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Einfluss von *NOD2*, intestinalem Mikrobiom und Vitamin D auf den klinischen Verlauf von chronisch-entzündlichen Darmerkrankungen

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Meiner lieben Ricke

1. Einleitung und Zielsetzung

Chronisch-entzündliche Darmerkrankungen (CED) bestehen hauptsächlich aus den Entitäten M. Crohn (MC) und C. ulcerosa (CU) (Abraham und Cho 2009). Die Pathogenese beinhaltet eine durch Darmbakterien induzierte Dysregulation des intestinalen Immunsystems bei genetisch prädisponierten Individuen (Baumgart und Sandborn 2012; Abraham und Cho 2009; Sartor 2008; Mayer 2010). Bei der Entstehung eines MCs sind Mutationen im *NOD2* Gen ein wesentlicher Risikofaktor (Hugot et al. 2001; Ogura et al. 2001; Cuthbert et al. 2002). Bei *NOD2* handelt es sich um einen intrazellulären „pattern recognition receptor“, welcher Bestandteile des Peptidoglykans (PGN), wie Muramyl Dipeptid (MDP) erkennt und es hierdurch letztendlich zu einer Aktivierung des Transkriptionsfaktors NF-kappa-B kommt (Strober et al. 2006). Mutationen im *NOD2* Gen sind bei MC Patienten vor allem assoziiert mit einem jüngeren Alter bei Erstdiagnose, Dünndarmbeteiligung, Ileozökalresektion und einer höheren Rate an postoperativen Rezidiven und Re-Operationen (Büning et al. 2004). Während die CED in der ersten Hälfte des 20. Jahrunderts noch eine seltene Krankheitsgruppe darstellten, kam es in der zweiten Hälfte des 20. Jahrhunderts zu einem dramatischen Anstieg mit in etwa einer Verdopplung der Inzidenz in jeder Dekade (Molodecky et al. 2012). Dieser Aspekt kann nicht allein durch genetische oder immunologische Faktoren erklärt werden, sondern es spielen hier möglicherweise auch Umweltfaktoren eine wichtige Rolle. In den letzten Jahren ist der Einfluss des intestinalen Mikrobioms auf die CED zunehmend in den wissenschaftlichen Focus gerückt. Das Darmmikrobiom – die Gesamtheit aller im Darm lebenden Mikroorganismen – zeigt eine unterschiedliche Zusammensetzung in verschiedenen Teilen des Gastrointestinaltraktes mit der höchsten Anzahl von Mikroorganismen im Kolon von 10^{11} oder 10^{12} Zellen/g Darminhalt und einem Gesamtgewicht von ca. 1 kg (Dave et al. 2012; Savage 1977). Verschiedene Studien legen einen Zusammenhang zwischen der intestinalen bakteriellen Komposition und dem Auftreten von CED nahe (Frank et al. 2007; Frank et al. 2011; Willing et al. 2010). In einem *NOD2*-Knockout Mausmodell zeigte sich, dass *NOD2* eine wichtige Rolle für die Zusammensetzung der intestinalen Bakterien spielt (Rehman et al. 2011). Darüber hinaus hat der NOD2 Rezeptor eine bedeutende Rolle in der Sekretion von Defensinen aus Paneth Zellen (Wehkamp et al. 2005). Inwieweit jedoch eine Veränderung der bakteriellen Zusammensetzung ursächlich oder vielmehr Folge einer mukosalen intestinalen Inflammation darstellt, bleibt eine noch offene Frage und ist weiterhin Gegenstand der aktuellen Forschung. Ein Umweltfaktor, welcher im Zusammenhang mit CED steht, ist das Vitamin D. Eine Hypovitaminosis D ist ein häufiges Phänomen bei CED Patienten (Ulitsky et al. 2011; Leslie et al. 2008). Neben seiner Wirkung auf den Calcium- und Phosphatmetabolismus sowie den Knochenstoffwechsel verdichtet sich die Datenlage, dass Vitamin D eine wichtige Rolle als Regulator des angeborenen und erworbenen Immunsystems darstellt (Cantorna und Mahon 2005; Cantorna et al. 2014). Dass es einen Zusammenhang zwischen Vitamin D und *NOD2* gibt, konnte in einer Studie gezeigt werden, in der nach

Vitamin D Stimulation eine höhere Expression des Vitamin D Rezeptors in humanen Monozyten und Epithelzellen nachgewiesen wurde (Wang et al. 2010). Darüber hinaus wurde in einer kürzlich publizierten genomweiten Assoziationsstudie nachgewiesen, dass Mutationen im Vitamin D Rezeptor mit spezifischen intestinalen mikrobiellen Profilen assoziiert waren (Wang et al. 2016).

Ziel der hier vorgestellten Arbeiten ist es, den Einfluss von *NOD2*, dem intestinalen Mikrobiom und Vitamin D auf den Verlauf der CED zu untersuchen.

2. Methoden

Alle Untersuchungen am Menschen und an Tieren wurden mit Zustimmung der jeweils zuständigen Ethik-Kommissionen, im Einklang mit nationalem Recht sowie gemäß der Deklaration von Helsinki von 1975 (in der aktuellen, überarbeiteten Fassung) durchgeführt. Von allen beteiligten Patienten liegt eine Einverständniserklärung vor.

Über die Durchführung der einzelnen technisch-apparativen Untersuchungen wird ausführlich in den einzelnen Manuskripten eingegangen.

3. Ergebnisse

3.1 Die Stimulation von *NOD2* durch ein von *S. aureus* hergestelltes Peptidoglykan wird durch eine *TLR2* Ko-Stimulation mit Lipoproteinen in dendritischen Zellen verstärkt

Die wichtigsten Liganden für NOD2 sind das PGN sowie dessen Bestandteil, das MDP. Diese Arbeit fokussierte sich insbesonders auf mehrfach verzweigte, polymere PGN (PGNpol) Fragmente in der Aktivierung des angeborenen Immunsystems (Schäffler et al. 2014). Es wurde der Effekt einer kombinierten NOD2 und TLR2 Stimulation im Vergleich zu einer alleinigen NOD2 Rezeptor-Stimulation untersucht. PGNpol wurde von einem Lipoprotein enthaltenden *S. aureus* Stamm (PGNpol) sowie von einem Lipoprotein-defizienten S. aureus Stamm (PGNpolΔlgt) isoliert. Während PGNpol sowohl als NOD2 als auch als TLR2 Ligand fungiert, handelt es sich bei PGNpolΔlgt um einen alleinigen NOD2 Liganden. Es kam nach Stimulation mit PGNpol in aus murinem Knochenmark gewonnen dendritischen Zellen (BMDC) zu einer starken Aktivierung und Reifung der dendritischen Zellen im Gegensatz zu PGNpolΔlgt. Eine deutliche Erhöhung pro-inflammatorischer Zytokine nach Stimulation mit PGNpol im Gegensatz zu PGNpolΔlgt konnte in verschiedenen Zellsystemen (BMDC, J774, MonoMac 6) nachgewiesen werden. Zusammenfassend kann festgestellt werden, dass eine kombinierte TLR2 und

NOD2 Stimulation zu einer deutlichen Aktivierung des Immunsystems im Gegensatz zu einer Stimulation des jeweils einzelnen Rezeptors allein führt.

Publikation: Schäffler, Holger*; Demircioglu, Dogan Doruk*; Kühner, Daniel; Menz, Sarah; Bender, Annika; Autenrieth, Ingo B. et al. (2014a): NOD2 stimulation by Staphylococcus aureus-derived peptidoglycan is boosted by Toll-like receptor 2 costimulation with lipoproteins in dendritic cells. In: *Infection and immunity*82 (11), S. 4681–4688. DOI: 10.1128/IAI.02043-14.

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3.2 Spezifische klinische Faktoren beeinflussen die Vitamin D Spiegel bei CED-Patienten

Die genauen Auswirkungen einer Hypovitaminosis D auf den Krankheitsverlauf bei CED Patienten sind bisher noch nicht vollständig geklärt, dieser Frage widmet sich die im Folgenden beschriebene Arbeit.

In einer retrospektiven Untersuchung wurde die Assoziation von Vitamin D Werten mit klinischen Parametern, wie z.B. medikamentöse, krankheitsgerichtete Therapie, anatomische Situation, Krankheitslokalisation und Krankheitsaktivität untersucht. Es wurden 208 Patienten in die Studie eingeschlossen (123 MC und 85 CU). Ein schwerer Vitamin D Mangel (25-OH-Vitamin D < 27,5 nmol/l) zeigte sich hochprävalent in den Wintermonaten von Januar bis April. Eine Therapie mit einem TNF-alpha hemmenden Wirkprinzip war bei MC Patienten mit signifikant höheren Vitamin D Werten assoziiert, unabhängig von einer möglichen Vitamin D Substitution. Patienten mit MC Befall im Dünndarm bzw. MC Patienten nach Dünndarmresektion zeigten signifikant niedrigere Vitamin D Spiegel. Bei Patienten mit CU war eine höhere Krankheitsaktivität mit niedrigeren Vitamin D Spiegeln assoziiert. Letztendlich kann aus der Arbeit geschlossen werden, dass ein Screening auf eine Hypovitaminosis D insbesondere in den Monaten Januar bis April sinnvoll ist, darüber hinaus bei Patienten mit einem Dünndarmbefall bzw. nach Dünndarmresektion.

Schäffler, Holger; Schmidt, Martin; Huth, Astrid; Reiner, Johannes; Glass, Änne; Lamprecht, Georg (2017c): Clinical factors are associated with vitamin D levels in IBD patients - a retrospective analysis. In: *Journal of digestive diseases. DOI:* 10.1111/1751-2980.12565.

3.3 Einfluss von krankheitsspezifischen Faktoren auf das Mukosa-assoziierte Darmmikrobiom bei CED Patienten

Während die Untersuchung der intestinalen bakteriellen Komposition mittels Sequenzierung sowohl technisch als auch bioinformatisch aufwendig und kostenintensiv ist, können mittels einer Polymerase Kettenreaktion (PCR) einzelne bakterielle Stämme kostengünstig und zeitnah quantitativ erfasst werden. In dieser Arbeit wurde der Einfluss der Krankheitsentität (MC und UC), von Mutationen im *NOD2* Gen, der Therapie mit einem TNF-alpha Blocker sowie dem Vorhandensein einer endoskopisch

erkennbaren Inflammation auf die Abundanz von sieben bakteriellen Stämmen (*Bacteroides fragilis*, *Escherichia coli*, *Prevotella melaninogenica*, *Clostridium coccoides*, *Clostridium difficile*, *Bifidobacterium bifidum* und *Faecalibacterium prausnitzii*) untersucht, welche als wichtige Repräsentanten der jeweiligen Phyla möglicherweise eine Rolle in der Entstehung und im Verlauf von CED haben. Es zeigte sich, dass verschiedene klinische Situationen zu einer veränderten Abundanz von bakteriellen Stämmen führen können. Die Untersuchung einzelner bakterieller Stämme könnte zukünftig eine prädiktive Rolle im Krankheitsverlauf bei CED darstellen, bzw. es könnte hierdurch möglicherweise perspektivisch einen Ansatz einer zielgerichteten Therapie entstehen lassen.

Schäffler, Holger; Kaschitzki, Annika; Alberts, Christian; Bodammer, Peggy; Bannert, Karen; Köller, Thomas et al. (2016): Alterations in the mucosa-associated bacterial composition in Crohn's disease. A pilot study. In: *International journal of colorectal disease* 31 (5), S. 961–971. DOI: 10.1007/s00384-016-2548-z.

3.4 Mutationen im *NOD2* Gen sind assoziiert mit einem distinktiven Krankheitsphänotyp bei MC Patienten

In diversen vorherigen Studien konnte bisher für Patienten mit dem Vorliegen einer *NOD2* Mutation gezeigt werden, dass diese mit einem spezifischen Krankheitsphänotyp assoziiert sind (s.o.). Wir konnten an unserer MC Kohorte an der Universitätsmedizin Rostock zeigen, dass Patienten, bei denen eine Mutation im *NOD2* Gen vorliegt, signifikant häufiger ein Krankheitsbefall im Ileocolon vorhanden war, sowie diese Patienten signifikant häufiger eine Ileocoecalresektion oder einen strikturierenden bzw. perianalen Krankheitsverlauf aufwiesen. Patienten mit einer *NOD2* Mutation hatten seltener einen kolonischen MC sowie seltener eine Stoma-Anlage nach einer OP. Patienten mit einer Mutation im SNP13 zeigten häufiger einen perianalen Befall. Im klinischen Alltag wird der Therapieerfolg bzw. das Therapieversagen auf einen TNF-alpha Blocker mittels Durchführung eines therapeutischen Drug Monitorings (TDM) gesteuert. Interessanterweise war das Vorliegen einer *NOD2* Mutation assoziiert mit signifikant häufigeren TNF-alpha Spiegeln im subtherapeutischen Bereich und numerisch niedrigeren TNF-alpha Spiegeln im Vergleich zu Patienten ohne das Vorliegen einer *NOD2*-Mutation. Ein Fazit, welches aus dieser Studie gezogen werden kann, ist, dass Patienten mit dem Vorliegen einer *NOD2* Mutation möglicherweise von einem engmaschigeren TDM profitieren könnten, was ein proaktives Vorgehen anstelle eines reaktiven TDM Ansatzes nahelegt.

Schäffler, Holger; Geiss, David; Gittel, Nicole; Rohde, Sarah; Huth, Astrid; Glass, Änne et al. (2018a): Mutations in the NOD2-gene are associated with a specific phenotype and lower anti-TNF trough levels in Crohn's disease. In: *Journal of digestive diseases. DOI:* 10.1111/1751-2980.12677.

3.5 Eine Vitamin D Substitution führt zu einer Veränderung der intestinalen bakteriellen Komposition bei MC Patienten, jedoch nicht bei gesunden Kontrollen

Der Einfluss einer Vitamin D Administration auf die spezifische intestinale bakterielle Komposition bei MC Patienten im Vergleich zu gesunden Kontrollen wurde bisher noch nicht untersucht. In dieser Arbeit führten wir eine prospektive, longitudinale, kontrollierte Interventionsanalyse in Patienten mit MC und gesunden Kontrollen durch, indem wir unter Vitamin D Substitution die intestinale bakterielle Zusammensetzung untersuchten. Darüber hinaus wurde die Krankheitsaktivität sowie als Inflammationsmarker das fäkale Calprotectin miterfasst. Unter Vitamin D Applikation kam es zu keiner signifikanten Änderung der Krankheitsaktivität bzw. des fäkalen Calprotectinwertes. Es zeigte sich jedoch, dass es bei MC Patienten in der frühen Substitutionsphase zu einer spezifischen Veränderung der bakteriellen Zusammensetzung kam, welche im späteren Verlauf wieder revertierte. Bei den gesunden Kontrollen kam es zu keiner Änderung der Zusammensetzung der Darmbakterien. Interessanterweise kam es bei den MC Patienten unter Vitamin D Substitution zu einer Reduktion der „operational taxonomic units“ (OTU’s). Darüber hinaus konnte gezeigt werden, dass bestimmte Bakterienspezies unter Vitamin D Substitution eine höhere Abundanz hatten als in der Kontrollgruppe. Diese Studie legt einen direkten Effekt von Vitamin D auf die Zusammensetzung der intestinalen bakteriellen Zusammensetzung nahe.

