein-2 or plakoglobin in the region of the intercellular gaps. (Zeiss Axioskop 50. Magnification: ×200, ×400. Camera: Nikon coolpix 990.)

tests reached a significant level. Non-parametric correlation analysis was performed by Spearman's rank correlation test. All tests were two-sided, with a level of significance of $P < 0.05$.

Results

PATIENTS AND GERD-SPECIFIC HISTOMORPHOLOGICAL CHANGES

The three groups (control, NERD, and ERD) were similar with respect to mean age and *H. pylori* status Table 1A. Demographic data of the subgroups for immunohistochemical analysis were comparable, except for a higher mean age in the control group, which reached borderline significance ($P = 0.04$), and very low-level or absent *H. pylori* infection Table 1B. The histomorphological characterization of the study groups was described in detail recently.[31,32] Therefore, primary data are not given in this article. Briefly, a trend of increasing scores for activity and chronicity in patients with NERD or ERD versus controls was identified in the oesophageal mucosa. Histomorphological markers for GERD revealed significant changes for BCH, DIS, and to a lesser extent, elongation of papillae.[31,32]

UP-REGULATION OF DESMOSOMAL PROTEINS IN THE OESOPHAGEAL MUCOSA IN RELATION TO THE PRESENCE OF GERD

To evaluate the basal and GERD-related gene expression patterns of various desmosomal components, transcript levels of the genes for plakoglobin, desmog-

Table 3. Transcript levels of desmosomal components in the oesophageal mucosa

	Control	NERD	ERD	*P*-value (Kruskal–Wallis, ***$P < 0.05$) (Mann–Whitney *U*-test, *post-hoc* analysis)
Plakoglobin	0.068	0.22	0.13	***
NERD versus control		3.2-fold		**0.0013**
ERD versus control			1.9-fold	**0.0009**
ERD versus NERD				NS
Desmoglein-1	0.014	0.072	0.075	***
NERD versus control		6.5-fold		**0.0039**
ERD versus control			8.0-fold	**0.00001**
ERD versus NERD				NS
Desmoglein-2	0.0016	0.013	0.0047	***
NERD versus vontrol		8.1-fold		**0.0044**
ERD versus control			2.9-fold	**0.0017**
ERD versus NERD				NS
Desmoglein-3	0.012	0.021	0.034	***
NERD versus control		1.8-fold		NS
ERD versus control			2.8-fold	**0.0019**
ERD versus NERD				0.15

ERD, erosive reflux disease; NERD, non-erosive reflux disease; NS, not significant.
Data are presented as median artificial units, and x-fold change in relation to control or NERD, as indicated in the left column. Statistical analyses were performed with the Kruskal–Wallis and Mann–Whitney *U*-test for multiple and pairwise comparisons, respectively. *P*-values of significant differences are shown as ***($P < 0.05$) for the Kruskal–Wallis test or presented in bold for the Mann–Whitney *U*-test. *P*-values >0.2 are identified as NS.

lein-1, desmoglein-2 and desmoglein-3 were quantitatively analysed in biopsies obtained from the patient groups. Also, by immunohistochemistry, the cellular localization of protein expression was studied, and expression scored semiquantitatively.

Figure 1 shows primary data for the expression of plakoglobin and desmoglein-3 in the oesophageal mucosa. Corresponding data for other genes are summarized in Tables 3 and 4. All four genes were ubiquitously expressed in the oesophageal mucosa of controls (Table 3).

GERD led to a significant increase in transcript levels of all four genes, independently of the endoscopic appearance (NERD as well as ERD). As compared with controls, the transcription levels were increased 1.8 to 8.1-fold (Table 3). In line with this induced gene expression, a significant increase in semiquantitative immunohistochemical scores for desmosomal proteins in the oesophageal mucosa was identified (Table 4). Furthermore, changes in the intraepithelial and subcellular distribution of protein expression were observed. In controls, the expression of plakoglobin was membrane-associated in the basal and suprabasal layers, whereas GERD led to additional cytoplasmic expression, and extension of staining to the spinous layers was also observed.

In contrast, the expression of desmoglein-1, desmoglein-2 and desmoglein-3 in controls was accentuated in the spinous layers. In GERD, the expression of desmogleins extended into the basal and suprabasal layers, with a washy, broadened membranous pattern. Desmogleins were also observed, to a certain extent, in the cytoplasm.

Table 4. Comparison of immunohistochemical scores for desmosomal components in the oesophageal mucosa among patient groups

	Control	NERD	ERD	*P*-value (one-way ANOVA, ***$P < 0.05$) (LSD, *post-hoc* analysis)
Plakoglobin	4.9 ± 1.9	7.1 ± 2.9	8.4 ± 3.8	***
NERD versus control				**0.040**
ERD versus control				**0.012**
ERD versus NERD				NS
Desmoglein-1	2.1 ± 1.7	3.2 ± 1.7	4.2 ± 2.1	***
NERD versus control				0.13
ERD versus control				**0.024**
ERD versus NERD				0.19
Desmoglein-2	2.8 ± 1.8	4.8 ± 1.8	5.4 ± 1.8	***
NERD versus control				**0.016**
ERD versus control				**0.0024**
ERD versus NERD				NS
Desmoglein-3	5.2 ± 2.4	8.1 ± 2.6	7.6 ± 3.1	***
NERD versus control				**0.009**
ERD versus control				**0.049**
ERD versus NERD				NS

ERD, erosive reflux disease; NERD, non-erosive reflux disease; NS, not significant.
Data are presented as mean ± standard deviation. Statistical analyses were performed with one-way ANOVA and LSD as *post-hoc* analysis. *P*-values of significant differences are shown as ***($P < 0.05$) for ANOVA or presented in bold for the LSD test. *P*-values >0.2 are identified as NS.

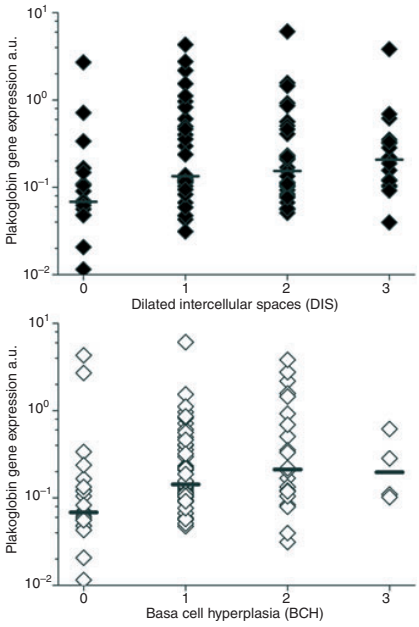

Figure 2. Correlation of plakoglobin with histomorphological changes in the oesophageal mucosa. In the left panel, the correlation of plakoglobin with dilated intercellular spaces is shown. (Median: 0, 0.07; 1, 0.13; 2, 0.15; 3, 0.21. $r = 0.19$, $P = 0.054$.) The right panel shows the correlation with basal cell hyperplasia. (Median: 0, 0.07; 1, 0.14; 2, 0.21; 3, 0.2. $r = 0.32$, $P = 0.001$.)

THE INDUCTION OF DESMOSOMAL GENES CORRELATES PARTIALLY WITH HISTOMORPHOLOGICAL CHANGES IN THE OESOPHAGEAL MUCOSA

Correlation analyses (exemplarily shown for plakoglobin; Figure 2) demonstrated significant associations of plakoglobin, desmoglein-1 and desmoglein-2 with DIS and BCH (Table 5). Papillary elongation did not correlate with any gene expression; no correlation with histomorphological changes was observed for desmoglein-3 (Table 5).

Discussion

Here, we demonstrate that GERD is associated with a general up-regulation of desmosomal components of the oesophageal epithelium, independently of its endoscopic entity (NERD or ERD). These alterations were partially associated with histomorphological alterations characteristic of GERD, such as DIS and BCH.[12] As previously shown in the same study population,[31] and by other investigators,[34–38] both NERD and ERD are characterized by mucosal infiltration by immune cells (mostly lymphocytes); induction of the Th1-type cytokines interleukin (IL)-1β and IL-8[31,34–36] and their receptors;[37] and the activation of several other pathways/mediators, such as NFκB,[34,38] PAR-2,[39] reactive oxygen species (ROS), and iNOS.[40–42] In animal models, the pathophysiological role of acidified and/or bile-containing refluxate in histomorphological changes (DIS and BCH) and the activation of inflammatory mediators has been demonstrated.[43–45] Yamaguchi *et al.* observed that acid-induced acute oesophagitis in Wistar rats resulted in increased expression of TNF-α and cytokine-induced neutrophil recruitment, which themselves produce ROS.[43,44] In animal models, exposure to acid, pepsin, bile acids and physical stress provokes DIS,[45,46] which can lead to reduced transepithelial electrical resistance and increased transepithelial permeability in rabbit and mouse models.[45,46] Although there is one study in humans that did not find impaired epithelial resistance/increased permeability after the infusion of hydrochloric acid,[47] overall there are reliable data showing that luminal exposure to mixed gastric or gastroduodenal refluxate can lead to cellular damage and oesophagitis.[6,48] Recently, on the basis of work with a rat model, Souza *et al.*[49] proposed an alternative

Table 5. Correlation between gastro-oesophageal reflux disease-specific histopathological alterations and gene expression level of desmosomal genes

	Plakoglobin *r*; *P*	Desmoglein-1 *r*; *P*	Desmoglein-2 *r*; *P*	Desmoglein-3 *r*; *P*
Basal cell hyperplasia	0.32; 0.001	0.29; 0.003	0.27; 0.006	0.11; NS
Elongation of papillae	0.07; NS	0.01; NS	0.06; NS	0.05; NS
Dilated intercellular space	0.2; 0.054	0.17; 0.08	0.24; 0.01	0.11; NS

NS, not significant.
The histopathological scores (Table 3) and transcript amounts (Table 4) were correlated for each gene, combining all three groups. Data (*r*-values and *P*-values) represent potential correlations between these parameters.

concept, suggesting that the release of cytokines by epithelial cells is the initial trigger for the infiltration of immune cells, starting in the lamina propria and spreading subsequently into the epithelial layer. Nevertheless, impaired epithelial barrier function of the oesophageal mucosa, which is affected either primarily by the refluxate or secondarily by the inflammation, remains a central pathophysiological process in GERD.

In line with the pathophysiological link between GERD and increased epithelial permeability, dysregulation of tight junction components has been described in animal models.[16–18] Notably, up-regulated IL-6 levels were identified as a mediator of impaired cell–cell contacts (tight junctions and desmosomes) in a rat model,[18] as well as of the motogenic activity of smooth muscle cells.[50] Despite the evidence for the pathophysiological role of cell–cell contacts in the development of GERD, there are only limited data from human studies. A few studies showed the involvement of various claudins in the pathogenesis of Barrett's metaplasia, in particular in the transition towards oesophageal adenocarcinoma.[19,51–53] So far, neither the role of these molecules nor the involvement of desmosomal proteins has been characterized in ERD and NERD.

For the first time, this study describes the up-regulation of desmosomal proteins in relation to GERD. With a few exceptions, significant induction of gene expression was present at both the transcriptional and protein levels, and in both endoscopic entities, NERD and ERD. On the basis of the structure of desmosomal complexes, we decided to investigate plakoglobin as a component of the intracellular 'desmosomal backbone' and desmoglein-1, desmoglein-2 and desmoglein-3 as 'contact-forming' members of desmosomes. The observed induction of the desmosomal genes encoding these proteins might be considered as a repair mechanism for the oesophageal mucosa, which, however, is not sufficient to restore the epithelial barrier function completely. Their intraepithelial and subcellular dislocation, which was observed in our immunohistochemical studies, might contribute to the impaired function of the desmosomal components. The observed correlation for plakoglobin, desmoglein-1, desmoglein-2 and BCH and DIS further supports the functional link between these desmosomal components and GERD. Lack of correlation between desmosomal gene expression and elongation of papillae is consistent with the histological characterization. The increase in BCH and DIS in patients with NERD/ERD as compared with controls was highly significant, whereas the elongation of papillae was at the borderline of significance.[31,32] Because of the descriptive nature of this study, we cannot answer the question of whether the molecular alterations in desmosomal gene expression contribute causally to GERD or merely represent a surrogate marker for the existing disease. The fact that the induction of these components was uniformly observed in ERD and NERD strengthens the hypothesis that desmosomes might have a general role in this disease. Although functional studies were not performed for the majority of our patients, the presence of reflux symptoms confirmed by questionnaire and endoscopic lesions (details published in Mönkemüller *et al.*[31]) is specific enough for the diagnosis of GERD.

Taken together, the findings of this study demonstrate a general up-regulation of desmosomal components in the oesophageal mucosa of patients with GERD, strongly implying a pathophysiological role for desmosomes in this disease. Future studies are required to show whether treatment with PPIs, which leads to the resolution of symptoms and healing of the oesophageal mucosa in the majority of patients, is accompanied by a normalization of desmosomal gene expression.

Disclosure/conflict of interest

The authors declare that none of them has financial interests in relation to this study. This work was partially supported by the NBL-3 programme of the 'Bundesministerium für Forschung und Technik' (01ZZ0407/PFG1) and the Deutsche Forschungsgemeinschaft (WE2170/8-1).