Schäffler, Holger; Herlemann, Daniel Pr; Klinitzke, Paul; Berlin, Peggy; Kreikemeyer, Bernd; Jaster, Robert; Lamprecht, Georg (2018a): Vitamin D administration leads to a shift of the intestinal bacterial composition in Crohn's Disease patients, but not in healthy controls. In: *Journal of digestive diseases. DOI:* 10.1111/1751-2980.12591.

3.6 Die mukosa-assoziierte intestinale bakterielle Komposition ist assoziiert mit der Krankheitsaktivität bei MC Patienten

CED sind assoziiert mit einer Veränderung der bakteriellen Zusammensetzung, auch genannt Dysbiose. Üblicherweise werden für Mikrobiomstudien Stuhlproben verwendet. In dieser Arbeit an einem Kollektiv von MC Patienten wurden mukosale Biopsate aus routinemäßig durchgeführten Koloskopien bezüglich der intestinalen bakteriellen Komposition untersucht und nach Krankheitsaktivität, Applikation eines TNF-alpha Blockers, lokalem Inflammationsstatus sowie dem Vorhandensein einer *NOD2*-Mutation stratifiziert. Es konnte gezeigt werden, dass Patienten mit einer höheren Krankheitsaktivität (gemessen anhand des CDAI, Crohn’s Disease Activity Index) eine signifikante Änderung der intestinalen bakteriellen Komposition zeigten. Darüber hinaus konnten einzelne bakterielle Spezies identifiziert werden, welche eine hohe Abundanz bei niedriger, mittlerer und hoher Krankheitsaktivität zeigten. Dies ist die erste Studie, welche einen Zusammenhang zwischen der

Krankheitsaktivität bei MC Patienten und einer Veränderung der bakteriellen Zusammensetzung beschreibt.

Schäffler, Holger*; Herlemann, Daniel Pr*; Alberts, Christian; Kaschitzki, Annika; Bodammer, Peggy; Bannert, Karen et al. (2016): Mucosa-attached bacterial community in Crohn's Disease coheres with the Clinical Disease Activity Index. In: *Environmental microbiology reports. DOI:* 10.1111/1758-2229.12411.

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3.7 Das Vorhandensein einer Mutation im *NOD2* Gen ist assoziiert mit der Entwicklung eines Darmversagens – unabhängig vom Vorliegen eines M. Crohns

Mutationen im *NOD2* Gen sind der wichtigste genetische Risikofaktor für das Vorliegen eines MCs. In einer Kohorte von 85 Patienten mit nicht-malignem Darmversagen wurde das Vorhandensein einer Mutation im *NOD2*-Gen und darüber hinaus im *ATG16L1* sowie *IL23R* Gen untersucht. Es konnte gezeigt werden, dass Patienten mit einem Darmversagen signifikant häufiger eine *NOD2* Mutation aufwiesen als die Kontrollgruppe. Bei MC Patienten mit Darmversagen war die *NOD2* Mutationsfrequenz zwischen Patienten mit und ohne Darmversagen nicht signifikant verschieden. Das Vorliegen einer *NOD2* Mutation war weder mit Faktoren, welche zu einer Darm- oder Multiviszeraltransplantation führen, noch mit einer früheren Indikationsstellung für eine solche Transplantation assoziiert. *NOD2* Mutationen scheinen somit die Entstehung von komplikationsträchtigen abdominellen Ereignissen, welche konsekutiv zu einem Darmversagen führen, zu begünstigen oder aber die Adaptation nach einem solchen Eingriff negativ zu beeinflussen.

Schäffler, Holger; Schneider, Nina; Hsieh, Chih-Jen; Reiner, Johannes; Nadalin, Silvio; Witte, Maria et al. (2013): NOD2 mutations are associated with the development of intestinal failure in the absence of Crohn's disease. In: *Clinical nutrition (Edinburgh, Scotland)* 32 (6), S. 1029–1035. DOI: 10.1016/j.clnu.2013.02.014.

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3.8 Zwei Patienten mit einem Darmversagen, Vorliegen einer *NOD2*-Mutation und einer Lymphknotentuberkulose

In dieser Fallserie werden zwei Patienten mit Darmversagen beschrieben. Beide Patienten bedürfen einer totalen heimparenteralen Ernährung, darüber hinaus liegt bei beiden Patienten eine *NOD2* Mutation vor. Es zeigte sich, dass bei beiden Patienten eine Lymphknotentuberkulose diagnostiziert wurde, welche im FDG-PET-CT positiv war. Beide Patienten erhielten eine tuberkulostatische Therapie und zeigten eine klinische Abheilung, die PET positiven Läsionen waren im Verlauf deutlich regredient. Als Schlussfolgerung kann aus dieser Fallserie gezogen werden, dass Patienten mit Darmversagen,

welche eine *NOD2* Mutation aufweisen, engmaschig auf das Vorliegen einer Lymphknotentuberkulose hin untersucht werden müssen.

Schäffler, Holger; Teufel, Matthias; Fleischer, Sabrina; Hsieh, Chih-Jen; Frick, Julia-Stefanie; Lamprecht, Georg (2014b): Two patients with intestinal failure requiring home parenteral nutrition, a NOD2 mutation and tuberculous lymphadenitis. In: *BMC gastroenterology*14, S. 43. DOI: 10.1186/1471-230X-14-43.

3.9 M. Crohn Patienten zeigen NOD2- und krankheitsspezifische Genexpressionsprofile in mononukleären Zellen des peripheren Blutes

In dieser Arbeit wurden die zwei Hypothesen getestet, dass mononukleäre Zellen aus dem peripheren Blut (PBMC's) ein geeignetes Medium sind, um Genexpressionsprofile bei CED Patienten zu untersuchen sowie dass MC Patienten – selbst im Zustand der klinischen Vollremission – eine krankheitsspezifische Genexpression in PBMC's aufweisen (Schäffler et al. 2018b). Zunächst wurde in Form eines hypothesenfreien Ansatzes mittels Microarray Technologie nach Vitamin D sowie PGN und LPS Stimulation in einem kleinen Kollektiv von 3 MC Patienten und 3 gesunden Probanden 267 Gene identifiziert, welche eine signifikant unterschiedliche krankheitsspezifische Regulation aufwiesen. Eine Eingrenzung dieser Gene und anschließende Verifikation in einem größeren Kollektiv an Patienten und Kontrollen mittels Real-Time PCR zeigte, dass drei Gene krankheitsspezifzisch (*CLEC5A*, *LYZ* und *TREM1*) sowie 6 Gene *NOD2*-abhängig (*CD101*, *CLEC5A*, *CXCL5*, *IL-24*, *ITGB2*, *LYZ*) reguliert waren. Wir sehen vor allem *TREM1* sowie *CLEC5A* in einem regulatorischen Netzwerk als vielversprechenden Baustein in der Pathophysiologie des MC.

Schäffler, Holger; Rohde, Maria; Rohde, Sarah; Huth, Astrid; Gittel, Nicole; Hollborn, Hannes et al. (2018b): NOD2- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients. In: *World journal of gastroenterology* 24 (11), S. 1196–1205. DOI: 10.3748/wjg.v24.i11.1196.

4. Diskussion

Die genaue Ätiopathogenese der CED ist nach wie vor nicht vollständig geklärt, eine wichtige Rolle in Ihrer Entstehung und im klinischen Verlauf haben einerseits Umweltfaktoren, wie z.B. das intestinale Mikrobiom sowie Vitamin D und andererseits genetische Marker, wie z.B. Mutationen im *NOD2*-Gen. Die vorliegenden Arbeiten beschäftigen sich einerseits mit dem Wechselspiel dieser Einzelfaktoren untereinander und andererseits mit deren Einfluss auf den Verlauf der CED. Übergeordnetes Ziel der hier durchgeführten Untersuchungen war es, den Krankheitsverlauf *ex ante* besser einschätzen zu können und somit – anhand der Bestimmung von sogenannten „Biomarkern" – ein präziseres Verständnis im Sinne einer personalisieren Medizin für den Patienten zu entwickeln und den Krankheitsverlauf möglicherweise günstig zu beeinflussen.

Der **Einfluss einer *NOD2* Mutation** auf den Verlauf von CED wurde bereits in mehreren Studien intensiv untersucht (Büning et al. 2004). Wir konnten in einer Genotyp-Phänotyp-Assoziationsstudie an MC Patienten in einem norddeutschen CED Zentrum einen Zusammenhang zwischen dem Vorhandensein einer Mutation im *NOD2* Gen und bestimmten klinischen Faktoren zeigen, u.a. waren *NOD2* Mutationen assoziiert mit ileokolonischem Verlauf sowie strikturierendem und auch penetrierendem Krankheitsverhalten. Insbesondere beim Vorliegen einer Mutation im SNP13 zeigte sich signifikant häufiger ein perianaler Befall. Dass NOD2 auch im klinischen Alltag eine wichtige Rolle spielt, konnten wir dadurch zeigen, dass Patienten mit einer *NOD2*-Mutation signifikant häufiger subtherapeutische anti-TNF Talspiegel aufwiesen bzw. numerisch signifikant niedrigere anti-TNF Talspiegel hatten. Patienten mit einer *NOD2* Mutation könnten somit von einem engmaschigeren TDM profitieren. Möglicherweise wäre in diesem „Hochrisiko-Kollektiv" auch ein Vorgehen im Sinne eines proaktiven – im Gegensatz zum üblicherweise und auch von den entsprechenden Leitlinien empfohlenen reaktiven TDM – zu diskutieren (Feuerstein et al. 2017). Während Mutationen im *NOD2* Gen als wichtigste genetische Risikofaktoren für die Entstehung eines MCs gelten, konnten wir in zwei weiteren Arbeiten zeigen, dass es eine Assoziation von Mutationen im *NOD2* Gen und dem Vorliegen eines Kurzdarmsyndromes (KDS) – unabhängig von der zugrundeliegenden Ätiologie – sowie der Entwicklung einer Lymphknotentuberkulose gibt. Im murinen Modell sowie anhand verschiedener Zellkulturen konnte darüber hinaus nachgewiesen werden, dass eine *TLR2* Ko-Stimulation eine wichtige Rolle in der *NOD2*-abhängigen Aktivierung des angeborenen Immunsystems darstellt.

Ein **Vitamin D** Mangel ist bei CED hochprävalent und mit einem schlechteren Outcome verknüpft (Gubatan und Moss 2018). Wir konnten zeigen, dass Vitamin D mit einem spezifischen

Krankheitsphänotyp bei CED Patienten (Schäffler et al. 2017) assoziiert ist, z.B. haben Patienten mit einem Dünndarmbefall bzw. nach Dünndarmresektionen signifikant niedrigere Vitamin D Spiegel als die entsprechenden Kontrollen. Wir haben gezielt in Form eines interventionellen Ansatzes untersucht, ob die Applikation von Vitamin D auch Auswirkungen auf die intestinale bakterielle Komposition hat. Interessanterweise kam es bei MC Patienten zu einer transienten Veränderung der intestinalen bakteriellen Komposition im Gegensatz zu den gesunden Kontrollen (Schäffler et al. 2018a). Unter Vitamin D Substitution kam es zusätzlich zu einer signifikant erhöhten Abundanz von einzelnen bakteriellen Stämmen, u.a. *Alistipes* und *Parabacteroides*. Es bleibt spekulativ und ein Gegenstand von zukünftigen Studien, ob möglicherweise ein Teil dieser Stämme an sich bzw. in Kombination mit Vitamin D einen positiven Einfluss auf die Krankheitsaktivität bei CED Patienten haben könnte. Mithilfe eines hypothesenfreien Ansatzes mittels Microarray Technologie konnten wir aus PBMC's von MC Patienten nach Vitamin D sowie PGN/LPS Stimulation 267 Gene identifizieren, welche bei MC Patienten krankheitsspezifisch reguliert waren. In einer weiteren Untersuchung in einem größeren Patientenkollektiv wurden verschiedene Gene nachgewiesen, welche krankheitsspezifisch bzw. *NOD2*-abhängig exprimiert wurden. Insgesamt bieten PBMC's ein vergleichsweise einfach zu generierendes Medium, um Genexpressionsprofile zu untersuchen, welche perspektivisch als Biomarker fungieren könnten. Weitere Genotyp-Phänotyp-Assoziationsstudien der identifizierten Gene in einem größeren Patientenkollektiv sowie funktionelle Studien folgen.

Das **intestinale Mikrobiom** zeigt einen engen Zusammenhang mit der Entstehung und dem Verlauf von CED. Verschiedene Studien mit endoskopisch gewonnenen mukosalen Proben bei CED Patienten konnten sowohl mittels RT-PCR als auch über „Next Generation Sequencing" (NGS) nachweisen, dass bestimmte klinische Konstellationen mit einem spezifischen Darmmikrobiom assoziiert sind (Schäffler et al. 2016; Schäffler et al. 2016). Unter anderem konnten wir nachweisen, dass die Krankheitsaktivität signifikant mit der bakteriellen Zusammensetzung im Darm korreliert. Einzelne Darmbakterien als „Markerkeime" konnten ebenfalls identifiziert werden und könnten perspektivisch als Biomarker bei CED Patienten eingesetzt werden. Weitere Studien sind hier notwendig, um das komplexe Zusammenspiel zwischen Darmmikrobiom und dem Krankheitsverlauf bei CED besser zu verstehen.

Wir konnten insgesamt in verschiedenen Studien nachweisen, dass sowohl Vitamin D als auch *NOD2* und das intestinale bakterielle Mikrobiom einen spezifischen Einfluss auf den Krankheitsverlauf von CED haben.

5. Zusammenfassung

Der Krankheitsverlauf bei CED Patienten lässt sich aktuell nur unzureichend vorhersagen, prädiktive Marker stehen nur eingeschränkt zur Verfügung. Die hier vorgestellten Arbeiten beschäftigen sich mit dem Zusammenhang zwischen Darmmikrobiom, Vitamin D und genetischen Risikofaktoren, wie Mutationen im *NOD2* Gen bei CED. Aus den hier durchgeführten Untersuchungen resultiert u.a. ein besseres Verständnis darüber, wie sich die einzelnen Faktoren gegenseitig beeinflussen und eine Auswirkung auf den Krankheitsverlauf bei CED haben. Dies alles trägt dazu bei, den Krankheitsverlauf bei CED besser zu verstehen und zukünftig anhand möglicher Biomarker präziser vorherzusagen.

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7. Liste der eigenen Publikationen

Der angegebene Impact-Faktor bezieht sich auf das Erscheinungsjahr der Arbeit. Für die im Jahre 2018 erschienenen Artikel wird der letzte verfügbare Impact-Faktor verwendet.

7.1. Originalarbeiten mit Impact-Faktor

Schäffler, Holger; Daraban, A. M.; Roggenbrod, S.; Schumacher, U.; Königsrainer, A.; Gregor, M.; Lamprecht, G. (2011): Characterization of refractory port-related blood stream infections in intestinal failure patients on parenteral nutrition. In: *Zeitschrift für Gastroenterologie* 49 (3), S. 335–339. DOI: 10.1055/s-0029-1245980.

IF: 1,612

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IF: 2,533

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IF: 1,623

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IF: 3,300

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IF: 1,623

Schäffler, Holger; Geiss, David; Gittel, Nicole; Rohde, Sarah; Huth, Astrid; Glass, Änne et al. (2018a): Mutations in the NOD2-gene are associated with a specific phenotype and lower anti-TNF trough levels in Crohn's disease. In: *Journal of digestive diseases. DOI:* 10.1111/1751-2980.12677.

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IF: 2,731

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IF: 5,496

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* contributed equally

IF: 3,256

Schäffler, Holger*; Herlemann, Daniel Pr*; Alberts, Christian; Kaschitzki, Annika; Bodammer, Peggy; Bannert, Karen et al. (2016): Mucosa-attached bacterial community in Crohn's Disease coheres with the Clinical Disease Activity Index. In: *Environmental microbiology reports. DOI:* 10.1111/1758-2229.12411.

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IF: 0,806

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7.2. Übersichtsartikel mit Impact-Faktor

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IF: 4,019

7.3. Korrespondenz / Kasuistiken mit Impact-Faktor

Warnke, P.; Kiefel, V.; **Schäffler, H.**; Podbielski, A. (2013): Transfusion reaction due to Klebsiella pneumoniae-contaminated red blood cells. A case report. In: *Transfusion medicine (Oxford, England)* 23 (6), S. 445–446. DOI: 10.1111/tme.12078.

IF: 1,798

Schäffler, Holger; Lamprecht, Georg; Witte, Maria (2017b): Peristomal Lesions in Crohn's Disease. Are They Always Fistulae? In: *Deutsches Ärzteblatt international* 114 (38), S. 634. DOI: 10.3238/arztebl.2017.0634.

IF: 3,890

7.4 Sonstige Korrespondenz / Kasuistiken / Kommentare

Schäffler, Holger; Huth, Astrid; Lamprecht, Georg; Anders, Olaf (2017a): Vedolizumab Treatment for Ulcerative Colitis in an Elderly Multimorbid Patient with Hemophilia A. In: *Case reports in gastroenterology* 11 (3), S. 774–779. DOI: 10.1159/000485372.

8. Danksagung

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9. Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die Habilitationsschrift selbständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Rostock, den 04.10.2018

Dr. med. Holger Schäffler

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NOD2 Stimulation by *Staphylococcus aureus*-Derived Peptidoglycan Is Boosted by Toll-Like Receptor 2 Costimulation with Lipoproteins in Dendritic Cells

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Mutations in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) play an important role in the pathogenesis of Crohn's disease. NOD2 is an intracellular pattern recognition receptor (PRR) that senses bacterial peptidoglycan (PGN) structures, e.g., muramyl dipeptide (MDP). Here we focused on the effect of more-cross-linked, polymeric PGN fragments (PGNpol) in the activation of the innate immune system. In this study, the effect of combined NOD2 and Toll-like receptor 2 (TLR2) stimulation was examined compared to single stimulation of the NOD2 receptor alone. PGNpol species derived from a lipoprotein-containing *Staphylococcus aureus* strain (SA113) and a lipoprotein-deficient strain (SA113 Δ*lgt*) were isolated. While PGNpol constitutes a combined NOD2 and TLR2 ligand, lipoprotein-deficient PGNpolΔ*lgt* leads to activation of the immune system only via the NOD2 receptor. Murine bone marrow-derived dendritic cells (BMDCs), J774 cells, and Mono Mac 6 (MM6) cells were stimulated with these ligands. Cytokines (interleukin-6 [IL-6], IL-12p40, and tumor necrosis factor alpha [TNF-α]) as well as DC activation and maturation parameters were measured. Stimulation with PGNpolΔ*lgt* did not lead to enhanced cytokine secretion or DC activation and maturation. However, stimulation with PGNpol led to strong cytokine secretion and subsequent DC maturation. These results were confirmed in MM6 and J774 cells. We showed that the NOD2-mediated activation of DCs with PGNpol was dependent on TLR2 costimulation. Therefore, signaling via both receptors leads to a more potent activation of the immune system than that with stimulation via each receptor alone.

Crohn's disease is a systemic inflammatory disease, and together with ulcerative colitis, it forms the complex of inflammatory bowel diseases (IBD). Mutations within the NOD2 gene, encoding nucleotide-binding oligomerization domain-containing protein 2 (NOD2), have been identified as risk factors for the development of Crohn's disease (1–3). NOD2 is an intracellular pattern recognition receptor that senses peptidoglycan (PGN) fragments, such as muramyl dipeptide (MDP), derived from Gram-positive bacteria, to activate a cascade of reactions which consecutively lead to the activation of the transcription factor NF-κB (4).

Dendritic cells (DCs) are important professional antigen-presenting cells (APCs) in the intestine (5, 6) and are crucial for T cell activation and polarization (7, 8). Depending on the antigen, DCs can promote either inflammation or tolerance (9). DCs are thought to contribute to the pathogenesis of Crohn's disease (10, 11) by inducing T cell activation via antigen presentation. As an example, colonic CD11c^{+} DCs isolated from inflamed parts of the gut from IBD patients showed an increased expression of Toll-like receptor 2 (TLR2), TLR4, and the costimulatory molecule CD40 compared to DCs from noninflamed areas or DCs from healthy controls (12). Also, the ability of DCs to induce tolerogenic regulatory T cells might be lost in Crohn's disease patients (13).

PGN fragments with a low degree of cross-linking, such as PGN monomers, have been shown to be natural ligands for the NOD2 receptor (14). However, the role of more-cross-linked PGN fragments, so called polymeric PGN (PGNpol), and their effect in stimulating immune cells are still unclear, as even highly purified PGNpol might be contaminated with potent immune-stimulating lipoproteins (15). Using *Bacillus anthracis* peptidoglycan, it has been shown that polymeric PGN is a more potent activator of innate immune cells than MDP or monomeric PGN (16).

The importance of PGN in inflammation was recently demonstrated in mice infected with wild-type (WT) *Staphylococcus aureus* and corresponding *O*-acetyltransferase A (OatA) mutants. In *S. aureus*, PGN is modified by an *O*-acetyltransferase at the C-6 OH position of *N*-acetylmuramic acid (17). This modification, which occurs only in pathogenic staphylococcal strains (18), confers complete resistance of PGN to lysozyme, while mutations of the *oatA* gene result in a PGN that is sensitive to lysozyme (17, 19). In comparing PGNpol from WT *S. aureus* with PGN from the *oatA*-deficient mutant (*S. aureus* Δ*oatA*), it turned out that WT PGNpol strongly suppressed interleukin-1β (IL-1β) secretion and inflammasome activation, while the PGNpol of the *S. aureus* Δ*oatA* strain strongly increased IL-1β secretion and inflammasome activation (20). In the absence of *oatA*, PGN is degraded

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by lysozyme, resulting in a number of PGN breakdown products which boost inflammation. Thus, modification of PGN by *O*-acetylation in *S. aureus* is an efficient immune escape mechanism.

The interplay between the NOD2 and TLR2 pathways is complex, and interactions between both receptors seem to contribute to activation or inhibition of the immune system (21). NOD2 was shown to be part of an inhibitory system which blocks TLR2-mediated inflammation, resulting in less Th1 cytokine production after stimulation (22). Additionally, activation of mouse peritoneal macrophages with the NOD2 ligand MDP resulted in downregulation of TLR2/1 signaling-mediated IL-1β expression (23). However, different studies suggest a synergistic role for NOD2 and TLR2 (24).

Additionally, it is still controversially discussed whether PGN interacts with TLR2. PGN isolated from either Gram-negative or Gram-positive bacteria is reported not to be sensed by TLR2 (25). However, stimulation of primary mouse keratinocytes (MKs) with PGNpol from *S. aureus* SA113 resulted in internalization of the molecule and colocalization with NOD2 and TLR2 receptors and induced subsequent host immune responses (26). Additionally, lipoproteins of *S. aureus* have been shown to activate TLR2 (26–29).

To further elucidate the interaction between the NOD2 and TLR2 pathways, we investigated the innate immune sensing of two different types of naturally occurring *S. aureus*-derived PGN polymers. PGN of *S. aureus* SA113 contains traces of lipoproteins. This PGN is referred to as the wild-type PGN. The other PGNpol fraction was derived from the *S. aureus* SA113 Δ*lgt* mutant, which is unable to lipidate prolipoproteins and whose PGNpol therefore does not contain lipoproteins (30).

By studying the effects of PGNpol from the wild type and that from the Δ*lgt* mutant (PGNpolΔ*lgt*) on DC maturation and activation as well as cytokine secretion, we showed that PGNpol is a potent stimulator of the immune system, through both NOD2 and TLR2, while PGNpolΔ*lgt*, as a selective NOD2 ligand, does not induce host immune responses. However, the addition of the synthetic TLR2 ligand Pam3Cys (P3C) to PGNpolΔ*lgt* resulted in activation of the host immune response. Costimulation with the NOD2 ligand PGNpolΔ*lgt* and the TLR2 ligand P3C had a synergistic effect on cytokine production, suggesting that NOD2-dependent activation of DCs with PGN requires TLR2 costimulation by lipoproteins.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). $NOD2^{-/-}$ mice were a kind gift from Tilo Biedermann (Department of Dermatology, Eberhard Karls University, Tübingen, Germany). All mice were housed under specific-pathogen-free conditions at the animal facilities of the University of Tübingen according to German law and European guidelines. All experiments were approved by the local authorities (Regierungspräsidium Tübingen; Anzeigenummer 1.12.11).

Isolation of BMDCs. Bone marrow-derived dendritic cells (BMDCs) were isolated by flushing the bone marrow from the femurs and tibias of 8- to 14-week-old WT and $NOD2^{-/-}$ mice according to a previously described method (31), with minor modifications.

Cells were harvested at day 8 and used to evaluate the effects of stimulation with different bacterial PGN products on cytokine release and expression of surface markers, as described below.

Stimulation of BMDCs. DCs were stimulated with different ligands, e.g., P3C for TLR2, lipopolysaccharide (LPS) for TLR4, or MDP and PGNpolΔ*lgt* for NOD2, or stimulated with the combined NOD2 and TLR2 ligand PGNpol, at a dose of 1 μg/ml if not otherwise mentioned. The different PGN types were used at a concentration of 10 μg/ml if not otherwise mentioned. After 24 h, supernatants were harvested and analyzed for tumor necrosis factor alpha (TNF-α), IL-6, and IL-12p40 cytokine concentrations. Additionally, the expression of DC activation and maturation surface markers major histocompatibility complex class II (MHC-II) and CD40 was determined by flow cytometry.

Cytokine analysis by ELISA. Concentrations of murine IL-6, IL-8, IL-12p40, and TNF-α in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BD Bioscience, Heidelberg, Germany).

Fluorescence-activated cell sorter (FACS) analysis. Immature DCs were harvested and stimulated for 24 h with different NOD2 or TLR ligands. Cells were washed with phosphate-buffered saline (PBS) and 1% fetal calf serum (FCS). Fc-Block was used to prevent nonspecific binding of antibodies. DCs were incubated for 30 min at 4°C with a fluorochrome-conjugated antibody. The following antibodies were used for staining: allophycocyanin-conjugated anti-mouse CD11c, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD40, CD80, and CD86, phycoerythrin (PE)-conjugated anti-mouse TLR2 and TLR4 (all antibodies from BD Pharmingen), and appropriate isotype controls. After another washing step (twice), the cells were fixed with 4% paraformaldehyde (PFA). A total of 5×10^4 cells were analyzed using a FACS LSR Fortessa flow cytometer (BD Bioscience, Heidelberg, Germany). Data were analyzed with FlowJo 7.6.4 (TreeStar Inc.).

Culture and stimulation of J774 cells and MM6 cells. J774 cells were grown in VLE-RPMI 1640 medium with stable glutamine (Biochrom, Berlin, Germany), 10% FCS, 1% nonessential amino acids, 1% sodium pyruvate, and 0.5% mercaptoethanol. A total of 1×10^6 cells were seeded per well and incubated for 1 h. J774 cells were stimulated with Pam3Cys or PGNpolΔ*lgt* for 48 h. After stimulation, supernatants were collected, and cytokine (TNF-α) concentrations were determined by ELISA (BD Bioscience, Heidelberg, Germany). Mono Mac 6 (MM6) cells were cultured in VLE-RPMI 1640 medium with stable glutamine (Biochrom, Berlin, Germany), 10% FCS, 1% nonessential amino acids, and 1% penicillin-streptomycin. After stimulation of the Mono Mac 6 cells with Pam3Cys and PGNpolΔ*lgt* for 48 h, the supernatants were collected, and the concentration of IL-8 was analyzed by use of an ELISA kit (BD Biosciences) according to the manufacturer's instructions.

Strains and growth conditions. *Staphylococcus aureus* SA113 (wild type) and the Δ*lgt* mutant (no expression of mature lipoproteins) were grown in tryptic soy broth (Sigma, Steinheim, Germany) at 37°C with aeration for 16 h. The optical density at 578 nm was 12 (Heλios α spectrophotometer; Thermo Scientific).

Isolation of polymeric peptidoglycan (PGNpol). The isolation of ultrapure PGN, free of DNA, RNA, proteins, wall teichoic acids, lipoteichoic acids, and salts, was done according to the method of de Jonge et al. (32), with some modifications. These modifications included the usage of three different buffers (buffers A to C). The pellet of a 50-ml overnight culture was boiled in 10 ml buffer A (2.5% SDS in 0.1 M Tris-HCl, pH 6.8) for 20 min at 100°C. The SDS was removed by several washing steps with double-distilled water at 4,700 rpm at room temperature. The pellet was resuspended with 20 ml 0.1 M Tris-HCl, pH 6.8. Cells were disrupted by vortexing with glass beads (150 to 212 μm). The supernatant was subsequently incubated with buffer B for 1 h (10 μg/ml DNase and 50 μg/ml RNase in 0.1 M Tris-HCl, pH 6.8), with buffer C overnight (50 μg/ml trypsin in double-distilled water), and, finally, with hydrofluoric acid (HF) for 4 h. HF was washed out with double-distilled water, and the PGN was lyophilized overnight.