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ORIGINAL ARTICLE

Chronic Mucosal Inflammation of the Gastric Cardia in Gastroesophageal Reflux Disease Is Not Regulated by FOXP3-Expressing T cells

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Abstract *Introduction* Chronic inflammation at the cardia occurs in gastroesophageal reflux disease (GERD), as well as in the presence of *Helicobacter pylori*. Regulatory T cells have been demonstrated for *H. pylori*-induced gastritis, whereas their role has not been studied in GERD. *Methods* We prospectively analyzed the expression of FOXP3, a marker of various regulatory T cells, as well as the mucosal transcript levels of TGF-β1 and IL-10. RNA and protein levels have been determined in cardiac biopsies of 70 patients stratified according to GERD ($n = 22$), controls ($n = 17$), and *H. pylori* ($n = 31$). *Results* GERD presented with chronic inflammation and reduced FOXP3-mRNA in the cardiac mucosa (−84%), whereas *H. pylori*-positive patients revealed a 25.1-fold increase of FOXP3 gene expression. These results were verified by the regulatory cytokines IL-10 and TGF-β1, and by the immunohistochemical detection of intramucosal FOXP3-expressing T cells. *Conclusion* Chronic inflammation at the cardia associated with either GERD or *H. pylori* differs concerning the presence of FOXP3-expressing T cells. In contrast to *H. pylori*, FOXP3-expressing T cells are not associated with GERD-associated carditis.

Keywords Cardia · Carditis · FOXP3 · GERD · Interleukin-10 · Regulatory T cells

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Introduction

Both gastroesophageal reflux disease (GERD) and *Helicobacter pylori* lead to inflammatory changes at the gastric cardia [1–3]. They represent distinct etiologic models of inflammation, being gastric refluxate in GERD and infectious disease for *H. pylori*. GERD-related inflammation is characterized by a proinflammatory TH_1-derived cytokine milieu, specifically by increased IL-8 gene expression [4, 5], NFκB activation [5, 6], and the upregulation of chemokine receptors and COX-2 [7]. Besides these molecular changes, the reflux-associated mucosal inflammation at the gastric cardia has not been comprehensively analyzed in the context of regulatory T cells.

The *H. pylori*-associated chronic cardiac inflammation is characterized by similar histopathological changes as demonstrated for the antrum-predominant gastritis [1, 2]. Also, *H. pylori*-induced cardiac inflammation is associated with increased cytokine levels, including IL-8, MCP, RANTES [8], and additionally IL-10 and TGF-β1 considered as immunosuppressive cytokines [9]. There are several studies in humans [9–12] and animals [12, 13] describing the presence of regulatory T cells that seem to be of critical for the immunopathogenesis of gastritis and persistence of the bacteria. For the gastric cardia as well as for the antrum, an infiltration of FOXP3-expressing immune cells was found in the context of *H. pylori* infection [9].

No data exists about the involvement of regulatory T cells and GERD-associated carditis so far. The aim of the study was to prospectively analyze the expression of the transcription factor FOXP3, a marker expressed on various

types of regulatory T cells, and the regulatory cytokines IL-10 and TGF-β1 in the cardiac mucosa of patients with GERD.

Methods

Study Design

All patients were included after giving their written informed consent approved by the local ethics committee and government authorities. Patients were selected from a cohort of 225 patients that were enrolled in a prospective study design (2001–2004) to characterize inflammatory changes at the gastroesophageal junction in the context of GERD and *H. pylori*. Exclusion criteria were defined as the presence of any malignancy, continuous acid suppressive medication and/or antibiotic therapy within the last four weeks, use of non-steroidal anti-inflammatory drugs (NSAIDs), the presence of specialized intestinal metaplasia in the distal esophagus (Barrett's esophagus) or atrophic corpus gastritis, alcohol or drug consumption, and pregnancy. Retrospectively, six patients were excluded from the analysis because of Crohn's disease ($n = 2$), use of NSAIDs ($n = 2$), celiac disease ($n = 1$), and chronic atrophic gastritis ($n = 1$).

Finally, 70 patients were selected and stratified into the following groups: 'reference' [no GERD, no *H. pylori* infection, $n = 17$], 'GERD' [GERD, no *H. pylori* infection, $n = 22$], and '*H. pylori*' [$n = 31$] (Table 1). Notably, 22 of those 31 patients presented with erosive reflux disease (ERD). Note that the patient groups were not matched for age and gender.

All *H. pylori*-infected patients presented with antrum-predominant gastritis, revealing higher inflammatory scores and colonization densities in the antrum compared to the corpus mucosa.

Since paraffin-embedded tissue specimens were only available from a few of the 70 patients, an additional set of unrelated tissue specimens were obtained from the Institute of Pathology for immunohistochemical analysis. Forty-five tissue samples were selected and stratified in GERD and *H. pylori* based on the records (Table 1B).

Upper Gastrointestinal Endoscopy

Endoscopy was performed with a standard endoscope (type GIF Q 145, Olympus Optical Europe, Hamburg, Germany). During upper gastrointestinal endoscopy, three mucosal biopsies were taken each from the antrum, corpus, and cardia. Antral specimens were taken 3–5 cm proximal to the pylorus at the lesser curvature. Biopsies from cardiac mucosa were obtained just below the esophageal-gastric junction at the upper margin of gastric folds. One of the biopsies was subjected to quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The other two biopsies were fixed in formalin. No samples from the distal esophagus were obtained.

Determination of GERD and *H. pylori* Infection

GERD was diagnosed based on the presence of typical reflux symptoms (e.g., heartburn, regurgitation) according to the Montreal classification [14] and/or endoscopic signs of reflux esophagitis according to the Los Angeles classification.[15]

H. pylori infection was determined by the rapid urease test (HUT®, AstraZeneca, Wedel, Germany)[16] and histopathological analysis that included the semiquantitative grading of inflammation and *H. pylori* colonization as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) in the context of the updated Sydney classification [17]. Patients were regarded as *H. pylori*-positive if one of the two tests were positive; negative *H. pylori* status is based on the negativity of both tests.

Table 1 Demographic data of patients stratified to the presence of gastroesophageal reflux disease (GERD) and *Helicobacter pylori*

A	Reference, $n = 17$	GERD, $n = 22$	*H. pylori* , $n = 31$
Gender (male/female)	4/13	11/11	14/7
Age (mean ± SD; range)	49.6 ± 14.9 years; 20–75 years	56.4 ± 11.3 years; 33–75 years	52.9 ± 12.4 years; 24–76 years
B (immunohistochemistry)	–	GERD, $n = 24$	*H. pylori*, $n = 21$
Gender (male/female)	–	10/14	9/12
Age (mean ± SD; range)	–	47.8 ± 15.9 years; 23–79 years	52.5 ± 17.2 years; 23–76 years

Data are presented as means, standard deviation (SD), range, and the proportion of males and females

Quantitative RT-PCR Analysis

Total RNA extraction and quantitative RT-PCR were performed using standard protocols as described previously [18] with the following primers: FOXP3 (5′-ACA-AGG-GCT-CCT-GCT-GCA-TCG-3′; 5′-ATG-AGC-GTG-GCG-TAG-GTG-AAA-GG-3′), TGF-β1 (5′-CCG-CGT-GCT-AAT-GGT-GGA-AAC-3′; 5′-TAA-CCA-CTC-TGG-CGA-GTC-GCT-G-3′), IL-10 (5′-ACC-TGG-GTT-GCC-AAG-CCT-TGT-C-3′; 5′-AAA-TCG-ATG-ACA-GCG-CCG-TAG-C-3′), β-actin (5′-CAT-GCC-ATC-CTG-CGT-CTG-GAC-C-3′, 5′-ACA-TGG-TGG-TGC-CGC-CAG-ACA-G-3′). The amplified cDNA fragment of each primer set included at least one exon–intron splice sites. The length of the amplicons were 210 bp (FOXP3), 265 bp (TGF-β1), 165 bp (IL-10), and 400 bp (β-actin). Since the size of all amplicons matched the expected length from the cDNA sequence, a genomic-derived amplification can be ruled out and expression data can be exclusively attributed to the mRNA pool of the sample.

Immunohistochemical Analysis of FOXP3 and CD45 (Leukocyte Common Antigen)

Immunohistochemistry was performed using the avidin-biotin complex immunostaining method and the automated immunohistochemistry slide staining system by Ventana NexES (Ventana Medical Systems, Strasbourg, France), as previously described [9]. Incubation with anti-FOXP3 (mouse monoclonal antibody, ab20034, dilution 1:40, Biozol, Eching, Germany) and anti-CD45 (mouse monoclonal antibody, M0701, dilution 1:300, DakoCytomation, Hamburg, Germany) was conducted at 37°C for 32 min and was followed by PBS washing. Positive immunohistochemical reactions were revealed by the iVIEW^{TM} DAB Detection Kit (Ventana, Tucson, Arizona, USA). Counterstaining was performed with hematoxylin. For negative controls, primary antibody was either replaced by irrelevant IgG1 antibody or omitted; both procedures revealed no signals. Samples were examined by two different observers (A.K., D.K.) blinded to the group assignment. The numbers of FOXP3- and/or CD45-expressing cells were counted in five independent high-power fields (Zeiss Axioskop 50; magnification: ×400; camera: Nikon Coolpix 990).

Statistical Methods

The data were entered into a database using the Microcal Origin^{TM} 6.0 software package (Northampton, MA, USA) and SPSS 12.0. The data are expressed as raw, median, mean ± standard error (SE), or 95%-CI (confidence intervals), if not stated otherwise. Statistical analysis of gene expression values among subgroups was performed by non-parametric tests. Comparisons of all three groups were performed by the Kruskal–Wallis test. Using closed testing procedure arguments for unadjusted pairwise comparisons for 'reference' vs. 'GERD' and 'reference' vs. '*H. pylori*', the Mann–Whitney *U*-test was added. Histopathological parameters were analyzed by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test as post-hoc analysis for pairwise comparisons. Differences in the total count and the percentage of FOXP3 immunoreactive T cells were compared using a parametric *t*-test. Correlation between paired gene expression data or between gene expression data and histopathological scores were calculated by non-parametric Spearman's rank correlation. All tests were applied two-sided with a significance level of $P < 0.05$.

Results

Patient Characteristics and Histopathological Changes in Respect to GERD

Pairwise analysis revealed significant differences for gender and age between the 'reference' and '*H. pylori*' groups ($P < 0.05$, Table 1).

Table 2 Histopathological evaluation of inflammation at the cardia according to the updated Sydney classification from 0 (normal) to 3 (markedly abnormal)

	Reference	GERD	*H. pylori*	*P*-value [one-way ANOVA] [post hoc analysis]
Activity	0.27 ± 0.12	0.40 ± 0.13	1.23 ± 0.09	*
Reference versus GERD				0.46
Reference versus *H. pylori*				<0.001
GERD versus *H. pylori*				<0.001
Chronicity	1.0 ± 0.0	1.25 ± 0.12	1.83 ± 0.07	*
Reference versus GERD				0.065
Reference versus *H. pylori*				<0.001
GERD versus *H. pylori*				<0.001

Values are presented as mean ± standard error (SE)

* $P < 0.001$

The inflammatory scores among the three groups differed significantly (Table 2). While '*H. pylori*' revealed higher scores for activity and chronicity compared to the two other groups, the activity scores between the 'reference' and 'GERD' groups were found to be similar. For chronicity, 'GERD' was found to have an increased score, although significance levels were marginally missed ($P = 0.065$).

Gene Expression of FOXP3, IL-10, and TGF-β1 at the Gastric Cardia Related to the Presence of GERD

The gene expression pattern of FOXP3 demonstrated significant differences among the three groups ($P < 0.001$; Fig. 1a). The presence of GERD alone was associated with a reduction of FOXP3-mRNA amounts by 84% in the patients compared to the 'reference' that was not of statistical relevance ($P = 0.12$). *H. pylori*-infected patients presented with 25.1-fold ($P = 0.03$) and 142-fold ($P < 0.01$) elevated gene expression levels compared to the 'reference' and 'GERD' groups, respectively.

The mucosal transcript levels of IL-10 and TGF-β1 were similar between the three groups, except the 2–3-fold increase of TGF-β1 gene expression of '*H. pylori*,' missing statistical relevance. Although the gene expression analysis of IL-10 and TGF-β1 did not reveal major changes, the global correlation analysis, which was applied to the complete set of data, demonstrated a significant association between FOXP3 gene expression and the transcript levels of both cytokines (Fig. 2). Analyzing patients with GERD only, this positive correlation was not confirmed.

Identification of Intramucosal FOXP3-Expressing T Cells at the Gastric Cardia

To relate the changes of mRNA transcript levels representing a lack of FOXP3-expressing T cells in the cardia of patients with GERD, immunohistochemical staining and analysis of FOXP3-expressing T cells were performed. Using a different set of samples (Table 1b), the presence of FOXP3-expressing lymphocytes was quantified immunohistochemically in the cardiac mucosa of patients with either GERD or *H. pylori* infection (Fig. 3).