Lyophilized PGN (10 mg/ml) was digested with 500 U mutanolysin of *Streptomyces globisporus* ATCC 21553 (Sigma) in a 12.5 mM phosphate buffer (pH 5.5) at 37°C for 16 h. The sample was boiled for 3 min and centrifuged for 5 min at 10,000 rpm. The supernatant was reduced with sodium borohydride in 0.5 M borate buffer (pH 9) for 20 min at room

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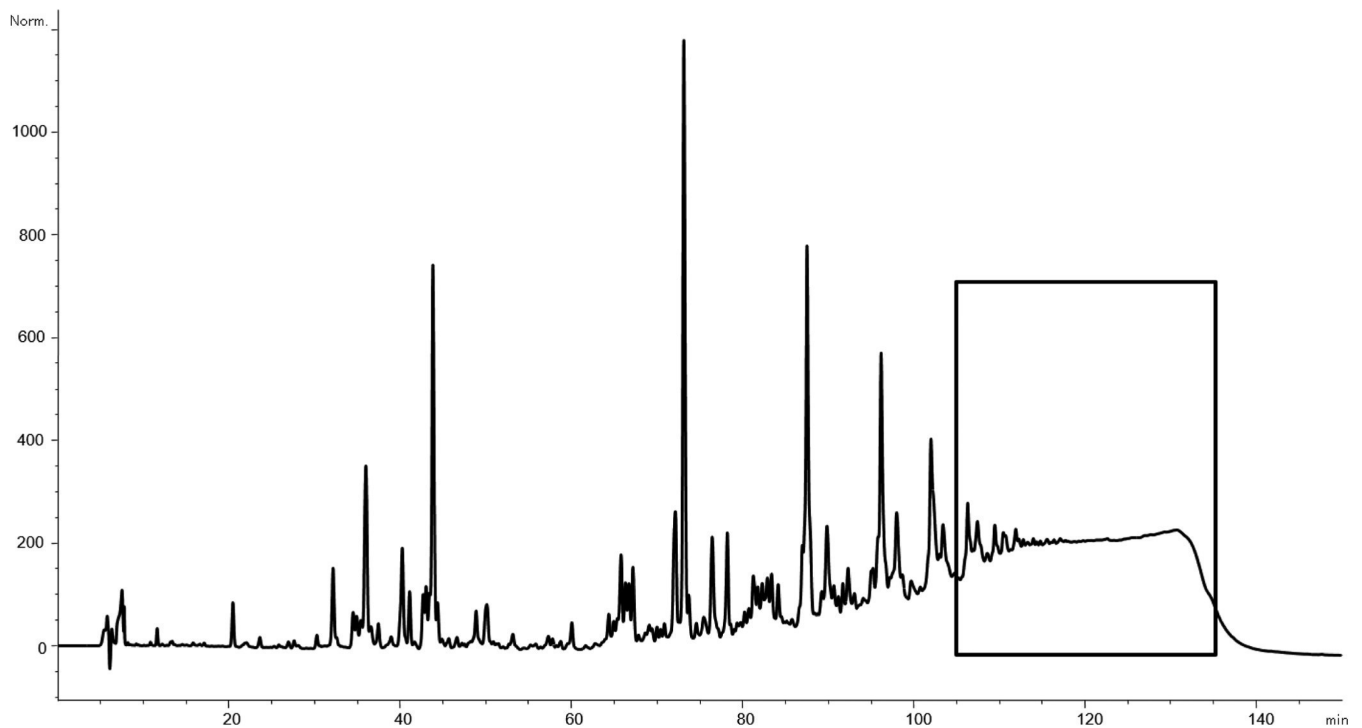

FIG 1 Separation and analysis of polymeric PGN. Separation and analysis of the peptidoglycan polymer were performed using an Agilent 1200 analytical HPLC system. The corresponding polymer peak (r_t = 105 to 135 min) was collected and desalted. Remaining LPS impurities were removed. Shown is the pattern for muramidase-digested PGN from an *S. aureus* SA113 wild-type overnight culture. The boxed sequence indicates the collected polymeric PGN fragments.

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temperature. The pH was subsequently adjusted to 2 with orthophosphoric acid. Samples were further processed or stored at −20°C.

The separation and analysis of the peptidoglycan polymer were performed by high-pressure liquid chromatography (HPLC), based on the method of Glauner (33), using an Agilent 1200 analytical HPLC system. A 250- by 4.6-mm reversed-phase column (Prontosil 120-3-C18 AQ; Bischoff) guarded by a 20- by 4.6-mm precolumn was used. The samples were eluted at a flow rate of 0.5 ml/min, using a linear gradient starting from 100% buffer A (100 mM NaH_2PO_4, 5% [vol/vol] methanol, pH 2.5) to 100% buffer B (100 mM NaH_2PO_4, 30% [vol/vol] methanol, pH 2.8) within 155 min. The column temperature was set to 52°C. The eluted muropeptides were detected by UV absorption at 205 nm. The corresponding polymer peak (r_t = 105 to 135 min) (Fig. 1) was collected and desalted on the same column, using a water-acetonitrile gradient.

LPS contamination was checked using an Endosafe-PTS system (Charles River, Sulzfeld, Germany). Remaining LPS impurities were removed using an Endo Trap Red endotoxin removal kit (Hyglos). Very low LPS contents could be detected (<0.005 endotoxin unit [EU]/mg).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA). Parameters were analyzed by a nonparametric one-way analysis of variance (ANOVA) model for repeated measurements, with the Bonferroni adjustment (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). P values of <0.05 were considered significant. Error bars represent standard errors of the means (SEM). If not otherwise mentioned, figures show means ± SEM of values from three experiments per group. Stimulated DCs from WT mice were compared to unstimulated DCs from WT mice, and stimulated DCs from NOD2$^{-/-}$ mice were compared to unstimulated DCs from NOD2$^{-/-}$ mice.

RESULTS

Purification of polymeric peptidoglycan (PGNpol) from *S. aureus* and its Δ*lgt* mutant. To address the importance of lipopeptide impurities within the PGN fractions in signaling, we isolated polymeric PGN from two *S. aureus* SA113 strains. On the one hand, we isolated PGN from wild-type SA113, containing residual mature lipoproteins within the polymeric PGN meshwork. On the other hand, we isolated PGN from SA113 Δ*lgt*, which is considered lipoprotein free because of its inability to produce mature lipoproteins. A typical pattern for PGN from WT SA113 after muramidase digestion is shown in Fig. 1. The pattern for PGN from SA113 Δ*lgt* was identical to the WT pattern and is therefore not shown.

Activation and maturation of DCs by PGNpol are due to residual lipoproteins. In order to elucidate the role of *S. aureus*-derived polymeric PGN in the NOD2-mediated activation of BMDCs, these cells were stimulated with PGN isolated from either WT *S. aureus* SA113 (PGNpol) or SA113 Δ*lgt* (PGNpolΔ*lgt*). PGNpol is a ligand of NOD2, but while PGNpolΔ*lgt* is lipoprotein free, the PGNpol preparation contains lipoproteins of *S. aureus*, which are known to be ligands of TLR2 (30).

For control purposes, Pam3Cys (a synthetic triacylated lipopeptide), LPS, and MDP were used as specific ligands for TLR2, TLR4, and NOD2, respectively. Incubation of DCs with increasing concentrations of PGNpolΔ*lgt* (up to 100 μg/ml) did not stimulate secretion of IL-6, as indicated in Fig. 2A. In contrast, PGNpol (10 μg/ml) strongly stimulated IL-6 secretion, to a degree that was comparable with that induced by LPS or Pam3Cys. Furthermore, incubation with PGNpolΔ*lgt* (up to 100 μg/ml) did not induce secretion of IL-12p40 (Fig. 2B), while PGNpol (10 μg/ml) strongly stimulated IL-12p40 secretion.

In order to find out whether PGNpol and PGNpolΔ*lgt* lead to DC maturation, DCs were stimulated with PGNpolΔ*lgt* or PGNpol and afterwards analyzed for MHC-II and CD40

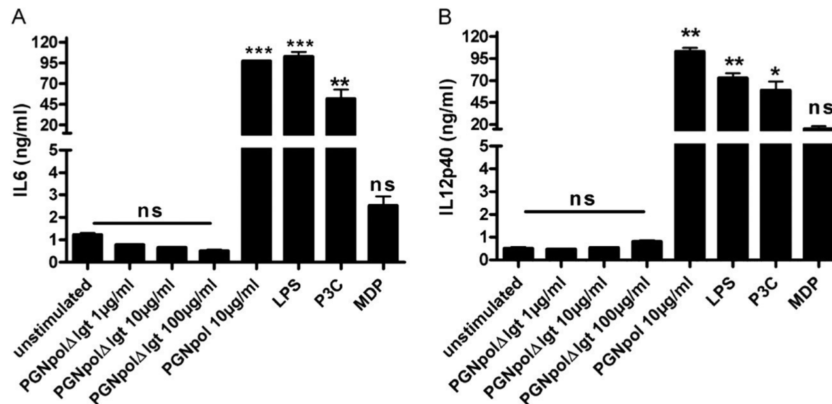

FIG 2 Stimulation with PGNpol, but not PGNpolΔ*lgt*, leads to significant secretion of IL-6 and IL-12p40 in DCs. Incubation of DCs with PGNpol, but not PGNpolΔ*lgt*, stimulated IL-6 (A) and IL-12p40 (B) secretion, which was comparable to that induced by LPS or Pam3Cys. DCs were stimulated with PGNpolΔ*lgt* at concentrations of up to 100 μg/ml. Stimulation with 10 μg/ml PGNpol led to high levels of secretion of IL-6 and IL-12p40. A comparison was made between unstimulated cells and each stimulant by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment. ns, not significant.

surface expression by flow cytometry. DCs stimulated with PGNpol expressed high levels of MHC-II and CD40, resulting in highly activated and mature DCs. In contrast, expression of these surface molecules was nearly unaffected in DCs exposed to PGNpolΔ*lgt*, suggesting a reduced ability of PGNpolΔ*lgt* to activate and mature DCs (Fig. 3).

Stimulation with PGNpol leads to increased activation and maturation of DCs compared to the case with PGNpolΔ*lgt*. To further elucidate the effect of a bacterial NOD2 ligand and the potentially costimulatory effect of a TLR2 ligand, DCs from $NOD2^{-/-}$ mice were compared to DCs from WT mice.

First, secretion of IL-12p40 was tested after stimulation with PGNpolΔ*lgt* and PGNpol. Consistent with the data shown in Fig. 2A, there was no detectable secretion of IL-12p40 after stimulation with PGNpolΔ*lgt* in DCs from either WT mice or $NOD2^{-/-}$ mice.

In contrast, stimulation with PGNpol induced a significantly smaller IL-12p40 signal in DCs from $NOD2^{-/-}$ mice than in DCs from WT mice (Fig. 4). These data strongly suggest that a combined NOD2 and TLR2 signal is necessary to induce a strong secretion of IL-12p40 and that the smaller signal in the DCs from $NOD2^{-/-}$ mice was the result of the sole activation of TLR2. Similar results were obtained with IL-6 after stimulation of DCs from $NOD2^{-/-}$ mice with PGNpolΔ*lgt* and PGNpol (data not shown).

To further test the effect of combined TLR2 and NOD2 stimulation on DC activation and maturation, we analyzed the expression of the DC surface markers MHC-II and CD40 by flow cytometry. In $NOD2^{-/-}$ DCs, incubation with PGNpol led to a significantly smaller proportion of $MHC\text{-}II^{high}$ cells than the case in stimulated WT DCs (Fig. 5A). Furthermore, there was an increased expression of CD40 in DCs after stimulation with PGNpol compared to PGNpolΔ*lgt* (Fig. 5B). Taken together, our data strongly suggest that a combined stimulation of the TLR2 and NOD2 pathways by PGNpol is important for the activation and maturation of DCs.

Combined stimulation of DCs with PGNpolΔ*lgt* and Pam3Cys reveals synergistic effects on TNF-α secretion pattern. TNF-α is a key cytokine in IBD and is well known for its role in mediating innate immune responses (34). To confirm our hypothesis that combined stimulation with TLR2 and NOD2 ligands

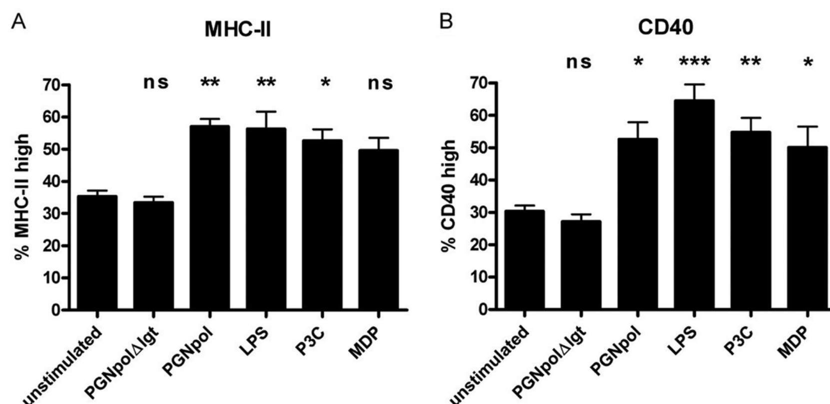

FIG 3 Stimulation with PGNpol, but not PGNpolΔ*lgt*, leads to increased MHC-II and CD40 expression in DCs. Immature BMDCs were activated with several NOD2 or TLR2 ligands. Maturation was quantified by FACS analysis to assess the levels of MHC-II (A) and CD40 (B). The quantification was based on isotype controls. Stimulation of DCs with PGNpol (10 μg/ml) led to strong MHC-II and CD40 signals. In contrast, stimulation of DCs with PGNpolΔ*lgt* (10 μg/ml) did not lead to increased expression of MHC-II and CD40. A comparison was made between unstimulated cells and each stimulant by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment.