The total count of intramucosal FOXP3-expressing lymphocytes in 'GERD' was only 0.78 ± 0.15 per HPF compared to 2.65 ± 0.44 in *H. pylori*-infected subjects (Fig. 3a, c; $P < 0.001$). The number of infiltrating lymphocytes was visualized by immunohistochemical staining of CD45, which is expressed on all lymphatic cells (Fig. 3b). The ratio of FOXP3-expressing lymphocytes among all lymphocytes was also significantly decreased in 'GERD' compared to '*H. pylori*' (Fig. 3b, d; $2.65 \pm 0.61\%$ vs $8.14 \pm 1.38\%$; $P < 0.001$). Displaying an association between inflammation and regulatory T cells in the tissue samples, positive correlations between the numbers of FOXP3-expressing T cells and the degree of activity ($r = 0.466$, $P = 0.002$) and chronicity ($r = 0.326$, $P = 0.040$) were identified (not illustrated).

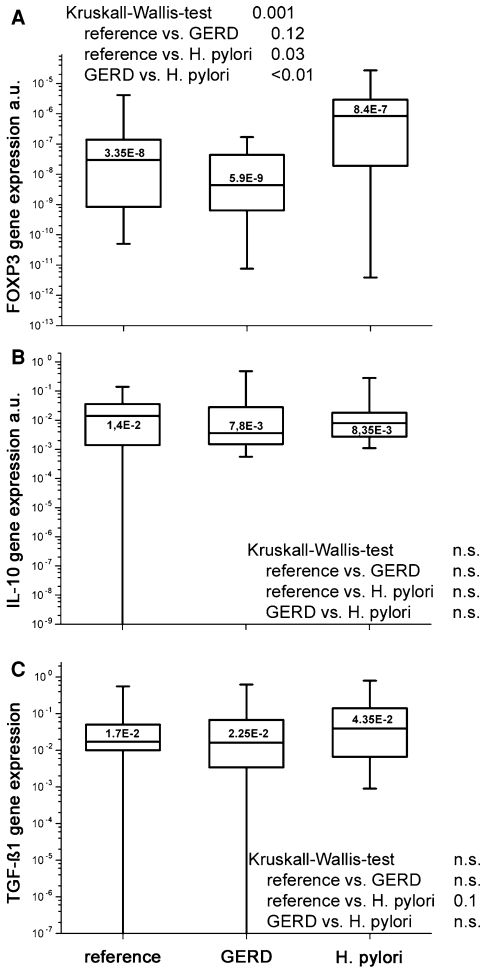

Fig. 1 Gene expression of FOXP3, IL-10, and TGF-β1 according to the diagnosis 'GERD,' '*H. pylori*,' and 'reference.' The data are presented by box plots illustrating 25th and 75th percentiles, median, and the 5th–95th range. Panel **a** represents FOXP3 gene expression; IL-10 and TGF-β1 gene expression are illustrated in panels **b** and **c**. 'GERD' is characterized by a slight decrease of FOXP3 transcript levels compared to 'reference' and by significantly decreased gene expression compared to '*H. pylori*'

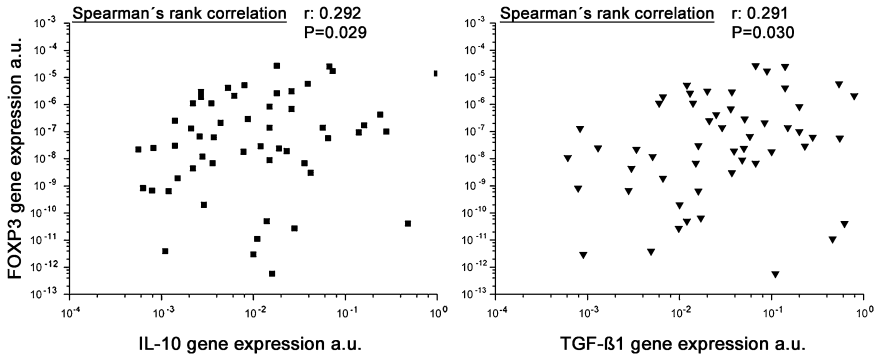

Fig. 2 Spearman's rank correlation between FOXP3 transcript levels and gene expression of the regulatory cytokines IL-10 and TGF-β1. The data illustrate the correlation between FOXP3 transcript levels in cardia mucosa and the gene expression of IL-10 and TGF-β1 in the same tissue sample when including the complete set of data

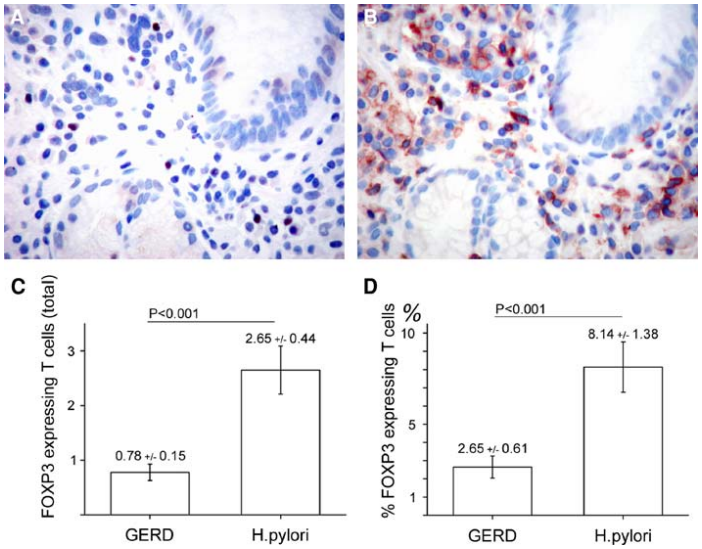

Fig. 3 Immunohistochemical analysis of FOXP3-expressing T cells at the gastric cardia related to 'GERD' and '*H. pylori*.' Panels **a** and **b** illustrate the immunohistochemical staining of FOXP3-immunoreactive lymphocytes and CD45-expressing lymphocytes (magnification ×400; Zeiss Axioskop 50, camera: Nikon Coolpix 990). The data are presented as columns displaying the means of the total number per high-power field (**c**) and the relative proportion of FOXP3-expressing T cells of all intramucosal lymphocytes (**d**) (HPF; magnification ×400) ± standard error (SE)

Discussion

In GERD-related chronic inflammation, FOXP3-expressing regulatory T cells are not present among the mucosal immune cells. This differs from the increased infiltration of regulatory T cell populations observed in *H. pylori*-induced inflammation [9]. As previously described [1, 3], the cardiac inflammation in GERD was mild compared to *H. pylori*-induced inflammation, which was more pronounced by other studies [15, 19, 20].

The quantification of FOXP3, IL-10, and TGF-β1 transcript levels were not upregulated in GERD-associated inflammation, contrary to what we found in *H. pylori* infection. The identified conjunction between FOXP3 gene expression and transcript levels of IL-10 and TGF-β strongly implies a functional role of FOXP3-expressing regulatory T cells, although we did not directly assess the suppressive activity of these cells in this study.

FOXP3 was described to be a specific transcription factor of $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cells and to be essential for their differentiation and function [21, 22]. Also, other activated (non-regulatory) T cells were recently described to express FOXP3 as well [23, 24]. Though there is still a 90% correlation of FOXP3 expression with other

specific human cell surface markers that have been used for the characterization of regulatory T cells [25, 26], therefore, FOXP3 can be considered to be a molecular marker for this cell lineage.

Based on the study design, biopsy protocol, and methods, our study has several potential limitations. First, GERD was solely diagnosed based on typical reflux-related symptoms and/or endoscopic signs of reflux disease. The pattern of gastroesophageal reflux episodes was not further characterized by diagnostic values of pH-metry or combined multichannel impedance/pH-metry. Therefore, the results and conclusion of this study are limited to the presence of clinically and endoscopically proven GERD (as defined above), but cannot be linked directly to the exposure of acid or non-acid gastric contents to the mucosa. Second, due to whole biopsy specimens, we can not directly link the FOXP3 and gene expression levels of both cytokines on a single-cell level. The isolation of infiltrating immune cells from a biopsy and the subsequent cell-based analysis by cytofluorometric approaches was not feasible. Certainly, stromal and epithelial cells contribute to mucosal IL-10 and TGF-β1 expression as well, and, therefore, we cannot directly prove the functional activity of FOXP3-expressing T cells in the cardiac mucosa. The analysis of FOXP3 expression on the transcript and protein levels was performed on different sets of patients. Since patients with GERD presented to be similar in both sets—with respect to clinical appearance and histopathological parameters—this issue should not have a major impact on the finding. Third, the analysis is restricted to FOXP3-positive cells that represent the major subset of all regulatory T cells but do not include all types of immunosuppressive regulatory T cells. It should be mentioned that functionally similar regulatory cell types were identified also among myeloid and dendritic cells [27, 28].

In *H. pylori*-induced gastritis, the role of regulatory T cells for the mucosal inflammation has been extensively studied [9–13]. Beside their role for the persistence of the infection [9, 11, 13], several studies identified regulatory T cells as critical for the development of gastric diseases, such as adenocarcinoma [29] and peptic ulcer disease [30].

Taken into consideration erosive and non-erosive GERD, no significant differences were found for FOXP3 expression in the cardiac mucosa between both groups. However, the mucosa of the distal esophagus also needs to be investigated in further prospective studies.

In conclusion, we demonstrate that the chronic inflammation at the cardia associated to GERD is not related to an infiltration of FOXP3-expressing regulatory T cells. This supports the concept that chemically induced inflammatory responses in the gastrointestinal tract differ in their immune regulation from other infectious-mediated inflammation [31]. Moreover, the distinct involvement of FOXP3-positive T cells in GERD- and *H. pylori*-associated inflammation might give rise to new biomarkers that help to differentiate between the two etiologies in cardiac inflammation and contribute to a better clinical management of cardia-related pathologies.

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Publikation IX

ESOPHAGUS

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Proteinase-Activated Receptor-2 in the Pathogenesis of Gastroesophageal Reflux Disease

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OBJECTIVES: **The proteinase-activated receptor-2 (PAR-2) is activated by serine proteases and has been demonstrated to induce proinflammatory and neuroinflammatory effects. It is considered to alter transepithelial resistance and mediates visceral hypersensitivity. This study aimed to evaluate the expression of PAR-2 in human esophageal mucosa of patients with gastroesophageal reflux disease (GERD) in relation to mucosal alterations.**

METHODS: **The study included 123 patients with GERD stratified to erosive reflux disease ($n=50$), non-erosive reflux disease ($n=46$), and reflux-negative patients as controls ($n=27$). Endoscopic and histopathological characterization was performed according to the Los Angeles classification and modified Ismail-Beigi criteria, respectively. PAR-2 expression was analyzed by quantitative reverse transcription (RT)-PCR and immunohistochemistry. The gene expression levels of interleukin (IL)-8 were determined by quantitative RT-PCR and correlated to PAR-2 expression in each patient. Performing *in vitro* studies, esophageal squamous cell lines (KYSE 150, KYSE 450) were incubated, adjusted to different pH (7.0, 6.0, and 5.0), and exposed to bile acids and PAR-2-activation peptide (SLIGKV-NH_2).**

RESULTS: **PAR-2 gene expression was 7- to 10-fold upregulated ($P<0.0001$) in the mucosa of patients with GERD and correlated positively with IL-8 expression and with histomorphological alterations (dilated intercellular spaces, papillary elongation, basal cell hyperplasia (BCH); $P<0.01$). Immunohistochemistry showed an intense staining of PAR-2 throughout all epithelial layers in patients with GERD compared with controls ($P=0.0005$). *In vitro* studies revealed a 1.5- to 20-fold induction of PAR-2 gene expression in esophageal squamous cells by acidified medium ($P<0.01$), but not by additional bile acids. The activation of PAR-2 leads to expression and secretion of IL-8.**

CONCLUSIONS: **This study provides evidence of the functional importance of PAR-2-mediated pathways in the pathogenesis of GERD and GERD-associated mucosal alterations and inflammatory changes.**

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INTRODUCTION

In up to 60% of the patients with gastroesophageal reflux diseases (GERD), GERD-related symptoms are not associated with mucosal lesions being detectable with conventional upper gastrointestinal endoscopy (non-erosive reflux disease—NERD) (1). Minimal abnormalities observed using high-resolution endoscopy have not been proven to be sufficiently sensitive and specific for the diagnosis of NERD (2,3). Histopathological and ultrastructural changes, such as dilated intercellular spaces (DIS), papillary elongation (PE), and basal cell hyperplasia (BCH), have been frequently reported to be associated with GERD (4–6) and therefore have been considered as an additional hint for GERD in the absence of mucosal breaks (NERD) (7). Besides the pH of the refluxate (acid, weakly acidic, and weakly alkaline), the exposure to several other intestinal contents, such as bile acids, intestinal proteases, or pancreatic trypsin, have been shown to induce mucosal damage and are suggested to be responsible for the generation of characteristic symptoms (8,9).