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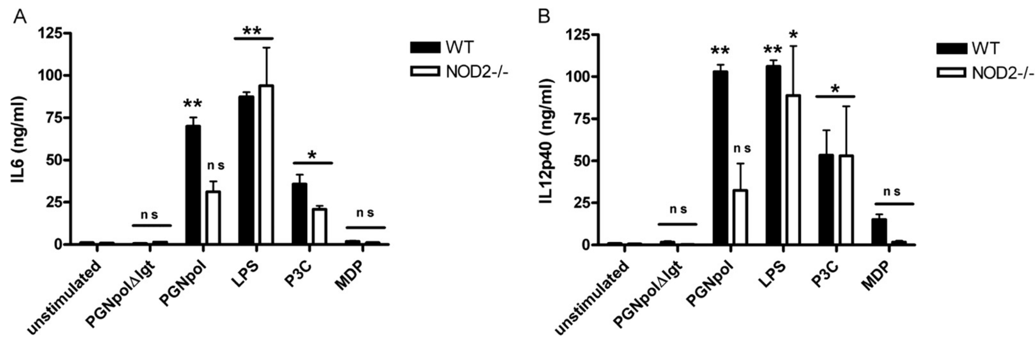

FIG 4 Stimulation with PGNpol in DCs is dependent on a NOD2 costimulus. Stimulation with PGNpol led to significantly increased secretion of IL-6 (A) and IL-12p40 (B) in DCs from WT mice compared to unstimulated controls. In contrast, DCs derived from NOD2$^{-/-}$ mice failed to show increased IL-6 and IL-12p40 secretion upon stimulation with PGNpol. Stimulated DCs from WT mice were compared to unstimulated DCs from WT mice, and stimulated DCs from NOD2$^{-/-}$ mice were compared to unstimulated DCs from NOD2$^{-/-}$ mice.

acts synergistically on DC cytokine secretion, we costimulated DCs with the NOD2 ligand PGNpolΔ*lgt* and the TLR2 ligand Pam3Cys and determined the secretion of TNF-α. In line with our hypothesis, the costimulation induced a significantly enhanced expression of TNF-α in DCs compared to the case in PGNpolΔ*lgt*- or P3C-monostimulated DCs (Fig. 6).

To further confirm these findings, the experiments were extended to additional immune cells, i.e., a murine macrophage cell line (J774 cells) (Fig. 7) and a human monocyte cell line (MM6 cells) (Fig. 8). In both cell lines, the synergistic costimulation with PGNpolΔ*lgt* and P3C resulted in an increased expression of proinflammatory cytokines, indicating that the observed effect is not restricted to mouse DCs but seems to be relevant for the activation of different types of innate immune cells.

DISCUSSION

NOD2 mutations play an important role in Crohn's disease (1, 2). However, how exactly these mutations contribute to this specific disease still remains uncovered. NOD2, as an important intracellular receptor of the innate immune system, senses bacterial cell wall products, such as MDP, and activates NF-κB (35). While MDP was the first NOD2 ligand described (36, 37), it later became clear that polymeric (16, 38) as well as monomeric (14) PGN also activates NOD2. DCs are the most potent APCs in the intestinal mucosa and have important functions in the mucosa-associated immune system (39). Because of their important role in activating and also regulating the immune system, they also seem to play an important role in Crohn's disease (11). Therefore, we studied the role of NOD2-mediated activation of DCs via natural *S. aureus*-derived PGN. In the present study, we focused on the role of polymeric PGN, which is an important part of the bacterial cell wall. Defined isogenic *S. aureus* mutant strains were used to elucidate the effect of NOD2 ligand (PGNpolΔ*lgt*) monostimulation or the synergistic effect of NOD2 and TLR2 ligand (PGNpol) costimulation. While monostimulation with a natural NOD2 ligand (PGNpolΔ*lgt*) did not lead to activation and maturation of DCs, costimulation with a NOD2 and TLR2 ligand (PGNpol) led to strong activation and increased cytokine secretion (IL-6 and IL-12p40) of DCs *in vivo*. This effect was seen not only in isolated DCs but also in J774 cells (macrophages) and MM6 cells (monocytes). In addition to already published work by Müller-Anstett et al. (26), our data indicate that singular NOD2 activation seems to

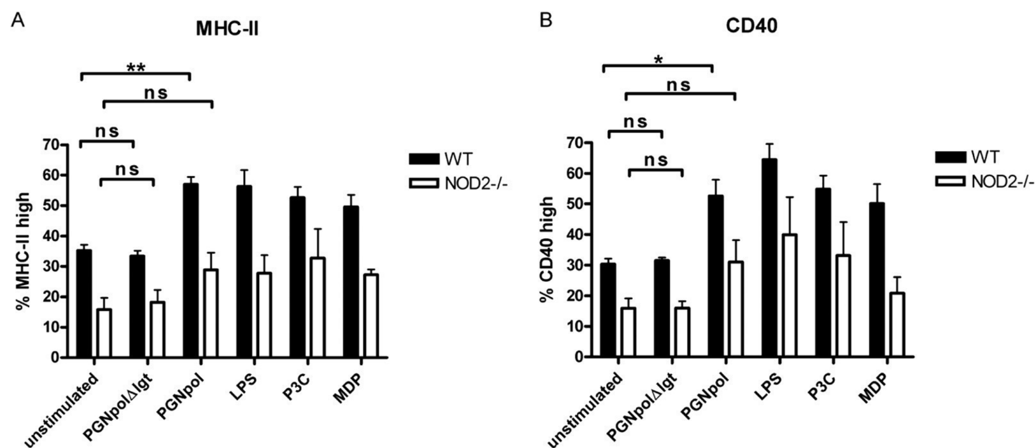

FIG 5 DC stimulation with PGNpol in WT mice leads to increased expression of MHC-II and CD40 compared to that in NOD2$^{-/-}$ mice. Stimulation of DCs from NOD2$^{-/-}$ mice with PGNpol (10 μg/ml) did not lead to significantly more expression of MHC-II (A) or CD40 (B), indicating that the costimulatory effect of NOD2 and TLR2 is necessary for effective maturation of DCs. Stimulated DCs from WT mice were compared to unstimulated DCs from WT mice, and unstimulated DCs from NOD2$^{-/-}$ mice were compared to stimulated DCs from NOD$^{-/-}$ mice. In addition, a comparison between DCs from WT and NOD2$^{-/-}$ mice was made by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment.

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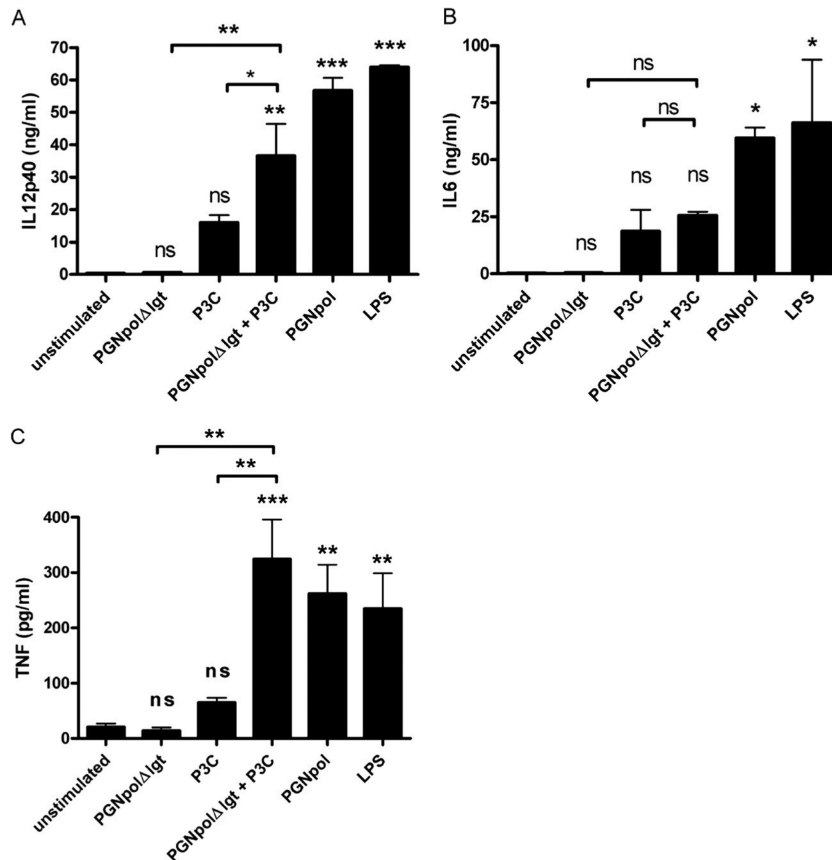

FIG 6 Combined stimulation of DCs with NOD2 and TLR2 ligands leads to stronger secretion of IL-12p40 and TNF than stimulation with each of the stimuli alone. DCs were stimulated with PGNpolΔ*lgt* (10 μg/ml), Pam3Cys (1 μg/ml), and LPS (1 μg/ml). After stimulation with both PGNpolΔ*lgt* and Pam3Cys, there were significantly higher signals for IL-12p40 (A) and TNF (C) than the case for stimulation with the single components alone. (B) There was not a significantly higher signal of IL-6 than that for stimulation with the single components. Unstimulated DCs from WT mice were compared to stimulated DCs from WT mice by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment.

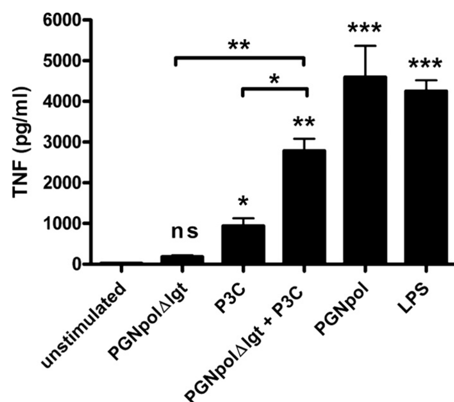

FIG 7 Combined stimulation of J774 cells with NOD2 and TLR2 ligands leads to significant TNF-α secretion. J774 cells were stimulated with Pam3Cys (1 ng/ml), PGNpolΔ*lgt* (10 μg/ml), PGNpol (10 μg/ml), and LPS (1 μg/ml) for 48 h. After stimulation with both PGNpolΔ*lgt* and Pam3Cys, there was a significant increase of TNF-α, which seemed to be higher than stimulation with the single components. Unstimulated DCs from WT mice were compared to stimulated DCs from WT mice by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment.

play a minor role in activating immune cells. However, synergistic costimulation of the NOD2 and TLR2 pathways results in potent activation of DCs, macrophages, and monocytes.

In patients with Crohn's disease, an exaggerated Th1-mediated immune response may contribute to mucosal inflammation (40). *In vivo*, MDP itself is not able to induce a Th1 cytokine profile but rather invokes a Th2 immune response (41). Additionally, in support of our results, it has been demonstrated that combined stimulation with MDP plus a TLR2 or TLR4 ligand leads to a synergistic release of IL-6 and IL-12p40 in BMDCs, which is abolished in $NOD2^{-/-}$ DCs (41). While stimulation of the innate immune system with a sole NOD2 ligand, in our case *S. aureus*-derived polymeric PGNpolΔ*lgt*, does not lead to an immune response, such a response is markedly enhanced after dual stimulation via the NOD2 and TLR2 pathways. The complex interplay between NOD2 and TLR2 signaling might have an influence on intestinal homeostasis.

However, how can these results be translated into the clinical entity of Crohn's disease? Activation of NOD2 by MDP protects mice from experimental colitis (42), thus promoting intestinal homeostasis. Additionally, PGN from specific lactobacilli features anti-inflammatory effects and thus seems to be crucial for the probiotic action of these specific strains (43). These studies further support the hypothesis that stimulation of NOD2 alone—as op-

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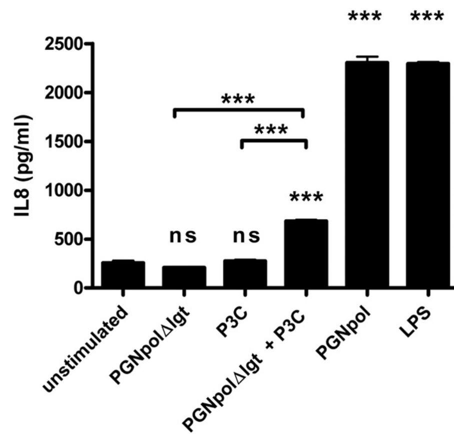

FIG 8 Combined but not single stimulation of MM6 cells with NOD2 and TLR2 ligands leads to a significant increase of IL-8. MM6 cells were stimulated with Pam3Cys (10 ng/ml), PGNpolΔ*lgt* (10 μg/ml), PGNpol (10 μg/ml), and LPS (1 μg/ml) for 48 h. After stimulation with both PGNpolΔ*lgt* and Pam3Cys, there was a significant increase of IL-8, whereas stimulation with single components did not lead to IL-8 secretion. Stimulated DCs from WT mice were compared to unstimulated DCs from WT mice. Unstimulated DCs from WT mice were compared to stimulated DCs from WT mice by using a nonparametric one-way ANOVA model for repeated measurements, with the Bonferroni adjustment.

posed to costimulation of NOD2 and TLR2—can possibly lead to downregulation of inflammatory pathways. A "loss of function" in the NOD2 gene might therefore be an important part of the pathogenesis of Crohn's disease.

In summary, we showed that in DCs, synergistic costimulation of the NOD2 and TLR2 signaling cascades leads to an increased activation and maturation of DCs, and also to increased cytokine secretion, compared to the case with monostimulation. These results might be important for a better understanding of the complex interplay between these receptors in maintaining homeostasis in the intestinal immune system.

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Original article

Clinical factors are associated with vitamin D levels in IBD patients: A retrospective analysis

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OBJECTIVE: There is growing evidence that vitamin D deficiency plays a role in the development and the course of inflammatory bowel disease (IBD). However, the correlation between vitamin D deficiency and clinical parameters in IBD is still not completely understood.

METHODS: A retrospective study of IBD patients was performed. Vitamin D values were analyzed, regardless of vitamin D substitution administration, and correlated with clinical parameters such as medical therapy, anatomical situation, location of the disease and disease activity. Level of 25-hydroxyvitamin D [25(OH)D] <50 nmoL/L was regarded as vitamin D deficiency and <75 nmoL/L as insufficiency.

RESULTS: In total, 208 IBD patients were analyzed, including 123 with Crohn's disease (CD) and 85 with ulcerative colitis (UC). Therapy with azathioprine did not affect the vitamin D values of either disease entity. But CD patients benefited from therapy with tumor necrosis factor-α inhibitor and exhibited significantly higher vitamin D levels than those without. Furthermore, significantly lower vitamin D levels were found if CD was located in the small bowel or if the small bowel had been resected. Moreover, significantly lower levels of vitamin D were detectable for high disease activity (reflected by high simple clinical colitis activity index values) in patients with UC.

CONCLUSIONS: Vitamin D deficiency is common in patients with IBD. However, certain clinical situations lead to significantly lower vitamin D levels and may therefore require close monitoring for vitamin D deficiency.