The mechanisms involved in epithelial damage are poorly understood in GERD. Among several putative candidates, the proteinase-

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activated receptor-2 (PAR-2) has been proposed for inflammatory and neuroinflammatory epithelial response (10,11). PAR-2 is specifically activated by serine proteases, including trypsin and mast cell-derived tryptase, and belongs to the family of 7-transmembrane G-protein-coupled receptor family. PAR-2 is activated by cleaving the N-terminal sequence of the extracellular receptor domain serving as a tethered ligand (12,13). PAR-2 activation results in a proinflammatory response in human esophageal cell lines, such as interleukin (IL)-8 secretion (14,15), induces neuroinflammatory effects by releasing substance P and calcitonin gene-related peptide (10), and mediates visceral hypersensitivity and pain (11). Considering the established pathophysiological role of PAR-2 in inflammation and hypersensitivity and the presence of serine proteases in the gastroesophageal refluxate, we raised the hypothesis that the increased expression of PAR-2 and its activation by endoluminal proteases contributes to esophageal mucosal abnormalities associated with GERD. Therefore, the expression of PAR-2 was studied in patients with GERD, including NERD as well as erosive reflux disease (ERD), and a reflux-negative reference group. PAR-2 gene expression was correlated with the degree of endoscopic esophagitis, esophageal inflammation, and GERD-related histomorphological changes. Furthermore, *in vitro* studies with esophageal squamous cell lines were used to confirm a regulatory link between acidic pH and gene expression of PAR-2. PAR-2-mediated activity was further evaluated by the expression of IL-8.

METHODS

Study design and patients

This study was approved by the local ethics committee and government authorities, and was conducted according to the ethical guidelines of the declaration of Helsinki (revised in 2000). All patients provided written informed consent, a detailed interview for history and GERD-related symptoms and physical examination.

The study included 123 randomly selected patients out of a large prospective cohort ($n=210$) aiming at the characterization of GERD. GERD was diagnosed according to the Montreal classification (16) including patients with typical reflux-related symptoms (heartburn or regurgitation) and/or endoscopic lesions. Patients were stratified by the endoscopic appearance of GERD: NERD ($n=46$), ERD ($n=50$), and patients without any clinical symptoms or endoscopic signs for GERD as controls ($n=27$). Erosive esophagitis was classified according to Los Angeles classification (17) comprising LA-A ($n=20$), LA-B ($n=24$), LA-C ($n=4$), and LA-D ($n=2$). For detailed demographic data see **Table 1A**. The patient groups were not matched for age and gender. Functional testing of reflux disease (pH metry, combined multichannel impedance (multichannel impedance/pH analysis)) was performed for few patients, only. Therefore, these data were not included in any of the statistical analysis.

Endoscopy

All patients underwent upper endoscopy (EGD under sedation (midazolam 2–5 mg intravenously) after an overnight fasting using a standard videogastroscope (GIFQ 160, Olympus Optical Europe, Hamburg, Germany). Endoscopic esophageal landmarks were defined as the gastroesophageal junction with the beginning of the gastric folds and the Z-line as the squamocolumnar junction. Erosive esophagitis was characterized according to Los Angeles classification. In the distal esophagus, three biopsies were taken 2 cm above the squamous–columnar junction at the 3 o'clock position. In case of erosions, specimens were taken 2 cm above the tip of the erosion or mucosal break. One biopsy was separately snap-frozen in liquid nitrogen and subsequently stored in 0.5 ml TRIZOL reagent (Life Technologies, Carlsbad, CA) at −80 °C until usage for molecular analysis. The two other biopsies were used for histopathology and immunohistochemical analysis and fixed in 4% neutral-buffered formalin.

Table 1. Demographic data

	Controls, $n=27$	NERD, $n=46$	ERD, $n=50$
(A)			
Gender (male/female)	8/19	7/39	27/23
Age (mean ± s.d.; range)	52.4±17.1 years; 20–79 years	48.2±14.4 years; 18–77 years	50.8±15.1 years; 20–79 years
(B) (Immunohistochemistry)			
	$n=10$	$n=14$	$n=14$
Gender (male/female)	3/7	6/8	9/5
Age (mean ± s.d.; range)	52.5±14.9 years; 20–76 years	46.0±13.3 years; 26–72 years	48.6±17.0 years; 20–77 years

ERD, erosive reflux disease; NERD, non-erosive reflux disease.

Quantitative RT-PCR analysis of PAR-2 and IL-8 gene expression

Total RNA extraction and complimentary DNA synthesis were performed using a two-step protocol as described previously (18). In each case, 500 ng of total RNA was transcribed using random hexanucleotides in a final volume of 40 µl, from which 1.2 µl was used for quantitative reverse transcription (RT)-PCR. The RT-PCR analysis was performed using an iCycler device (BioRad, Munich, Germany). A typical 30-µl reaction mixture consisted of 15-µl QuantiTect Sybr. Green Master Mix (Qiagen, Hilden, Germany), 1.2 µl of the RT-reaction, and 0.25 µM of the specific primers for PAR-2 or β-actin. Initial denaturation and activation of Taq-polymerase at 95 °C for 15 min was followed by 40 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Initial PAR-2 and β-actin transcript levels were calculated using the iCycler software and a standard curve obtained from a plasmid-derived standard curve containing the identical complimentary DNA fragment. The β-actin mRNA amounts were used for normalizing the complimentary DNA content for each sample. The resulting ratio is expressed as artificial units. The size of the resulting PCR product was verified by gel electrophoresis. The following primers were used for the RT-PCR analysis: PAR-2 (5′-CAC-CAT-CCA-AGG-AAC-

CAA-TAG-3′; 5′-AAT-TGG-AAG-GAA-GAC-AGT-GGT-C-3′, 169 bp), β-actin (5′-CAT-GCC-ATC-CTG-CGT-CTG-GAC-C-3′, 5′-ACA-TGG-TGG-TGC-CGC-CAG-ACA-G-3′, 400 bp); IL-8 (5′-TTG-AGA-GTG-GAC-CAC-ACT-GCG-3′, 5′-TGG-CAA-CCC-TAC-AAC-AGA-CCC-3′, 246 bp). As the amplified complimentary DNA fragment of PAR-2, IL-8, and β-actin included intron-spanning regions, the identified PCR products could be exclusively attributed to the corresponding mRNA pool of the sample.

Histopathology

The formalin-fixed biopsies were routinely processed, paraffin-embedded, and stained with hematoxylin and eosin and periodic acid-Schiff stain. Using standard light microscopy, histological evaluation of esophagitis was performed according to the Ismail-Beigi criteria (4) modified by Vieth *et al.* and previously published (19–21), including the (1) density of intraepithelial lymphocytes (chronic inflammation), (2) BCH, (3) presence of PE, and (4) the DIS. All histological parameters were semiquantitatively scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) (for details see **Table 2**).

Immunohistochemical analysis of PAR-2 expression

Immunohistochemistry was performed using the avidin–biotin complex immunostaining method and the automated immunohistochemistry slide staining system by Ventana NexES (Ventana Medical System, Strasbourg, France). Three-μm thick, formalin-fixed, paraffin-embedded serial sections were deparaffinized and dehydrated. For antigen retrieval, pretreatment was performed by microwave heating in 1 mM sodium citrate buffer (30 min, 600 W, pH 6.0). Incubation of each one series with anti-PAR-2 (rabbit polyclonal antibody no. SP4476P, dilution 1:100, Acris Antibodies GmbH, Herford, Germany) was conducted at 37 °C for 32 min and followed by phosphate-buffered saline washing. Positive immunohistochemical reactions were revealed using the iVIEW DAB Detection Kit (Ventana, Tuscon, AZ) as chromogen substrate. Specimens were counterstained with hematoxylin and mounted using DEPEX. For negative controls, primary antibody was replaced by irrelevant polyclonal rabbit serum that did not reveal specific signals. The proximal tubular epithelium of human kidney was used as positive control (data not shown).

Samples were examined by one experienced pathologist (D.K.) blinded to the group assignment. PAR2 immunoreactivity was scored for each sample in five representative high power fields (Zeiss Axioskop 50, Jena, Germany). For PAR-2, the staining intensity and the percentage of positive cells were semiquantitatively assessed using the following score: staining intensity was classified into 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong); percentage of positive cells: 0 (no positive cells), 1 (< 10%), 2 (10%–50%), 3 (51%–80%), and 4 (> 80%). For each slide, the immunoreactive score was calculated as (staining intensity×percentage of positive cells) with a possible maximum score of 12.

In vitro studies using esophageal cell lines

Squamous epithelial cell lines, KYSE 150 and KYSE 450, were purchased from American Type Culture Collection (Manassas, VA). Esophageal cells were maintained in 75 cm^2 cell culture flasks

Table 2. Histopathological changes related to NERD and ERD

	Controls	NERD	ERD	*P* value (one-way ANOVA) (*post hoc* analysis)
Chronicity	0.70±0.10	1.00±0.07	1.18±0.09	0.003
Control vs. NERD				0.034
Control vs. ERD				0.001
NERD vs. ERD				NS
Basal cell hyperplasia	0.56±0.11	1.04±0.09	1.46±0.12	<0.001
Control vs. NERD				0.006
Control vs. ERD				<0.001
NERD vs. ERD				0.005
Papillary elongation	1.30±0.15	1.54±0.13	2.20±0.11	<0.001
Control vs. NERD				NS
Control vs. ERD				<0.001
NERD vs. ERD				<0.001
Dilated intercellular spaces	0.74±0.13	1.46±0.15	2.10±0.13	<0.001
Control vs. NERD				0.001
Control vs. ERD				<0.001
NERD vs. ERD				0.001

ANOVA, analysis of variance; ERD, erosive reflux disease; NERD, non-erosive reflux disease; NS, not significant.

(NUNC GmbH, Wiesbaden, Germany) in a cell incubator at 37 °C and 5% CO_2. KYSE-150 cells were cultivated in 49% RPMI-1640, 49% Ham's F12 medium containing 2% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamycin. KYSE-450 cells were grown in 45% RPMI-1640, 45% Ham's F12 medium containing 10% fetal calf serum and antibiotics as KYSE-150. All reagents were purchased from PAA (Colbe, Germany).

Media were adjusted to pH values of 7.0, 6.0, and 5.0 using 0.1 M HCl solution. Dihydrocholic acid, cholic acid (CA), and deoxycholic acid were purchased from Sigma (Taufkirchen, Germany). Stock solution (each 1 M) was prepared in 100% dimethylsulfoxide (for dihydrocholic acid) and 75% dimethylformamide (for CA, deoxycholic acid). Final concentration used for incubation was 50 µM for all three bile acids. Incubation studies at different pH in the presence of bile acids were performed in 25 cm^2 culture flasks for 24 h. Cells were seeded at a concentration of 10^6 cells per 10 ml pH-adjusted medium with/without bile acids. After 24 h incubation, 1 ml medium was collected, subjected to centrifugation (12,000×*g*, 15 min, room temperature) and 0.5 ml of the resulting supernatant was stored at −80 °C until analysis. Cells attached to the flask were washed three times with phosphate-buffered saline (pH 7.4), and then harvested by phosphate-buffered saline (pH 7.4) using a cell-scraper, washed once and suspended in 1-ml phosphate-buffered saline (pH 7.4). Cells were collected by centrifugation and pellets were resuspended in RLT-buffer for RNA-extraction (Qiagen). For each experiment (pH 5–7 including individually dihydrocholic acid, CA, or deoxycholic acid) at least three independent experiments, including corresponding controls, were performed.

Aiming the interaction between PAR-2 expression/activation and IL-8, experiments focused KYSE-450 cell line and CA only. KYSE-450 cells (3.5×10^5) were seeded in 3 ml appropriate media (see above) in six-well culture plates (Nunc GmbH). Three independent experiments were performed using two wells for each setting (1× RNA, 1× protein lysate, and supernatant). Cells were cultivated for 24 h, before treatment with acidified media (with/without cholic acid) was initiated. Investigating PAR-2 activation in context to IL-8 expression, PAR2-activating peptide (PAR-2-AP) "SLIGKV-NH_2" (H-5042, Bachem Distribution Services GmbH, Weil am Rhein, Germany) was added at a concentration of 100 µM adjusted to pH 7.0, 6.0, and pH 5.0. Cells were harvested after 24 h incubation for analyses.

For analyzing IL-8 expression on protein levels, 1-ml cell culture supernatant was obtained and microcentrifuged (14,000×*g*, 4 °C) for 15 min, and the resulting supernatant was transferred into a new tube, and frozen at −80 °C until analysis. The corresponding cell pellet was resuspended in protein lysis buffer containing 0.5% sodium dodecyl sulfate, 0.5% Triton X-100, 0.5% Tween 20, 10% (v/w) glycerol, 62.5 mM TRIS (pH 6.8), and protease inhibitor cocktail (Roche, Mannheim, Germany), homogenized on ice using a homogenizer (Polytron, Kinematica, 500W, Luzern, Switzerland), subjected to sonification (3×20 s, 4 °C, 300 W) and incubated on ice for 15 min. The lysate was microcentrifuged (14,000×*g*, 4 °C) for 15 min, and the resulting supernatant was transferred into a new tube. The total protein content of the sample was analyzed using the Advanced protein assay (Tebu, Offenbach, Germany) according to manufacturer's protocol. Protein samples were aliquoted and stored at −80 °C until measurement. IL-8 levels in lysate and supernatant were quantified using the Quantikine IL-8 Kit (R&D Systems, Minneapolis, MN) as described by the manufacturer. Finally, cellular IL-8 levels were normalized using the protein content of the corresponding sample and expressed as pg per 10 µg total protein lysate, while corresponding levels from supernatant are presented as pg per 50 µl.

Statistical analysis

All data were entered into a database using the Microcal Origin 6.0 program package (Northhampton, MA) and SPSS 12.0. Data are expressed as raw, median, mean±s.e., or 95% confidence intervals, if not stated otherwise. Nonparametric Kruskal–Wallis test and Dunn's multiple comparisons for pairwise comparisons between groups were applied for gene expression analysis. Histopathological parameters were analyzed by one-way analysis of variance (as global test for multiple testing) and least significant difference as *post hoc* analysis for pairwise comparisons if global test reached significant level. Nonparametric correlation analysis was performed by Spearman's rank correlation test. All tests were applied two-sided with a level of significance of $P<0.05$.

RESULTS

Patients' characteristics and histopathological alterations in patients with GERD

As illustrated in **Table 1**, the three groups (controls, NERD, ERD) were similar with respect to age, but differed in their gender ratio. Male gender was represented significantly higher for ERD compared with NERD and controls ($P<0.05$). Demographic data of the subgroups selected for immunohistochemical analysis were concordant to those of the corresponding complete group (**Table 1**). Histopathological evaluation revealed very low activity scores (0–0.25) among the three groups (data not shown), and elevated scores for chronicity, DIS, PE, and BCH ($P=0.003$ to $P<0.001$; **Table 2**). It is to be noted that a gradual increase of all four scores was observed from controls toward NERD and ERD (**Table 2**).

GERD is characterized by elevated PAR-2 expression

In patients with GERD, normalized PAR-2 transcript amounts were 7- to 10-fold elevated in the esophageal mucosa of patients with GERD (**Figure 1a**, $P<0.0001$). No differences were found for PAR-2 mRNA levels between NERD and ERD. An analysis of patients with ERD only showed that the increase of PAR-2 gene expression was independent from the endoscopic severity (Los Angeles classification, data not shown).

To confirm transcriptional induction and to identify the cellular origin of PAR-2 expression in the esophageal mucosa, immunohistochemical analysis was performed in a representative subset of patients (**Table 1B**). Controls presented a weak intensity of PAR-2 immunoreactivity (**Figure 1c, e**), whereas corresponding samples of patients with GERD (NERD and ERD) showed an intense PAR-2 immunoreactivity throughout all epithelial layers, exemplarily

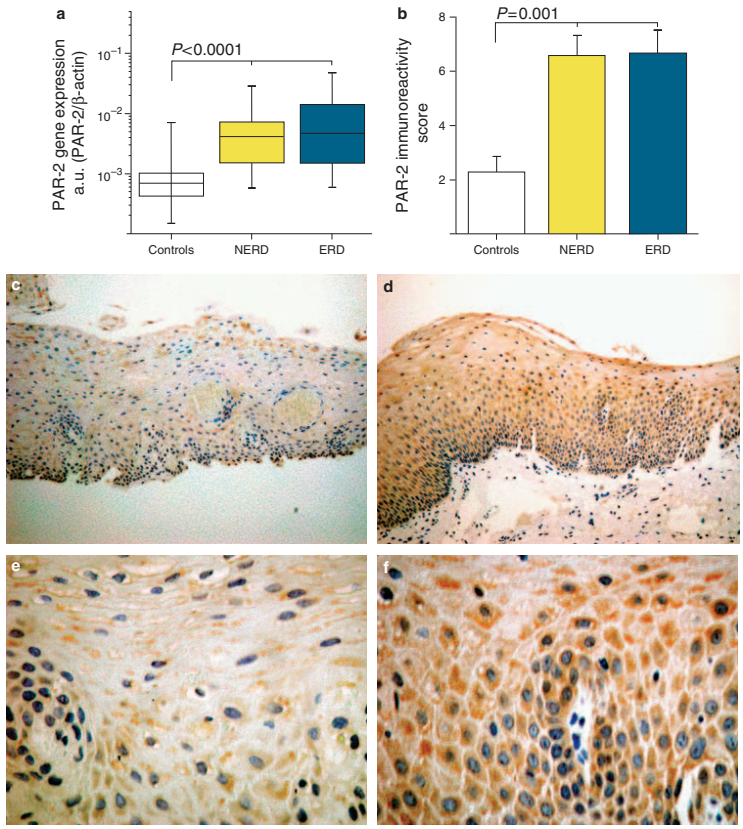

Figure 1. Proteinase-activated receptor-2 (PAR-2) is elevated in patients with non-erosive reflux disease (NERD) and erosive reflux disease (ERD). The upper panel presents the data of reverse transcription (RT)-PCR analysis (**a**) and immunohistochemistry (**b**) of PAR-2 expression. Data are presented as box plots illustrating 25th and 75th percentiles, median, and 5–95 range (**a**), and as columns illustrating means±s.e. of PAR-2 immunoreactivity (**b**). Immunohistochemical staining of PAR-2 in controls (**c, e**) and NERD (**d, f**) is displayed in the lower panels. High magnification (×400, **e**, **f**) illustrates an intense PAR-2 expression throughout all epithelial layers in NERD (**f**) compared with controls (**e**). In addition to membrane-associated staining, an intensive cytosolic staining of PAR-2 is visualized for NERD (**f**) (Zeiss Axioskop 50; original magnification: ×100, ×400; camera: Nikon coolpix 990 (Nikon, Düsseldorf, Germany)).

shown for one patient with NERD (**Figure 1d, f**). Detailed evaluation revealed the elevated PAR-2 protein expression identified at the epithelial cell surface as well as in the cytosolic cell compartment. Calculating a semiquantitative immunoreactivity score, patients with GERD presented significantly higher values compared with controls (**Figure 1b**; mean control: 2.3±0.52 vs. NERD: 6.57±0.75 vs. ERD: 6.64±0.88; $P=0.001$). No differences between the immunohistochemical scores of NERD and ERD were noted.

PAR-2 expression correlates with histopathological alterations and with IL-8 secretion

Correlation analysis including all 123 data sets of quantitative RT-PCR analysis revealed a positive correlation of increased PAR-2 transcript levels with the histopathological alterations DIS, PE, and chronicity (**Figure 2a–c**). This finding is further supported by corresponding data from 38 patients being analyzed by immunohistochemistry. Here, a correlation was identified for chronicity ($P<0.001$) and trends were observed for the two other parameters, which marginally missed statistical significance (P values <0.10, **Figure 2d–f**); limited by the smaller sample size for immunohistochemistry especially for advanced histopathological changes (grade 3).

Accentuating the functional importance of a PAR-2-mediated pathway in GERD, we were able to demonstrate a positive correlation of PAR-2 gene expression and mucosal IL-8 transcript levels for patients with GERD (**Figure 3**, $P=0.0006$).

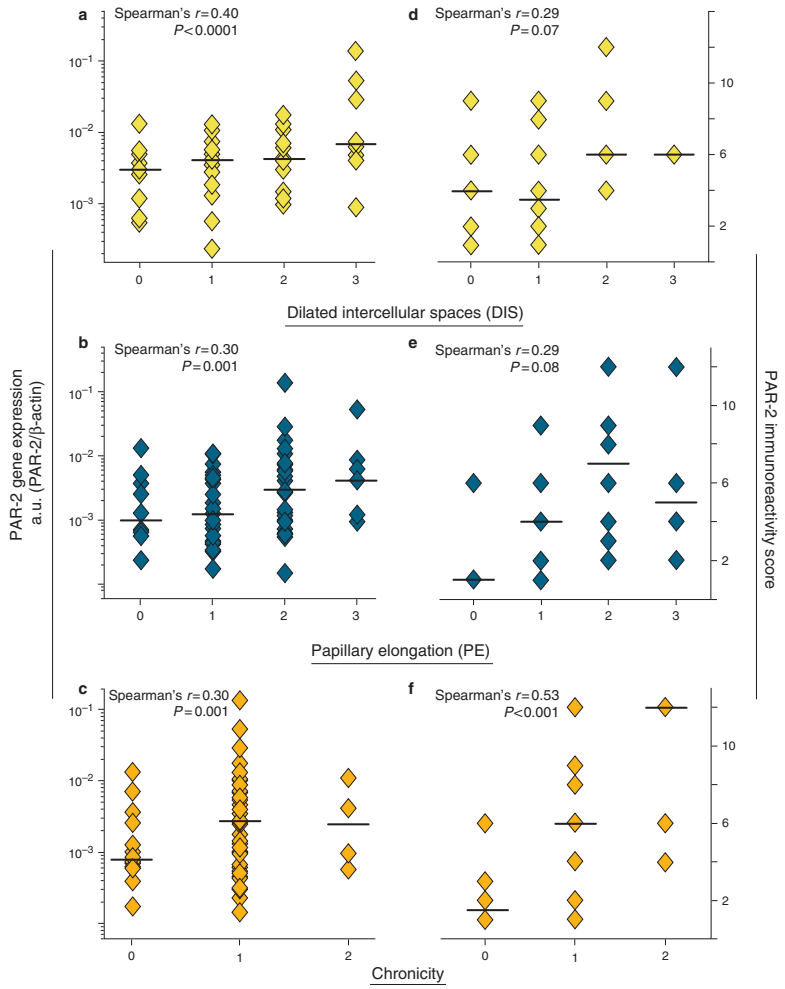

Figure 2. Positive correlation of proteinase-activated receptor-2 (PAR-2) with histopathological changes dilated intercellular spaces (DIS), papillary elongation (PE), and chronicity. The left panel presents quantitative reverse transcription (RT)-PCR analysis (**a**–**c**), whereas the results of PAR-2 immunoreactivity are illustrated at the right panel (**d**–**f**). Each set represents either the correlation of PAR-2 transcript levels (**a**–**c**) or PAR-2 immunoreactivity score (**d**–**f**) with DIS, PE, and chronicity of inflammation, respectively. Individual data are illustrated as squares, medians are presented as bars. Note that because of identical or closely overlapping values/scores not all individual data of the 123 patients (**a**–**c**) and 38 patients (**d**–**f**) are illustrated.

Acidification induces PAR-2 gene expression in esophageal cell lines independently from additional bile acids

To confirm *ex vivo* data regarding the induction of PAR-2 transcript levels in relation to GERD, the esophageal cell lines KYSE-150 and KYSE-450 were incubated at different pH and in combination with three different bile acids. As illustrated in **Figure 4a** and **c**, a 1.5- to 20-fold induction of PAR-2 gene expression by acidified medium was observed ($P<0.01$). Note that this effect was similarly seen in both cell lines, and found to be independent of the divergent basal PAR-2 expression levels (**Figure 4a, c**). The additional incubation with various bile acids at different pH did not result in a further increase of PAR-2 transcription (**Figure 4b, d**).

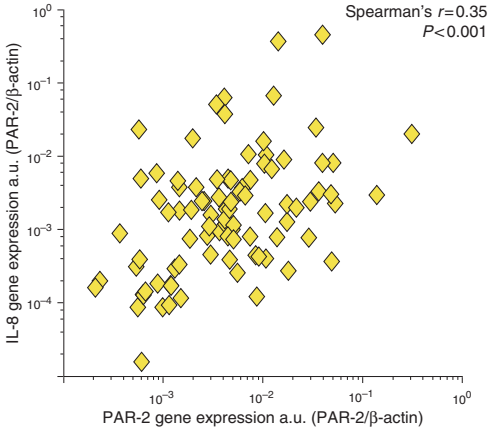

Figure 3. Proteinase-activated receptor-2 (PAR-2) expression is positively correlated with interleukin (IL)-8 in gastroesophageal reflux disease (GERD). Data are presented as scatters illustrating IL-8 transcript related to PAR-2 expression for each patient with GERD (erosive reflux disease and non-erosive reflux disease). a.u., artificial units.

PAR-2-activation mediates IL-8 secretion in esophageal cell line KYSE-450

To further establish the causality between increased PAR-2 expression and elevated IL-8 levels, KYSE-450 cells were exposed to acidic media in the presence of PAR-2-AP (**Figure 5**). The activation of PAR-2 led to a 2.9-fold increase of IL-8 transcript levels (**Figure 5a**) and 3.2-fold higher IL-8 secretion into supernatant (**Figure 5c**). In the cell lysate, IL-8 protein was found to be elevated at pH 6, independent from PAR-2-AP (**Figure 5b**). At pH 5, we could not demonstrate an induction of IL-8 expression/secretion. Experiments in the presence of 50 µM CA led to similar results (data not shown).