KEY WORDS: Crohn disease, inflammatory bowel diseases, tumor necrosis factor-alpha, ulcerative colitis, vitamin D.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic intestinal disorders that together form the complex of inflammatory bowel disease (IBD).[1] The pathogenesis of these diseases involves the inappropriate activation of the mucosal immune system, which is triggered by the intestinal microbiota in genetically predisposed individuals.[1–4] Mutations in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene encoding for

NOD2 have been shown to be a major risk factor in the development of CD.[5–8] In addition, environmental factors, such as vitamin D deficiency, have been found to play a role in the pathogenesis of IBD.[8,9] Vitamin D is commonly known to be an important regulator of calcium and phosphate metabolism and is therefore essential for bone health.[10,11] However, there is mounting evidence that vitamin D also plays an important role as a regulator of the innate and adaptive immune system.[11–13] In a murine colitis model the application of calcitriol was associated with reduced mucosal injury.[14] The prevalence of vitamin D deficiency is high in patients with IBD.[15,16] However, it is still not clear whether vitamin D substitution has a beneficial effect on the course of the disease. In a prospective study infliximab treatment was associated with positive effects on bone metabolism.[17] On the other hand, in a retrospective study in patients with CD an inverse association between vitamin D levels and intestinal inflammation was found.[18] In another prospective study the application of vitamin D led to a decrease in the C-reactive protein (CRP) level in UC patients, suggesting that vitamin D supplementation has a beneficial effect on the inflammatory activity of UC.[19] In several other interventional studies vitamin D substitution appeared to have beneficial effects on clinical activity in IBD.[20–22]

The aim of our study was to investigate the prevalence of vitamin D deficiency in a single, northern German IBD cohort and to correlate the vitamin D levels with different clinical variables (e.g. disease-specific medication, location of disease, anatomical situation after surgery and disease activity).

PATIENTS AND METHODS

Patients with UC and CD who were admitted to the Outpatient Clinic of the Division of Gastroenterology, University of Rostock Medical Center (Rostock, Germany) from January 2011 to September 2014 were retrospectively analyzed. Data were collected from patients' medical records, including 25-hydroxyvitamin D [25(OH)D] levels, 25-OH-vitamin D substitution, disease activity, disease-specific medication [azathioprine (AZA), tumor necrosis factor (TNF)-α inhibitor] and the anatomic situation (i.e. colectomy). Disease activity was assessed in patients with CD using the Harvey–Bradshaw index (HBI),[23] and in UC using the simple clinical colitis activity index (SCCAI)[24]. It was differentiated among the ileocecal region (the terminal ileum, defined as 15 cm proximal to the ileocecal valve and cecum) and small bowel (duodenum, jejunum, ileum other than terminal ileum).

The patients were divided into two groups, including one with vitamin D substitution and the other without. Vitamin D substitution was administrated with colecalciferol 20 000 IE. The vitamin D status of the patients was assessed based on serum 25(OH)D levels.[10] Vitamin D deficiency was defined as: severe deficiency, serum 25(OH)D level <27.5 nmoL/L; deficiency, <50 nmoL/L; insufficiency, <75 nmoL/L. Normal vitamin D levels are defined as values higher than or equal to 75 nmoL/L.[10,25–28]

Statistical analysis

All the data were entered into a Microsoft Access database and analyzed by using the SPSS 22.0 (IBM, Armonk, NY, USA). The average of all single vitamin D measurements per patient represented the individual level. Continuous variables were expressed as mean ± standard deviation, whereas categorical variables were expressed as numbers and percentages or frequencies. To compare the vitamin D level between the two groups, the *t*-test was performed in case of normal distribution; otherwise, the Mann–Whitney *U*-test was used for the analysis. Normal distribution of the variables was assessed by the Kolmogorov–Smirnov test. *P* value less than 0.05 was regarded as statistically significant.

RESULTS

Altogether 208 patients with IBD (123 CD and 85 UC) were included in the study. In total, 1520 25 (OH)D values were analyzed, including 936 values obtained from the CD group (56.8 ± 32.4 nmol/L, *n* = 123), of which 486 were without substitution, and 584 obtained from the UC group (61.5 ± 33.2 nmol/L), of which 288 were without substitution. There was no significant difference in the vitamin D levels between the CD (56.2 ± 20.6 nmoL/L, *n*= 123) and UC groups (59.5 ± 22.9 nmoL/L, *n* = 85).

Vitamin D status

Because vitamin D values vary along the year as a result of different exposure to the sun, we first analyzed participants' vitamin D status during the year. Vitamin D deficiency in patients with IBD was more common during the winter months than the summer months (Fig. 1). Severe vitamin D deficiency was particularly present from January until April. The highest number of normal vitamin D values was found in July and August. Fig. 1 also confirms that the vitamin

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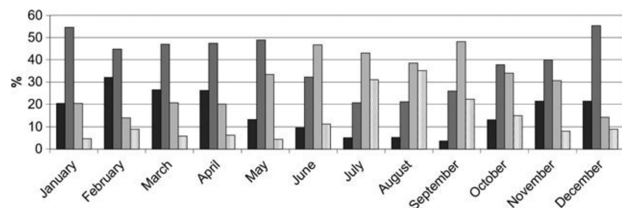

Figure 1. Vitamin D deficiency in patients with inflammatory bowel disease over the year. (■) Severe deficiency; (▩) deficiency; (▫) insufficiency; (□) normal.

D values in our cohort were obtained throughout the year. All patients (with and without vitamin D substitution) were included into this graph, since vitamin D substitution was relatively constant in all groups over the year.

Vitamin D substitution increased 25(OH)D levels

Vitamin D substitution with colecalciferol was administrated in patients with vitamin D deficiency. We compared the vitamin D values in the patients with and without substitution (Fig. 2). In the CD group, 450 vitamin D values were obtained with substitution (61.3 ± 21.6 nmol/L, n = 63) and 486 without substitution (42.6 ± 16.7 nmol/L, n = 60). In the UC group 296 vitamin D values were obtained with substitution (59.9 ± 22.2 nmol/L, n = 46) and 288 without substitution (41.8 ± 19.5 nmol/L, n = 39). In each of the IBD disease entities (CD and UC), the difference between vitamin D values with and without substitution was highly significant (P < 0.001). In addition, we examined whether higher vitamin D levels might also correlate inversely with a lower disease activity in CD or UC. However, the disease activity was not significantly different in both groups when comparing low or high vitamin D levels (data not shown).

Vitamin D values depended on the disease activity in UC

The average HBI in the CD group was 3.3 and the average SCCAI in the UC group was 2.9. Because disease activity may influence vitamin D levels, we stratified patients with CD and UC according to their disease activity (CD: HBI 0–3 *vs* >9; UC: SCCAI 0–2 *vs* >6; with HBI 0–3 and SCCAI representing disease remission and HBI >9 and SCCAI >6 for high activity) and compared the respective vitamin D levels.

We analyzed 439 vitamin D values (56.3 ± 21.2 nmol/L, n = 75) in the HBI 0–3 group in patients with CD, with 33 vitamin D values (58.3 ± 20.6 nmol/L, n = 9) in the HBI >9 group. No significant difference of vitamin D values between remission and highly active disease were detected (Fig. 3a).

Additionally, we compared 199 vitamin D values (64.3 ± 27.8 nmol/L, n = 40) in the SCCAI 0–2 group of patients with UC with the SCCAI > 6 group with 55 vitamin D values (44.8 ± 6.9 nmol/L, n = 10). We found significantly lower vitamin D levels in highly active disease in the UC group compared with that of the UC in remission (P = 0.008, Fig. 3b).

Disease-specific medications

To address the association of disease-specific medication with vitamin D status patients in the CD and UC groups were stratified according to their disease-specific medications (AZA or TNF-α inhibitor). TNF-α inhibitors infliximab and adalimumab were pooled because of their similar mode of action.

Altogether 39 patients in the CD group received anti-TNF therapy. UC patients receiving anti-TNF therapy were not included into the analysis due to their small sample size (n = 8). AZA was given to 37 patients with CD and 22 with UC. In total 14 patients received both anti-TNF agents and AZA.

Therapy with AZA did not affect the vitamin D values in patients with either CD (Fig. 4a) or UC (Fig. 4b): In the CD group 193 vitamin D values (58.3 ± 21.2 nmol/L, n = 37) in patients receiving AZA were compared with 743 vitamin D values from those not receiving AZA (55.5 ± 21.2 nmol/L, n = 86). In the UC group 136 vitamin D values from patients receiving AZA (68.6 ± 32.4 nmol/L, n = 22) were compared with 448 vitamin D values from patients who were not receiving AZA (56.9 ± 20.8 nmol/L, n = 63).

In contrast, therapy with TNF-α inhibitor was associated with significantly higher vitamin D values in the CD group. In the CD group 287 vitamin D values (63.2 ± 20.3 nmol/L, n = 39) were obtained under therapy with TNF-α inhibitor and compared with 649 vitamin D values from patients not receiving TNF-α inhibitor therapy (54.7 ± 21.3 nmol/L, n = 84; P = 0.014, Fig. 4c).

Disease location

CD can be localized in all the GI tract, from the mouth to the perianal region. We compared vitamin D values in the CD group in terms of the location of the disease (small bowel, ileocecal region, colon, or perianal region; Fig. 5).

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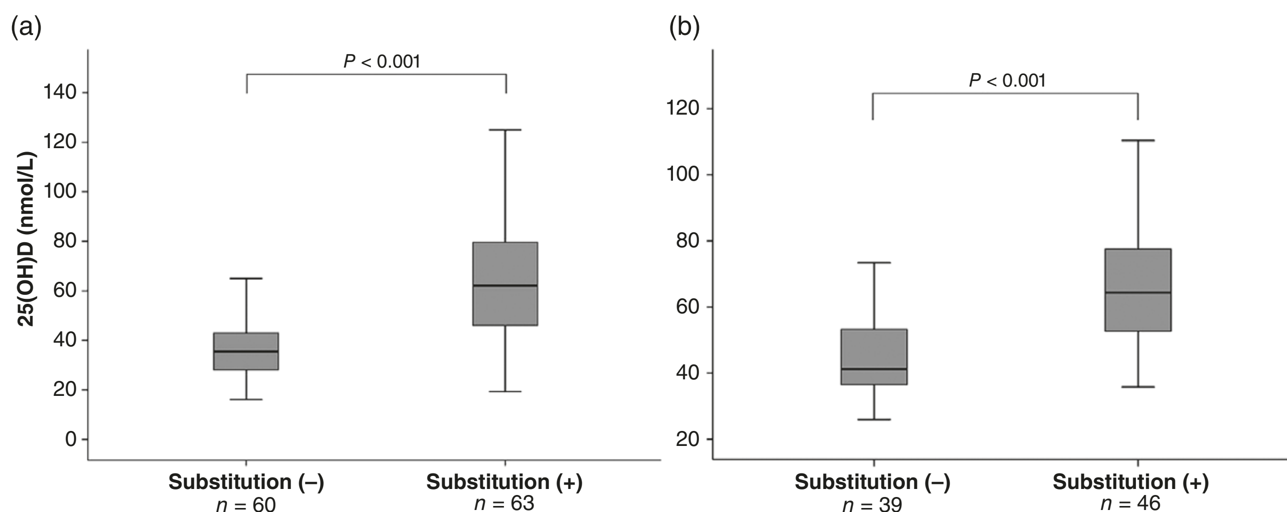

Figure 2. 25-hydroxyvitamin D [25(OH)D] values are significantly higher in the substituted group of patients with (a) Crohn's disease and (b) ulcerative colitis (both $P < 0.001$).

CD patients with disease involvement of the small bowel yielded 271 vitamin D values (48.5 ± 16.1 nmol/L, $n = 31$) that were significantly lower than the 665 vitamin D values from those without (58.8 ± 21.4 nmol/L, $n = 92$) ($P = 0.038$, Fig. 5a). The disease location of other parts of the intestine did not significantly affect the vitamin D values [terminal ileum involvement (55.1 ± 18.0 nmol/L, $n = 66$) *vs* no involvement (57.4 ± 23.4 nmol/L, $n = 57$); colonic involvement (53.7 ± 19.2 nmol/L, $n = 90$) *vs* no involvement of the colon (62.8 ± 23.2 nmol/L, $n = 33$); perianal involvement (60.7 ± 28.0 nmol/L, $n = 26$) *vs* no perianal involvement (54.9 ± 18.8 nmol/L, $n = 97$) (Fig. 5b–d)].

Vitamin D values after surgical resections

CD often requires surgical resection. Fig. 6 depicts vitamin D values in CD after surgical resection. The small bowel was defined as parts of the small bowel other than terminal ileum and the ileocecal region.

In patients with CD after resection of the small bowel 88 vitamin D values (44.1 ± 13.5 nmol/L, $n = 15$)

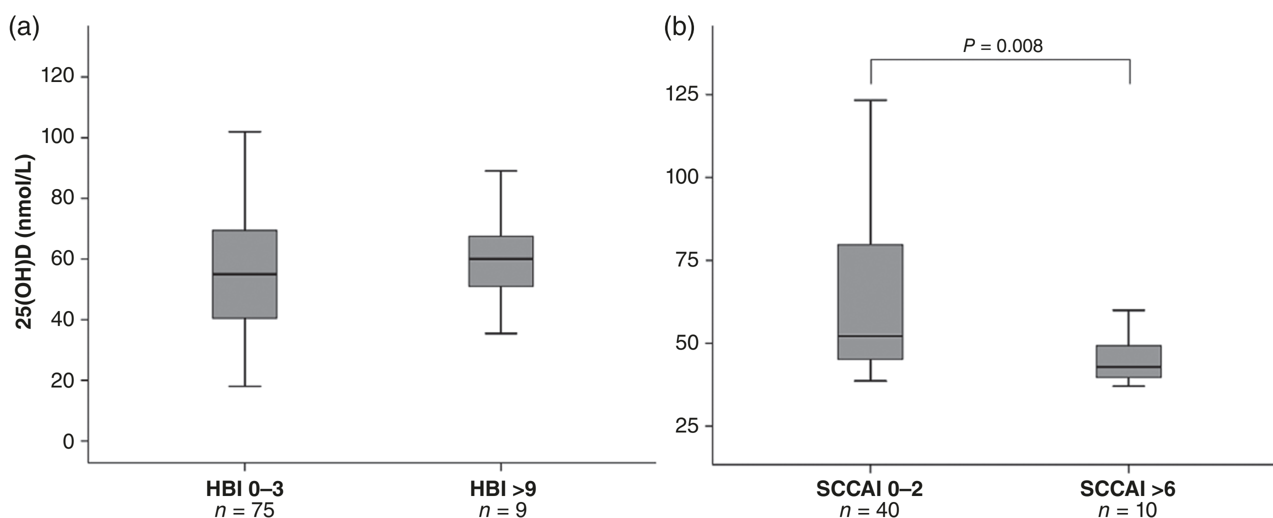

Figure 3. Disease activity in (a) Crohn's disease, measured using the Harvey–Bradshaw index (HBI) and (b) ulcerative colitis, measured using the simple clinical colitis activity index (SCCAI), is associated with vitamin D levels. 25(OH)D, 25-hydroxyvitamin D.

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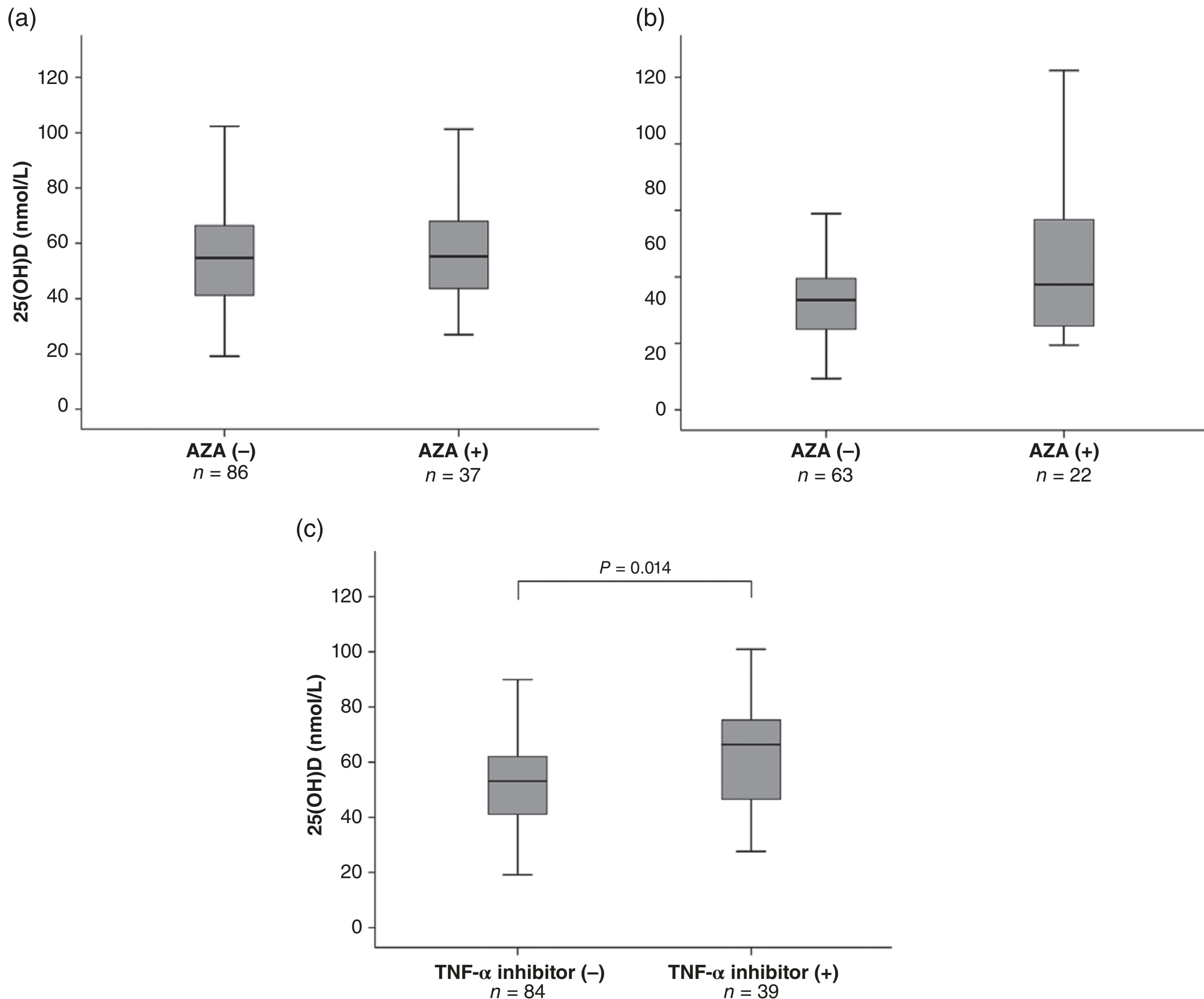

Figure 4. The effect of azathioprine (AZA) on the vitamin D values in (a) Crohn's disease and (b) ulcerative colitis; and (c) treatment with tumor necrosis factor (TNF)-α inhibitor on the vitamin D values in patients with Crohn's disease. 25(OH)D, 25-hydroxyvitamin D.

were compared with 848 vitamin D values from those without resection of the small bowel (57.1 ± 20.8 nmol/L, *n* = 108) with significantly decreased values (*P* = 0.023, Fig. 6a). Other resections do not lead to significant differences in the vitamin D values [ileocecal resections (55.0 ± 18.3 nmol/L, *n* = 43) *vs* no ileocecal resections (55.8 ± 21.7 nmol/L, *n* = 80); colonic resection (51.4 ± 18.1 nmol/L, *n* = 22) *vs* no colonic resection (56.4 ± 20.1 nmol/L, *n* = 101) (Fig. 6b,c)].

DISCUSSION

Vitamin D deficiency was found to be very prevalent in the general population[29,30] as well as in patients with IBD[15,16]. In our retrospective study in a single northern German IBD cohort we analyzed vitamin D values in patients with IBD (CD and UC) and correlated them with disease-specific parameters. According to other studies in patients without IBD, vitamin D deficiency is more common in the winter months than the summer months.[31] However, even in the summer months an optimal vitamin D value was achieved only in the month of July in just about 30% of all patients with IBD. The reasons for this include the low ultraviolet B radiation in the high latitude of northern Germany and low exposure to the sun. Based on our data, vitamin D deficiency should be tested and treated more aggressively from January until April. However, a high incidence of vitamin D deficiency can also be expected in the summer months.

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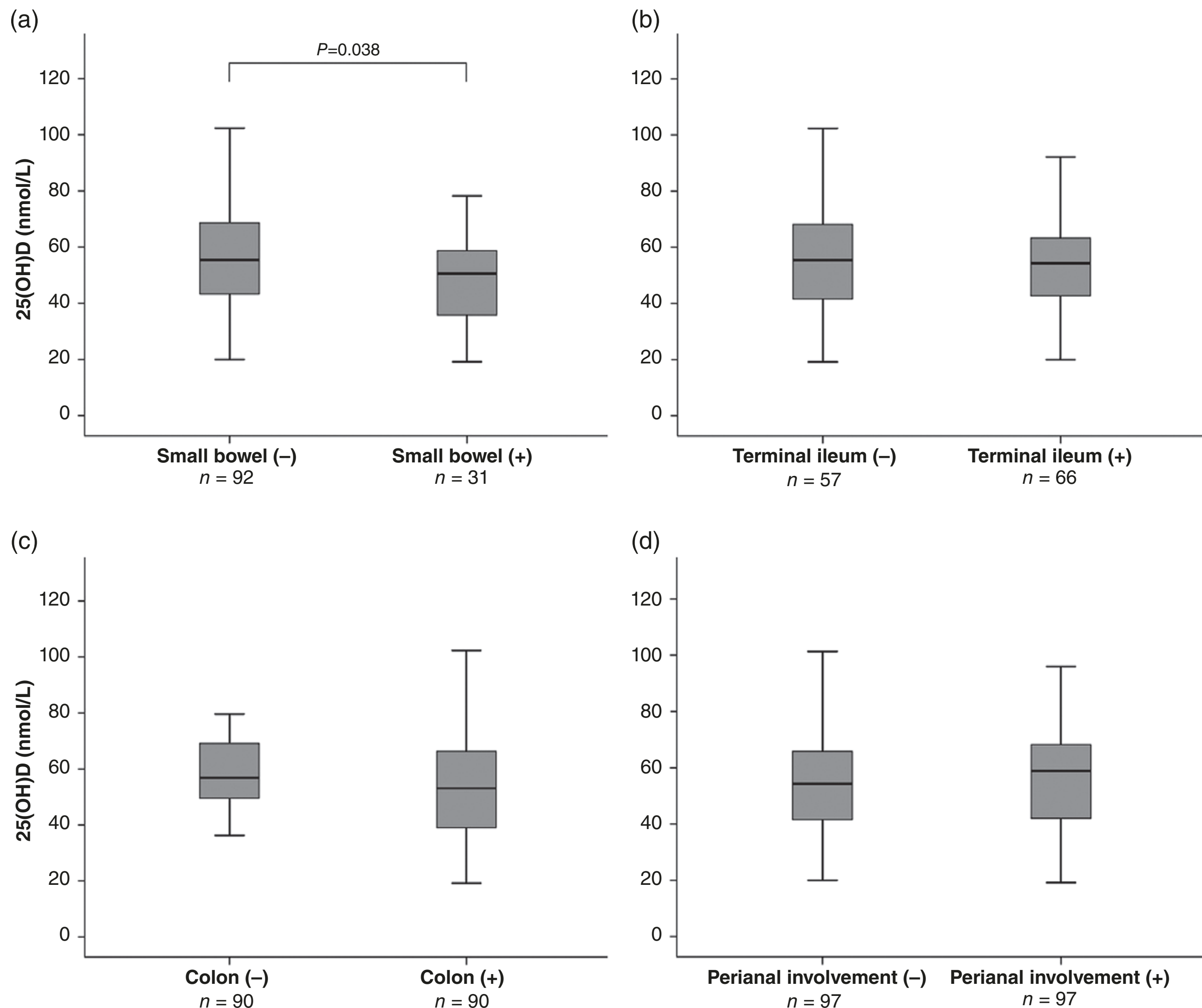

Figure 5. Vitamin D values depending the disease location in Crohn's disease in (a) small bowel, (b) terminal ileum, (c) colon and (d) perianal involvement. 25(OH)D, 25-hydroxyvitamin D.

Three major results were found in this study. First, patients under therapy with TNF-α inhibitor had significant higher vitamin D levels than those receiving other therapies. Second, disease activity correlated with vitamin D levels in patients with UC, but not in those with CD. Third, patients with CD located in the small bowel and after small bowel resection showed significant lower vitamin D levels.

We showed that there was a correlation between the use of TNF-α inhibitor and high vitamin D levels in CD. It would be reasonable to argue that patients on TNF-α inhibitor may have a better disease control and therefore, higher vitamin D levels (e.g. more exposure to sunlight, better absorption of vitamin D and a different diet). However, we could not detect a correlation between higher disease activity and lower vitamin D levels in patients with CD. Therefore, the effect of TNF-α inhibitor on the vitamin D levels appears to be independent of the disease activity in CD. The mechanism by which therapy with TNF-α inhibitor increases vitamin D levels cannot be elucidated from this study. In a recent article Winter *et al.* have shown that vitamin D levels may influence the initial response to TNF-α inhibitor.[32] Lower vitamin D levels prior to treatment were associated with a higher rate of IBD relapse. This result may be consistent with our study, where the application of TNF-α inhibitor was associated with high vitamin D levels. Additionally, we hypothesize that there is a strong

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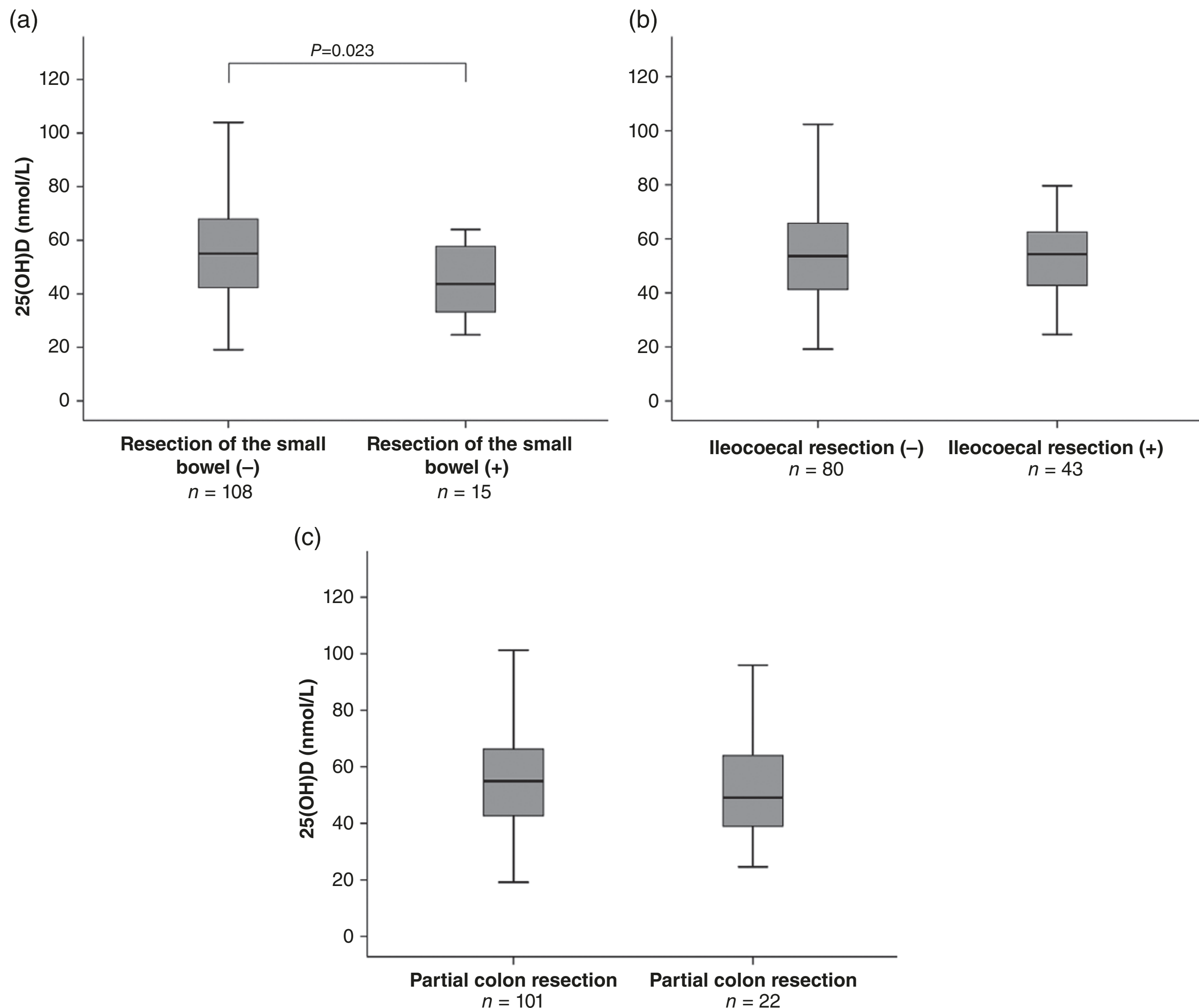

Figure 6. Vitamin D values in Crohn's disease after surgical resections in (a) the small bowel, (b) ileocecum and (c) colon. 25(OH)D, 25-hydroxyvitamin D.

link between intestinal inflammation, drugs affecting the intestinal mucosal inflammatory level, the patient's vitamin D status and intestinal microbial composition. In recent years there has been mounting evidence that the gut microbiota play a crucial role in the pathogenesis of IBD. Interestingly, the use of TNF-α inhibitors may also result in changes of the intestinal bacterial composition.[33] Previous studies using murine models show that vitamin D and vitamin D receptor are important regulators of the intestinal bacterial composition.[34–36] In a genome-wide association study, mutations in the vitamin D receptor gene were associated with different intestinal microbial profiles.[37] While different studies of patients with IBD have shown an altered microbial composition, that is, dysbiosis,[38–41] one can now speculate that there are potentially beneficial triggers in addition to the application of TNF-α inhibitor in changing the bacterial composition back into a state of remission, such as the substitution of vitamin D. However, prospective studies are needed to test the hypothesis whether vitamin D substitution leads directly to a change in intestinal bacterial composition.