DISCUSSION

PAR-2 expression is increased in the esophageal epithelial mucosa of patients with GERD, independently from endoscopic severity. Moreover, PAR-2 appears expressed at the same magnitude in patients with ERD and NERD. Although PAR-2 was found to be expressed only in the superficial strata of the mucosa in a recent study (22), we identified an intense PAR-2 expression in all epithelial layers of the esophageal mucosa. In addition to

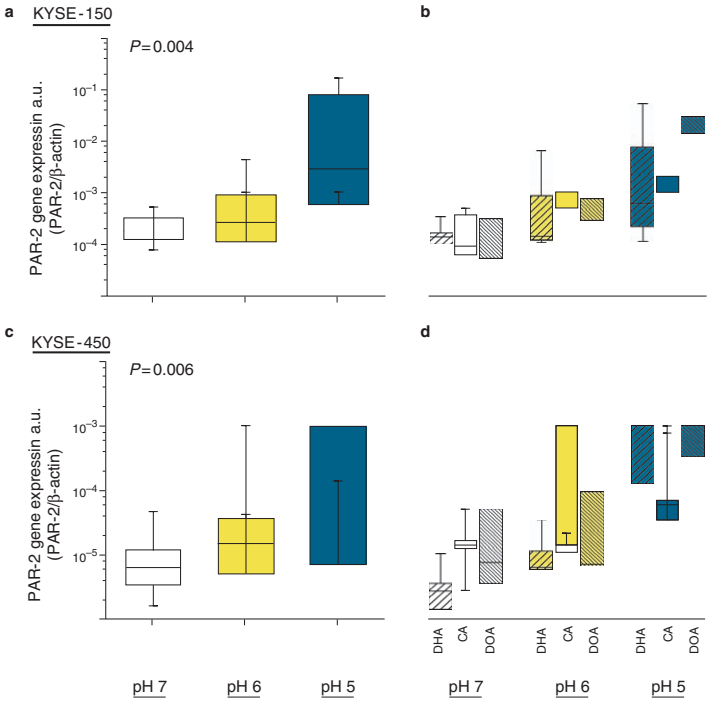

Figure 4. Induction of proteinase-activated receptor-2 (PAR2) gene expression in esophageal cell lines by acidified medium (**a**, **c**) and in combination with bile acids (**b**, **d**). KYSE 150 (**a**, **b**) and KYSE 450 (**c**, **d**) were cultivated at pH 7.0–5.0 and in the presence of 50 µM bile acids (dihydrocholic acid (DHA), cholic acid (CA), deoxycholic acid (DOA)) (**b**, **d**). Data are illustrated as box plots that represent the 25th, 50th, and 75th percentile values (horizontal lines of the box). Note that because of the logarithmic Y-scale, "0" (=0) cannot be shown, and that because of odd data distribution (e.g., missing expression) not all boxes are complete.

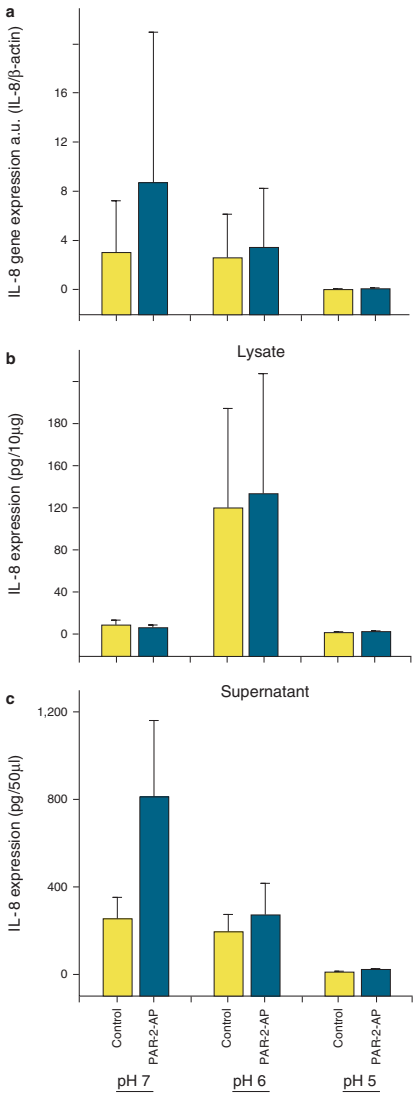

Figure 5. Proteinase-activated receptor-2 (PAR-2)-mediated interleukin (IL)-8 secretion in esophageal cell line (KYSE-450). KYSE-450 was exposed to acidified media (pH 7.0, 6.0, 5.0) in the presence of PAR-2-AP (100 µM). IL-8 expression is presented as columns illustrating means±s.e., demonstrating IL-8 gene expression at the left panel (**a**) and IL-8 protein amounts in the cell lysate (**b**) and supernatant (**c**).

the membrane-associated PAR-2 immunoreactivity on esophageal squamous cells, we detected an intense cytosolic staining of PAR-2. As PAR-2 undergoes a β-arrestin-dependent receptor endocytosis after its activation (23,24), we suggest that this cytosolic PAR-2 pattern indicates PAR-2 receptor activation as well as internalization and therefore PAR-2-mediated pathways being involved in the pathogenesis of mucosal abnormalities in GERD.

Previous studies demonstrated a marked inflammatory response to PAR-2 activation (25) with an induction of inflammatory cytokines (IL-8) and mediators (COX-2) in esophagus epithelial cell lines (15). Proinflammatory changes with increased IL-8 and IL-1β expression in the esophageal mucosa of patients with GERD have been reported earlier, but the mechanisms of cytokine release related to GERD has never been comprehensively investigated or linked to PAR-2 activity (20,26,27). Our study confirmed the previous *in vitro* findings (15). We complemented these findings by determining a positive correlation of PAR-2 between mucosal IL-8 gene expression and elevated PAR-2 transcript levels, which is associated with inflammatory changes in GERD. However, this association does not prove causality between the induction of PAR-2 and increased IL-8 levels *in vivo*. To extend the *ex vivo*-derived data, *in vitro* experiments with squamous epithelial cell lines were performed demonstrating an induced PAR-2 gene expression in response to acidic pH. The two cell lines KYSE-150, -450 were chosen as they represent the squamous epithelial phenotype, which correspond better to the *in vivo* situation than adenocarcinoma-derived cell lines often used in GERD-related *in vitro* studies.

IL-8 gene expression and secretion was found to be induced after PAR-2 activation. The variable induction of IL-8 in the investigated pH range might be explained by a pH-dependent activity of PAR-2-AP. These results imply different regulatory mechanisms of PAR-2-mediated IL-8 expression *in vitro* (transcription, translation, protein secretion) that were not further investigated in this study.

This model allows to speculate that PAR-2 and IL-8 expression are upregulated even by minor acidic reflux episodes (28,29) and IL-8 is released from esophageal mucosa by PAR-2-activation mediated by intraluminal serine proteases, such as pancreatic trypsin in the refluxate. These *in vitro* results still need to be proven for serine proteases *in vivo*. The presence of duodenogastroesophageal reflux has been suggested to be responsible for persistent symptoms in NERD patients not responding to standard acid suppressive medication (30,31). Besides bile acids, serine proteases, such as pancreatic trypsin or tryptase and cathepsin G (released by inflammatory cells), are components of the refluxate. The interaction of proteases with specific serine PARs, such as PAR-2 during reflux episodes, represents a possible pathophysiological mechanism for increased IL-8 secretion and proinflammatory changes in these patients. In this context, it is notable that functional testing has not been performed in all patients. Therefore, we cannot link the expression patterns of PAR-2 and IL-8 to the characteristcs of the refluxate in these patients. New prospective studies, which have been initiated, will address this question in future.

In our study, DIS, PE, and BCH have been detected as typical histomorphological changes besides chronic inflammatory changes (chronicity) (19–21) in patients with ERD and NERD (**Table 2**). DIS in animal modes have been demonstrated when the esophagus was exposed to acid, pepsin, bile acids, and physical stress (32–34). DIS was associated with a reduced transepithelial electric resistance and increased transepithelial permeability in the rabbit or mouse model, and therefore considered to be an important step in the pathogenesis of GERD (32,34). Regarding DIS and the transepithelial permeability, PAR-2 activation was demonstrated to lead to calmodulin-dependent phosphorylation of myosin light chain kinase (35) as well as to a rearrangement of junctional proteins and F-actin (36), leading to the dilatation of tight junctions at least in colonic epithelium, but further studies are warranted regarding PAR-2-mediated mechanisms involving cytoskeleton rearrangement and morphological changes in GERD.

An important issue of the study might be the definition of patients with NERD. Characterizing our patients, 24-h pH metry was not performed in all patients, but all patients presented with characteristic reflux-related symptoms according to the Montreal classification, and furthermore were responsive to proton pump inhibitor medication.

In summary, this is the first study that demonstrates an induction of PAR-2 expression in the esophageal mucosa of patients with GERD correlating with histomorphological changes (DIS, BCH, PE), inflammatory changes (chronicity), and elevated IL-8 expression. In an esophageal cell line model, PAR-2 expression was induced under acidic conditions, and IL-8 secretion was mediated by PAR-2 activation implying a reasonable mechanism for proinflammatory changes in GERD. The histomorphological changes such as DIS, PE, and BCH in GERD associating with PAR-2 suggest a functional role for the pathogenesis of these specific morphological changes—at least stating PAR-2 as a molecular marker for GERD. Further examinations need to investigate PAR-2-mediated effects on permeability, hypersensitivity, and generation of symptoms in patients with GERD as well as on the signaling pathway involved in the PAR-2-mediated upregulation of IL-8.

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CONFLICT OF INTEREST

Guarantor of the article: Peter Malfertheiner, MD.

Specific author contributions: Arne Kandulski: design of the study, acquisition of immunohistochemical data, statistical analysis and interpretation of data, and writing the article and revising it for important content; Thomas Wex: design of the study, acquisition of molecular data from *ex vivo* and *in vitro* experiments, statistical analysis and interpretation of data, and writing the article and revising it for important content; Klaus Mönkemüller and Lucia C. Fry: acquisition of clinical data, performing endoscopy, and revising the manuscript for important content; Doerthe Kuester: acquisition and interpretation of histological and immunohistochemical data, and revising it for important content; Albert Roessner: acquisition of histological data; Peter Malfertheiner: design of the study and revising the manuscript for important content.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Dilated intercellular spaces, papillary elongation, and basal cell hyperplasia are specific histomorphological alterations in gastroesophageal reflux disease (GERD).
- ✓ Results from animal models and irritable bowel syndrome studies provide evidence of intraluminal proteases and their receptors in the pathogenesis of disrupted epithelial barrier function, (neuro)inflammation, and symptom generation.

WHAT IS NEW HERE

- ✓ Proteinase-activated receptor-2 (PAR-2) is ubiquitously expressed in all layers of the esophageal mucosa.
- ✓ Patients with GERD (non-erosive reflux disease and erosive reflux disease) exhibit induced PAR-2 expression and activation of PAR-2-mediated pathways, which is documented by cytosolic translocation.
- ✓ Elevated PAR-2 expression correlates with histomorphological changes of GERD.
- ✓ Acidic pH induces PAR-2 and interleukin (IL)-8 gene expression in squamous epithelial cell lines.
- ✓ PAR-2-activation induces IL-8 secretion from epithelial cell lines.

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Publikation X

Gastroesophageal reflux disease—from reflux episodes to mucosal inflammation

Arne Kandulski and Peter Malfertheiner

Abstract | Gastroesophageal reflux disease (GERD) affects 20–30% of the population in Western countries, and is one of the most common clinical problems in daily practice. GERD-associated functional and structural abnormalities are caused by recurrent exposure of the esophagus to acidic and nonacidic refluxate of gastric contents (containing duodenal and intestinal proteases as well as acid and gastric pepsin) from the stomach. Major progress has been made in the understanding of the molecular pathogenesis of GERD-associated mucosal inflammation, suggesting a complex and multifactorial pathogenesis and immune-mediated effects. This Review summarizes the complexity of mucosal pathogenesis, including microscopic changes, mucosal inflammation and GERD-specific molecular mediators, in the context of the clinical features and pathophysiological characteristics of GERD. The abnormal exposure of the esophagus to luminal contents leads to chronic mucosal inflammation that is characterized by the release of IL-8 specifically, as well as other proinflammatory mediators, from the esophageal mucosa. Evidence from animal studies indicates a stepwise inflammatory response by the epithelium, which attracts immune effector cells to infiltrate the mucosa. From bench to bedside, these novel molecular findings might provide new treatment options beyond current acid-suppressive therapy and the principle of inhibition of transient lower esophageal sphincter relaxation.

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Introduction

Gastroesophageal reflux disease (GERD) is a chronic disorder that is caused by abnormal reflux with prolonged exposure of the distal esophagus to gastric (gastroduodenal) contents and leads to cardinal symptoms—heartburn and regurgitation. Substantial progress has been made in our understanding of the mucosal pathogenesis of GERD, with novel aspects of the underlying disease mechanisms and implications for medical management identified. This Review summarizes the complexities of mucosal pathogenesis in GERD and integrates the updated concepts of mucosal inflammation into the current model of GERD pathogenesis, with a focus on the disease in adults. Discussion of Barrett esophagus and its specific pathophysiology were excluded from this Review, but have been reviewed comprehensively elsewhere.[1,2]

Epidemiology

The prevalence of GERD in adults is high; in Western countries, up to 20–30% of the adult population have typical reflux-related symptoms (for instance, heartburn or regurgitation) at least twice a week.[3,4] This morbidity leads to a loss of quality of life[5,6] and represents a substantial burden for national health-care systems.[7] The accurate diagnosis of GERD represents a challenge as only 50% of patients with GERD present with the typical symptoms of heartburn and regurgitation,[8] whereas others with heartburn might not even have GERD. In a large Australian observational study,[8] GERD was accurately diagnosed by pH-metry over a prolonged 48 h period (using wireless pH monitoring). Heartburn and regurgitation were found in only 49% of confirmed GERD cases.[8] The prevalence of other troublesome symptoms in confirmed GERD cases were dyspepsia (discomfort or pain in the center of the upper abdomen) in 21.2%, bloating in 9.4% and abdominal pain or discomfort (not dyspepsia) in 9.9% of cases.[8] Extraesophageal symptoms were not reported in the study. Sensitivity and specificity for the accuracy of symptom-based diagnosis were 62% and 67%, respectively, for the RDQ questionnaire, 63% and 63%, respectively, for family practitioners and 67% and 70%, respectively, for gastroenterologists.

Up to 70% of patients with reflux symptoms have overlapping symptoms such as dyspepsia, constipation or diarrhea.[8] Extraesophageal manifestations might be causally related to, or even in some cases the only clinical presentation of, GERD.[9–11] In patients without abnormal reflux episodes during reflux monitoring and without a notable symptom association, the diagnosis is functional heartburn; that is, symptoms without any evidence of GERD.[12,13]

Competing interests
P. Malfertheiner declares associations with the following companies: Movetis, Novartis. See the article online for full details of the relationships. A. Kandulski declares no competing interests.

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Key points

- GERD-associated mucosal inflammation is characterized by epithelial release of IL-8 and other proinflammatory markers
- PAR2 expression is upregulated in patients with GERD and induced by acid conditions in cell-culture models; PAR2 activation leads to epithelial IL-8 release and contributes to the pathogenesis of GERD
- Structural abnormalities and microscopic changes in GERD are characterized by papillary elongation, basal cell hyperplasia, dilated intercellular spaces and an infiltrate of immune cells, and can even be identified using light microscopy
- Beyond PPI therapy, new pharmacological targets include mechanisms related to transient lower esophageal sphincter relaxation and mechanisms involved in symptom perception (TRPV1, cannabionoid receptors)

In Western countries, 30% of patients with GERD-related esophageal symptoms present with erosive changes to the esophageal mucosa (erosive reflux disease, ERD), whereas 60% of the patients have no visible lesions in the esophagus (nonerosive reflux disease, NERD).[14] 10% of patients with GERD-related symptoms have Barrett esophagus;[14] in these patients, the squamous epithelium in the distal esophagus is replaced by metaplastic columnar lined epithelium and is considered a preneoplastic lesion for distal esophageal adenocarcinoma (with an annual rate of 0.5–1%).[15–17] In the long term, the progression of the different endoscopic entities from NERD to ERD, and even to Barrett epithelium, is only observed in a small subset of adults and does not seem to have major clinical relevance.[18,19] Similar well-designed, longitudinal studies for infants and children are, however, lacking.[20,21]

GERD pathophysiology

Abnormal gastroesophageal reflux

GERD is caused by abnormal reflux and prolonged exposure of the distal esophagus to gastric (gastroduodenal) contents.[22] Established pathogenic mechanisms of gastroesophageal reflux include frequent episodes of transient lower esophageal sphincter relaxation (TLESR), which is associated with reflux events into the distal esophagus.[7,23] The presence of a hiatal hernia, obesity and medications that interfere with the function of smooth muscle cells (that is, nitrates, calcium-channel blockers, anticholinergic agents, among others) favor the development of GERD with abnormal TLESR and frequent reflux episodes.[24,25]

Limitations of conventional therapy

Erosive esophagitis and the induction of heartburn are thought to occur as a consequence of abnormal gastric acid exposure.[26] Patients with heartburn are usually responsive to treatment with adequate doses of PPIs, according to current international guidelines.[27,28] A substantial proportion of patients (up to 40%), however, especially those with NERD, continue to have symptoms even after optimal dosing of acid-suppressive medication.[29,30] Explanations for inadequate PPI responsiveness are inaccurate diagnosis (as heartburn can occur in conditions other than GERD), or insufficient effect of acid-suppressive therapy.[31] Experiments using intraesophageal perfusion of acidic solutions showed perception of heartburn already at pH 6 in 50% of the patients tested who had symptomatic gastroesophageal reflux.[32] Reflux episodes from exposure to acid levels of pH 4–7 (determined by a course of ambulatory 24 h pH monitoring) were associated with heartburn symptoms in about 30% of the patients with GERD.[33] Combined multichannel intraluminal impedance and pH (MII–pH) monitoring in patients with GERD revealed that even weakly acidic reflux episodes are capable of inducing heartburn and regurgitation.[34–38] Two independent studies, both including >100 patients with persistent GERD symptoms despite acid-suppressive therapy, showed a positive association with typical symptoms (such as heartburn) and nonacidic reflux episodes in approximately one-third of the patients.[39,40] In both studies, 50% of the patients' refractory symptoms were not associated with any reflux episodes at all and were considered functional heartburn.[38,40] Several sequential MII–pH-monitoring studies (with and without PPIs) demonstrated that PPIs do not reduce the total number, or the proximal extent, of reflux episodes, but instead affect only the acidity of the refluxate.[36,41] Other potential causes of PPI failure are insufficient inhibition of gastric acid secretion in a small subgroup of patients[42,43] and noncompliance to therapy.[44]

Mucosal aspects of GERD pathogenesis

Morphological changes to esophageal mucosa

In 1970, Ismail-Beigi and colleagues[45] first described basal cell hyperplasia and papillary elongation as histomorphological changes in the squamous epithelium of patients with GERD. Hopwood,[46] and subsequently Tobey and co-workers,[47,48] described a dilation of intercellular spaces in the GERD epithelium, and reintroduced these findings to the discussion of the pathogenesis of the disease. Several histological parameters and scoring systems for the diagnosis of GERD have been proposed, but no concordant view yet exists among different pathologists about their role in diagnosis, and the clinical relevance of these systems is also debated.[49–52] The histological findings of GERD were assessed by an international panel of gastrointestinal pathologists to determine common and standardized diagnostic criteria for GERD. A pairwise agreement of 73–97% for basal cell hyperplasia, papillary elongation, intraepithelial eosinophils, neutrophils and mononuclear cells, and a slightly lower agreement (64%) for dilated intercellular spaces, has been achieved for the histological definitions of GERD.[53] For the application of a combined severity score, the agreement reached a level of 77% with a κ value of 0.64, which was comparable with other accepted histological GERD definitions, but still needs further clinical validation.[53]

In the long term, after successful fundoplication or optimal acid-suppressive therapy, the 3-year follow-up of the LOTUS trial nicely demonstrated regression of the characteristic histological changes in patients with GERD after 1 year, which remained stable for 3 years.[54] In most patients, symptom relief persisted for 5 years, either on acid-suppressive medication or after fundoplication.[55]

Dilated intercellular spaces are typically present in individuals with ERD as well as those with NERD[47] and have been associated with impaired mucosal integrity and reduced transepithelial resistance. In animal models and also in healthy volunteers, dilated intercellular spaces are induced by acidic contents in the esophagus.[48,54–58] Interestingly, dilated intercellular spaces are also induced in the proximal esophagus that has not been exposed to acid.[59] Farré *et al.*[60] induced dilated intercellular spaces and impaired transepithelial resistance by acid infusion in a rabbit model. *In vivo*, these results correlated with reduced distal basal impedance that was determined by MII–pH monitoring before and after acid infusion; this reduced impedance was also confirmed in healthy human volunteers after acid perfusion.[60] The mechanisms that led to morphological alterations and rearrangement of the esophageal cytoskeleton were not evaluated in this study.

Mucosal inflammation

GERD-associated mucosal inflammatory cells include a moderate infiltration of lymphocytes, macrophages and mast cells in the esophageal mucosa.[48,61] Polymorphonuclear leukocytes (PMNs), which define this inflammatory activity, are present in up to 40% of patients with GERD.[62–65] The role of eosinophils—identified in some patients with GERD—needs to be critically re-evaluated.[63]

Over the past few decades, GERD has been assumed to be a caustic injury caused by damage to the squamous epithelium by acidic contents and peptidases, which is then followed by infiltration of inflammatory cells into the injured site to clear the cell detritus in the mucosa. The mucosal injury is traditionally believed to start at the luminal surface of the epithelium and to penetrate into deeper layers and submucosa, thus resulting in a proliferative response of the basal cell layers.[66] In this traditional model, hydrogen ions and gastric pepsin exert a corrosive effect on the surface of the esophageal mucosa and degrade junctional proteins, thereby destroying epithelial barrier function with the consequent induction of intramucosal inflammation.[65,66] The presence of chemokines and cytokines in the mucosa was traditionally explained to be released by infiltrating neutrophils and lymphocytes. The role of epithelial keratinocytes and other mucosal cell types (Table 1) has been ignored in GERD pathogenesis and the potential mechanisms and pathophysiological relationships between these cells remain insufficiently explained.

Mucosal cytokines and inflammatory mediators

The mucosal immune response in GERD is characterized by local cytokine release as the molecular pattern of inflammation. Different analytical methods (microarray, PCR, ELISA) demonstrate a predominant T-helper-1 (T_H1)-driven mucosal immune response in NERD and ERD that is characterized by the release of proinflammatory cytokines such as IL-8 and IL-1β. By contrast, Barrett esophagus is characterized by rather low levels of proinflammatory cytokines, with an enhanced expression of T-helper-2 (T_H2)-related cytokines, such as IL-4 and IL-10.[67,68] IL-10 and transforming growth factor β1 have been comprehensively studied for their effect on the activity and differentiation of T-regulatory (T_{REG}) cells. At the gastric cardia (non-Barrett epithelium), T_{REG} cells were not associated with GERD, but were typically found in the context of *H. pylori* infection of the cardia.[68]

Table 1 | Inflammatory mediators in GERD-associated mucosal inflammation

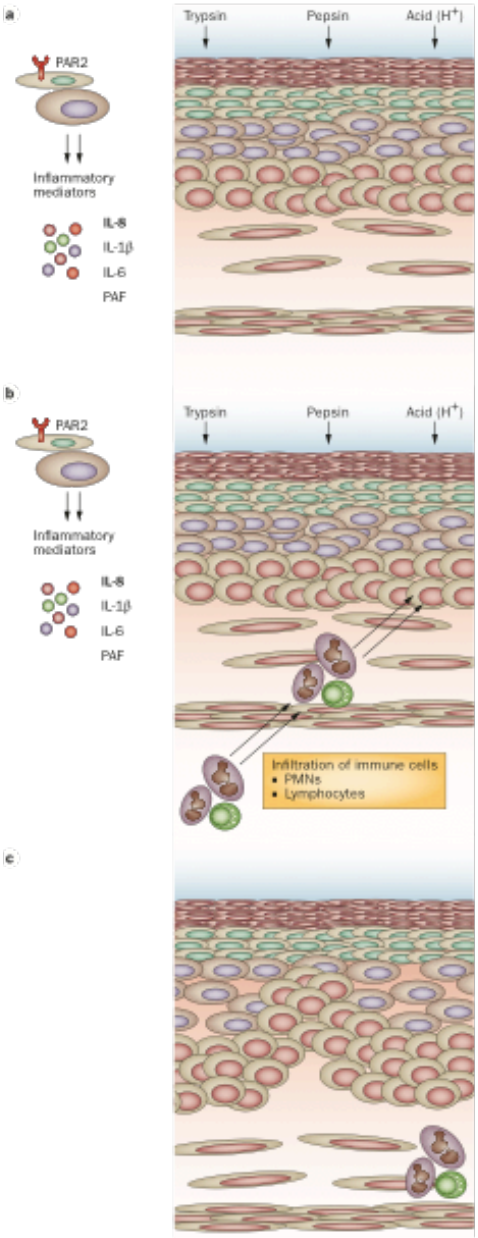
Inflammatory mediators	Source	Effect
IL-8; IL-1β; IL-6	Epithelial keratinocytes; immune cells (PMNs, lymphocytes, DCs); mesenchymal cells (fibroblasts, endothelium)	Chemoattraction; proinflammation
IL-4; IL-10	Immune cells (lymphocytes, T-regulatory cells, DCs)	Immunoregulation
PAF	Epithelial keratinocytes; mesenchymal cells (fibroblasts, endothelium)	Proinflammation; chemoattraction; activation of immune and nonimmune cells

Abbreviations: DCs, dendritic cells; GERD, gastroesophageal reflux disease; PAF, platelet-activating factor; PMNs, polymorphonuclear leukocytes.

The presence of IL-8, IL-1β and various other proinflammatory cytokines has been investigated in mucosal biopsy samples from patients with GERD, and they have been found to be localized in the esophageal keratinocytes in these patients,[70–72] and in animal models of GERD.[73–75] IL-8 and IL-1β are released at high levels in ERD and NERD.[70,72,76] Both cytokines correlate with endoscopic and histopathological severity and their levels decline after Nissen fundoplication or acid-suppressive therapy with PPIs.[69,70,72,76,77] IL-8 is a potent chemoattractant for, and activator of, PMNs and lymphocytes.[78] Other proinflammatory cytokines (such as IL-6) are increased in GERD, but inconsistent results are reported for levels of tumor necrosis factor (another key proinflammatory cytokine) in different animal models and in humans with GERD.[74,79] Platelet-activating factor is produced and released from esophageal mucosa after acid exposure and levels are increased in the mucosa of individuals with chronic esophagitis as well as in the circular muscle layer in a feline esophagitis model.[75,80,81]

Molecular signs of inflammation and mucosal inflammatory mediators are detected even before microscopic or macroscopic changes become apparent in those with GERD. In addition to immune cells, the different mucosal cell types (such as epithelial keratinocytes, mesenchymal cells and endothelial cells) are actively involved in mucosal inflammation and are an important source of inflammatory cytokines and mediators of inflammatory changes (Table 1).[82] The esophageal epithelium, when in contact with gastric acid contents, releases proinflammatory cytokines[71,75,83] and is the starting point for attracting and activating leukocytes in the esophageal mucosa and submucosa.

In 2009, Souza *et al.*[84] established a rat model of esophagitis induced by esophagoduodenostomy. They described the acid-induced secretion of epithelial IL-8 as an initial event in GERD, followed by an infiltration of lymphocytes and leukocytes into the submucosa and



◀ **Figure 1** | The inflammatory changes that occur in GERD pathogenesis. The schematic represents the changes that occur during reflux esophagitis in a rat model and are based on the findings of Souza and colleagues.[84] In the pathogenesis of GERD, **a** | an inflammatory response occurs in the squamous epithelium, induced by the release of inflammatory mediators, which **b** | leads to the subsequent chemoattraction and infiltration of immune cells and is **c** | followed by the proliferative response of the rat epithelium. Abbreviations: GERD, gastroesophageal reflux disease; PAF, platelet-activating factor; PAR2, protease-activated receptor 2; PMN, polymorphonuclear leukocytes.

later into the mucosa (Figure 1a,b). Subsequently, this inflammatory response is followed by a proliferative response of the mucosa (basal cell hyperplasia, papillary elongation; Figure 1c).[84] This hallmark histological study of esophagitis in a rat model of esophagoduodenostomy is not consistent with the previous concept of esophagitis, in which the initiating event is the caustic injury that originates from the epithelial surface. In the Souza *et al.*[84] experimental model, before esophageal erosions become apparent after 4 weeks, infiltration of lymphocytes is the initial microscopic event of mucosal inflammation, followed by the proliferative response with basal cell hyperplasia and papillary elongation, which implies an immune-mediated pathogenetic mechanism in GERD. On the basis of previous observations of increased mucosal cytokine expression, especially pro-inflammatory IL-8, in patients with GERD,[76,85] Souza and colleagues[84] hypothesized that a cytokine-mediated immune response is the primary mechanism that leads to inflammation and, finally, esophageal injury in GERD. In a cell-culture model with non-neoplastic, immortalized squamous epithelial cells, an increase of IL-8 and IL-1β secretion was observed at day 2 and 4 after exposing the cells to acidic bile salt media.[84] Yamaguchi and co-workers[73] described IL-8 secretion by epithelial keratinocytes in a cell-culture model and rat model of esophagitis, providing further support to the concept that the cytokine-mediated infiltration of inflammatory cells is the initial event in the pathogenesis of reflux esophagitis.

Role of PAR2 in the pathogenesis of GERD

Proteinase-activated receptor 2 (PAR2) is expressed on the surface of epithelial cells of the gastrointestinal and respiratory tract as well as by neuronal cells.[86,87] PAR2 is specifically activated by serine proteases such as trypsin and mast-cell-derived tryptase. These proteases cleave the *N*-terminal sequence of the PAR2 extracellular domain (which serves as a tethered ligand) and subsequently leads to receptor activation.[88,89] The specific activation of PAR2 in human esophageal cell lines leads to the release of IL-8.[71] PAR2 activation has been implicated in inflammatory and neuroinflammatory effects (by release of substance P and calcitonin gene-related peptide),[86,90] the modulation of visceral hypersensitivity and pain generation,[91,92] and increasing epithelial permeability.[93,94] Patients with erosive esophagitis and NERD have been found to have high levels of PAR2 expression on the cell surface as well as in the cytoplasm of esophageal cells throughout all epithelial layers of the esophageal mucosa.[83] Moreover, PAR2 expression was found to correlate positively with mucosal amounts of IL-8 (r 0.35; P<0.001). Establishing these associations *in vitro*, addition of weakly acidic media (pH 5 or 6) led to an up to

Table 2 | Drugs in progress for GERD therapy

Compound	Effect
$GABA_B$ agonism: baclofen; lesogaberan*; arbaclofen placarbil*	Decreased TLESR (increased basal LES tone)
mGluR5 antagonism: ADX10059*	Decreased TLESR
$5HT_4$ receptor agonism: mosapride	Increased basal LES tone
Cannabinoids (CB1): rimonabant	Decreased meal-induced TLESR; increased postprandial LES tone
TRPV1 antagonism: AZD1386	Reduction of thermal hyperalgsia; no effect on acid hyperalgesia

*Drug development discontinued. Abbreviations: $5HT_4$, 5-hydroxytryptamine receptor 4; GABA, γ-aminobutyric acid; GERD, gastroesophageal reflux disease; LES, lower esophageal sphincter; mGluR5, metabotropic glutamate receptor 5; TLESR, transient lower esophageal sphincter relaxation; TRPV1, transient receptor potential cation channel subfamily V member 1.

20-fold increase in *PAR2* expression in esophageal epithelial cell lines.[83] The addition of a PAR2-activating peptide in different pH-adjusted media to these cell cultures resulted in increased IL-8 gene expression and secretion in the supernatant. These findings support a possible mechanism of PAR2 upregulation in esophageal squamous epithelium by acid and weakly acidic reflux episodes.[83] Additional PAR2 activation by serine proteases (such as trypsin) in the refluxate can stimulate epithelial IL-8 secretion and subsequently attract immune cells to the esophageal mucosa and submucosa.[95] These results support the novel concept that GERD is an immune-mediated injury with early involvement of esophageal squamous epithelium.[95] Whether the reduction of acid exposure by adequate PPI therapy might decrease mucosal levels of PAR2 and also reduce the inflammatory response of the epithelium in individuals with GERD remains a question to be addressed in the future.

In a rat model, in which the pathophysiological role of trypsin for the induction of inflammatory changes in the esophageal epithelium had been studied, PAR2 activation has been suggested as a mediator for these inflammatory effects.[96] Camostat mesilate—a specific protease inhibitor—blocked the trypsin-mediated infiltration of immune cells, basal cell hyperplasia and COX2 expression substantially, whereas acid suppression with rabeprazole (a PPI) did not.[96] PAR2 is, therefore, a potential target for therapeutic development in GERD, especially for the population of patients who are refractory to a PPI and have predominantly weakly acidic reflux episodes.

Proinflammatory and neuroinflammatory aspects

The interaction of PAR2 with acid-sensitive receptors, such as the capsaicin-sensitive TRPV1 (transient receptor potential cation channel subfamily V member 1), is involved in the inflammatory epithelial response (including neuroinflammatory effects), that is supposed to be involved in GERD-related symptom generation. PAR2-dependent sensitization of TRPV1 was demonstrated in dorsal root ganglia,[97] and TRPV1 expression in primary afferent intramucosal neurons was demonstrated in patients with NERD.[98,99] Increased levels of TRPV1 have been found by western blot analysis in the mucosa of patients with erosive esophagitis as well as in those with NERD.[100] TRPV1 is a nonselective cationic channel that is activated by heat, capsaicin or hydrogen ions during inflammation.[101] The installation of capsaicin in the human esophagus causes the sensation of heartburn.[102] TRPV1-activation in primary afferent neurons evokes the sensation of burning pain and induces inflammatory and neuroinflammatory effects by release of substance P and calcitonin gene-related peptide, similar to PAR2.[103] TRPV1 expression was also shown in feline esophageal mucosa,[104] and in human epithelial cell lines in which TRPV1 activation mediates the release of platelet-activating factor.[105] Neuroinflammatory changes in the mucosa of patients with GERD include increased intramucosal concentrations of neurotropic factors such as nerve growth factor and glial-derived neurotrophic factor.[106] How faithfully these animal models resemble human disease is unclear, but novel data from experimental and human studies integrate several findings into a plausible model of immune-mediated effects in GERD pathogenesis.

Treatment perspectives

Refractory GERD remains a major challenge in clinical practice and studies unraveling different pathomechanisms have enabled the identification of several targets for drug development.[16] Several compounds that aim to reduce TLESR and reflux episodes have been investigated over the past few years (Table 2). Historically, baclofen—a γ-aminobutyric acid B receptor ($GABA_B$) agonist—was the first agent to be used as a therapy for refractory GERD and reduces the number of TLESR episodes and reflux episodes by >40%.[107,108] Baclofen also increases the basal tone of the lower esophageal sphincter in patients with GERD, but its application is limited by its adverse effects on the central nervous system.[107,108] Therefore, pharmacological development has led to the testing of an active *R*-isomer of baclofen, arbaclofen placarbil,[109] and a peripherally active agent, lesogaberan,[110] in clinical studies. Both formulations reduce the total number of TLESR episodes and reflux episodes documented by MII–pH monitoring with favorable tolerability and safety profiles.[109,110] For both formulations, the clinical efficacy and therapeutic effects have been somewhat modest and the further development of these compounds has been halted by the drug companies.

The metabotropic glutamate receptor 5 antagonist ADX10059 has a different pharmacodynamic approach to TLESR reduction than the above medications, and has been shown to reduce both acidic and nonacidic reflux events.[111–113] Although ADX10059 reached the clinical assessment phase of drug development,[111–113] after an increase in abnormal laboratory test results (liver enzymes) and a few cases of hepatic failure, the further

development of ADX10059 was also discontinued. The role of TLESR inhibition as the underlying mechanism of GERD warrants further extended studies in broader populations for it to be developed as an add-on therapy to PPIs.

The mixed $5HT_4$ receptor agonist and $5HT_3$ receptor antagonist mosapride has been shown to influence esophageal and gastric motility in humans;[114,115] in rats, it reduces visceral hypersensitivity.[114,115] However, although mosapride enhances esophageal bolus transit, it did not affect lower esophageal sphincter basal tone or even the number of TLESR episodes when compared with placebo.[116]

Animal studies confirmed a potential role of the cannabinoid receptor 1 (CB1) in the modulation of TLESR.[117,118] A mixed CB1–CB2 receptor agonist, Δ^8-tetrahydrocannbinol, enhanced lower esphageal spincter basal tone and reduced meal-induced TLESR in dogs and healthy volunteers, but is associated with notable adverse effects on the central nervous system.[119] Similar results have been obtained for rimonabant, a selective CB1 antagonist that notably enhances postprandial lower esophageal sphincter pressure.[120] Additionally, the total number of postprandial TLESR episodes and acid reflux episodes was markedly reduced in healthy volunteers after treatment with rimonabant.[120] Visceral pain modulators, such as selective serotonin reuptake inhibitors and tricyclic antidepressants, have become part of the treatment armamentarium to be considered for refractory GERD, as esophageal pain can be improved and the threshold of pain sensation in noncardiac chest pain is altered by these agents.[121]

On the basis of molecular data, targeting TRPV1 might have therapeutic use for refractory GERD and the first data in humans on their effects on esophageal pain and heat thresholds have been published.[122] Although AZD1386 (a TRPV1 antagonist) showed analgesic effects after thermal stimuli, a direct analgesic effect on acid-induced pain (pH and volume) was not observed.[122] Additional potential drug targets will require further experiments to show that reflux esophagitis can be prevented by specific inhibition of the cytokines and mediators mentioned above.

Conclusions

The complexity of GERD pathogenesis has been extended by novel findings. In addition to acidic or nonacidic reflux episodes, proteolytic components of the refluxate are involved in damaging the esophageal mucosa by modulating the immune response and esophageal inflammation and injury. Animal models and molecular data have deepened our understanding of inflammatory changes in the pathogenesis of GERD. Several models have extended the current knowledge of mechanisms that explain the distinct effects of acid and other components of the refluxate on the esophageal mucosa. Histomorphological aspects have been added to our understanding of the pathophysiology of GERD and have been proposed as useful criteria for diagnostic assessment. Close collaboration between gastrointestinal experts and pathologists is required to include histopathology in future diagnostic guidelines for GERD. For patients with GERD who are refractory to PPIs, new compounds are expected to fill the current therapeutic gaps. New drugs with effects on the antireflux barrier as well as on inflammatory mediators are awaited and might arise from our deepened and new understanding of the basic mechanisms involved in the pathogenesis of GERD.

Review criteria

Relevant literature on the pathophysiological mechanisms and clinical epidemiology of GERD were systematically identified by searching the PubMed database for articles published up to September 2011. The search terms used in combination with "GERD" were: "inflammation", "histology", "dilated intercellular spaces", "diagnosis", "TLESR", "IL-8", "TRPV1" and "PAR2". All papers identified were English-language, full-text original research articles published in peer-reviewed journals.

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Author contributions
A. Kandulski researched data for the article. Both authors contributed equally to discussions of content, writing, reviewing and editing the manuscript.