Screening for vitamin D deficiency is recommended by German CD guidelines.[42] However, whether vitamin D substitution has a beneficial effect on the disease course is still not clear. In a recent prospective study in patients with UC by Gubatan *et al.*,[43] lower

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vitamin D values were associated with a higher risk of relapse in the following 12 months. In our study, patients with a high UC disease activity showed lower vitamin D levels; however, we did not find such an association in patients with CD. From these studies one can hypothesize that vitamin D status is a surrogate marker of intestinal inflammation in UC.

Different factors could play a role in the pathogenesis of vitamin D deficiency in patients with IBD. These include low exposure to sunlight, a different diet, impaired absorption, impaired conversion of vitamin D into its active metabolites and an increased metabolism of vitamin D.[44–46] Vitamin D is fat-soluble and therefore requires bile acids for its uptake. Changes in the anatomy after surgical resection of the small bowel can cause interruptions of the enterohepatic circulation. This may also play a role in vitamin D deficiency. In our study a clear correlation was found between CD with small bowel involvement and low vitamin D levels. According to this result, resection of the small bowel also resulted in significantly lower vitamin D level. Other resections did not lead to significant changes in the vitamin D levels, therefore the small bowel plays an important role in the uptake of vitamin D, particularly in IBD. As a consequence, patients with CD located in the small bowel and also after small bowel resections may benefit from a close screening for vitamin D deficiency.

In this study, we have demonstrated that certain clinical conditions may have beneficial or adverse effects on vitamin D levels in patients with IBD. However, more prospective studies are needed to assess the consequence of vitamin D deficiency and vitamin D supplementation on the disease course of patients with IBD.

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ORIGINAL ARTICLE

Alterations in the mucosa-associated bacterial composition in Crohn's disease: a pilot study

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Abstract
Introduction Changes in the intestinal bacterial composition seem to play a major role in the pathogenesis and in the clinical course of inflammatory bowel diseases (IBD), which consist of Crohn's disease (CD), and ulcerative colitis (UC). Mutations in the NOD2 gene are the most important genetic risk factors for the development of CD. In this study, the association between mucosal biopsies and the mucosa-associated bacterial composition from CD and UC patients regarding their genetic risk factors (mutations in the *NOD2* gene), their endoscopic activity, and their medical therapy (TNF-α blocking therapy) was examined.
Material and methods Seventy biopsies from routine colonoscopies from 33 IBD patients (26 CD and 7 UC) were obtained. Disease activity and clinical characteristics were assessed. Seven different bacterial strains (*Bacteroides fragilis*, *Escherichia coli*, *Prevotella melaninogenica*, *Clostridium coccoides*, *Clostridium difficile, Bifidobacterium bifidum*, and *Faecalibacterium prausnitzii*) were quantified using real-time PCR. *NOD2* genotyping from patients with CD was performed.
Results Five of the 24 patients were positive for at least one mutation in the *NOD2* gene. The bacterial composition was different in CD compared to UC, in macroscopic healthy compared to macroscopic inflamed biopsies, in *NOD2* mutated compared to *NOD2* wildtype patients, and in patients receiving TNF-α blocking therapy compared to patients without this treatment.
Conclusion This study further characterizes the mucosa-associated bacteria in IBD patients. Different clinical situations lead to an altered mucosa-associated bacterial composition. The analyzed bacteria could be promising targets for cost-effective surveillance or therapies in IBD patients.

Keywords Crohn's disease · Ulcerative colitis · IBD · *NOD2* · Mucosa-associated bacteria · RT-PCR

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic intestinal disorders and together form the complex of inflammatory bowel diseases (IBD) [1]. Until today the pathogenesis of these disease entities is not completely understood; however, it involves an inappropriate activation of the mucosal immune system triggered by the intestinal microbiota in genetically predisposed individuals [1–4]. This model is supported by several findings: In IBD inflammation is mainly localized to areas in the gut with high bacterial quantities. Antibiotics have a beneficial effect in some patients, e.g., those with fistula or luminal activity [5]. Surgical diversion of the fecal stream is beneficial in some IBD patients [6, 7]. In several mouse models of colitis, bacteria are needed for the development of inflammatory changes [8]. An association of adherent-invasive *Escherichia coli* in ileal samples of patients with CD has been described [9]. Additionally, there are

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genetic polymorphisms in CD, e.g., mutations in the *NOD2* gene, which activate the innate immune system in the process of bacterial recognition. *NOD2* is expressed intracellularly in antigen presenting cells, e.g., macrophages and dendritic cells, and to a lesser extent in intestinal epithelial cells and T cells. A link between mutations in the *NOD2* gene and CD was first described in 2001 by two groups [10, 11]. Additionally, there are also clinical associations with mutations in the *NOD2* gene independent of CD, for example graft-versus-host-disease (GvHD) [12–14], septicemia [15], spontaneous bacterial peritonitis in liver cirrhosis [16], worsened outcome after intestinal transplantation [17], and the development of short bowel syndrome in the absence of CD [18]. In contrast to this increasing number of clinical conditions which are associated with mutations in the *NOD2* gene, the exact function of *NOD2*, and how mutations contribute to the clinical manifestation of CD is still under debate [19, 20]. One aspect is, that NOD2 as a receptor senses parts of the bacterial cell wall, which might be an important link between the innate immune system and the intestinal bacterial composition. Muramyl dipeptide (MDP) has been described as the classic ligand for the NOD2 receptor [21, 22]. However, the receptor also senses peptidoglycan (PGN), which is an important part of the bacterial cell wall [23, 24]. The NOD2 receptor has also been shown to play an important role in the secretion of defensins from Paneth cells. *NOD2*$^{-/-}$ mice and patients with certain mutations in the *NOD2* gene display a decreased expression of α-defensins in the Paneth cells [25, 26]. In another study in mice, it was shown that there is bilateral interaction of the NOD2 receptor and the intestinal bacteria, such that *NOD2* is also a regulator of the commensal gut microbiota [27].

Furthermore, Rehman et al. provided evidence for a distinct role for *NOD2* in the development of the bacterial composition: In *NOD2*$^{-/-}$ mice, an increased number of commensal microbiota was found. Additionally, the microbial composition in *NOD2*$^{-/-}$ mice was already significantly altered at a very early weaning stage [28].

Nevertheless, it is currently not known whether the changes in the gut microbiota in IBD are due to a disturbed local environment because of the inflammation or are a primary cause for the development of these disease entities.

Like in other mammals, the human gut microbiota consists of different phyla, mainly Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria [29–31]. Most taxa from the intestinal microbiota belong to the phyla Firmicutes and Bacteroidetes.

From a clinical point of view, dysbiosis—potentially linked to *NOD2* mutations and the resulting alteration in the interplay with the gut microbiota—may play an important role in the pathogenesis of CD [32–34]. Also a greater abundance of Enterobacteriaceae (phylum proteobacteria), mainly *E. coli*, was noted [9]. Other strains, like the butyrate-producing *Faecalibacterium prausnitzii*, are thought to play a beneficial role in the intestinal mucosa due to anti-inflammatory capacities [35–37]. Additionally, CD is also linked to a reduced diversity of the microbiota [38].

To investigate the relationship between the mucosa-associated bacteria and IBD, biopsy samples from a well-defined cohort of CD and UC patients were examined in our study. Real-time PCR technology was used to quantify seven bacterial strains as important representatives of the four main bacterial phyla in the human gut. The abundance of these strains was investigated and the results were correlated to clinical data.

Biopsy samples were investigated from CD and UC patients from macroscopically healthy and inflamed mucosa of the colon. Additionally, the CD patients were analyzed regarding mutations in the *NOD2* gene and these results were correlated to their clinical course.

Material and methods

Thirty-three IBD patients were recruited from the University Medical Center Rostock, Germany. Twenty-six patients had CD and seven patients had UC. The following clinical parameters were recorded: age, sex, disease activity, and disease-specific medication. The disease activity was determined via Crohn's Disease Activity Index (CDAI) in CD and via the Mayo score in UC. Colonoscopy was performed for a clinical indication and biopsies were taken from macroscopically non-inflamed and from inflamed mucosa. Altogether, 70 biopsies were analyzed. An EDTA blood sample was drawn from the patients with CD for analysis of mutations in the *NOD2* gene.

The study was approved by the ethics board of the University of Rostock (A 2012–0121). Written informed consent was obtained from each participant prior to enrollment.

DNA extraction

The biopsy specimens were collected in ATL buffer (Qiagen, Hilden, Germany) and snap frozen at −80 °C immediately. The DNA isolation was performed with the DNA stool extraction kit (Qiagen, Hilden, Germany). Prior to DNA isolation, tissue samples were homogenized with the tissue disruptor (Qiagen, Hilden, Germany).

PCR inhibition control

Exclusion of potential inhibitory matrix effects was performed utilizing real-time PCR targeting human ß-Globin sequences according to Klaassen et al. with minor modifications [39]. Primers βGlo_up (5′-GGGCAACGTGCTGGTCTG) and βGLO_down2 (5′-ATACTTGTGGGCCAGGGCAT) were purchased at TIB MOLBIOL (Berlin, Germany). The reaction mix consisted of 12.5 μl SYBR Green real-time PCR Master

Mix (Applied Biosystems, Foster City, CA, USA), 175 nM βGlo_up, 175 nM βGLO_down2 as well as 2 μl template and was adjusted to 20 μl per reaction with nuclease free water. Samples underwent the following PCR-program: one cycle of 95 °C for 10 min and then 40 cycles of 95 °C for 15 s, 60 °C for 60 s, followed by one cycle of 95° for 15 s, 60° for 20 s and 95 °C for 15 s. Amplified DNA was detected after each elongation step. Samples with a cycle threshold less than 33 cycles were classified as "non-inhibited."

PCR analysis

The abundance of seven bacterial strains as representatives of their corresponding phylum (Table 1) was analyzed (*E. coli*, *Bacteroides fragilis, Bifidobacterium bifidum*, *Prevotella melaninogenica*, *Clostridium difficile*, *Clostridium coccoides*, *Faecalibacterium prausnitzii*). Real-time qPCR was performed using an ABI ViiA 7 (Applied Biosystems, Waltham, Massachusetts, USA). Primer sequences are shown in Table 1.

The PCR amplification program was defined as one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. After each run, a melting point analysis was performed to control the amplicon specificity. To ensure the functionality of the primers, bacterial probes from each strain were ordered from the Leibniz-Institut–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Additionally, primer efficacy was optimized for each primer pair using serial dilutions of bacterial DNA in known concentrations. The optimal primer efficacy was confirmed using the bacterial reference probes from above (Table S1: Primer efficacies).

To analyze the bacterial composition between different clinical settings, we used the Delta CT relative quantification method as described elsewhere [47, 48]. Briefly, CT values of the specific bacterial strains were normalized to beta-actin (Delta CT value). Delta CT values from different settings were directly compared.

NOD2 genotyping

An EDTA blood sample was drawn from the patients with CD for analysis of mutations in the *NOD2* gene. The three major mutations in the *NOD2* gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844 and SNP 12; G908R, NCBI reference SNP ID: rs2066845 and SNP 13; 1007 fs, NCBI reference SNP ID: rs2066847) were detected as described previously in genomic DNA extracted from whole blood [18]. Briefly, whole blood was collected in EDTA-anticoagulated tubes and DNA was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The Taqman MGB biallelic discrimination assay was applied using the two pre-made assays c_ _ 11717468_20 and c_ _11717466_20 for the R702W and the G908R point mutations in *NOD2* as well as a custom developed assay for

Table 1 16S rRNA gene-targeted group and species-specific primers used in this study

Bacteria	Phylum	Primer name	nM	Primer sequence (5′–3′)	Reference
E. coli	Proteobacteria	E.coliF395Fw	200	CAT GCC GCG TGT ATG AAG AA	[40]
		E.coliR470Rev	300	CGG GTA ACG TCA ATG AGC AAA	[40]
B. fragilis	Bacteroidetes	Bact-F285	300	GGT TCT GAG AGG AGG TCC C	[41]
		Univ-R338	200	GCT GCC TCC CGT AGG AGT	[41]
Prevotella spp.	Bacteroidetes	Prevo-F449	300	CAG CAG CCG CGG TAA TA	[42]
		Prevo-R757	300	GGC ATC CAT CGT TTA CCG T	[42]
C. coccoides	Firmicutes	g-Ccocc-F	300	AAA TGA CGG TAC CTG ACT AA	[43]
		g-Ccocc-R	300	CTT TGA GTT TCA TTC TTG CGA A	[43]
C. difficile	Firmicutes	Cdifficile-F	300	TTG AGC GAT TTA CTT CGG TAA AGA	[44]
		Cdifficile-R	200	CCA TCC TGT ACT GGC TCA CCT	[44]
Bifidobacterium bifidum	Actinobacteria	g-Bifid-F	200	CTC CTG GAA ACG GGT GG	[45]
		g-Bifid-R	200	GGT GTT CTT CCC GAT ATC TAC A	[45]
Faecalibacterium prausnitzii	Firmicutes	PrausF480F	300	CAG CAG CCG CGG TAA A	[42]
		PrausR631R	300	CTA CCT CTG CAC TAC TCA AGA AA	[42]
β-Actin		ActinF	200	GCT GTG CTG TCC CTG TAT GCC TCT	[46]
		ActinR	200	CCT CTC AGC TGT GGT GGT GAA GC	[46]
β-Globin		βGLO_up	175	GGGCAACGTGCTGGTCTG	[39]
		βGLO_down2	175	ATACTTGTGGGCCAGGGCAT	[39]

the 1007 frame shift mutation (forward primer, GTCCAATAACTGCATCACCTACCT; reverse primer, CAGACTTCCAGGATGGTGTCATTC and VIC-labeled probe, CAGGCCCCTTGAAAG; FAM-labeled probe, CAGGCCCTTGAAAG. Applied Biosystems, Waltham, MA, USA).

Twenty nanograms of extracted genomic DNA were applied with 10 µl of TaqManUniversal PCR Master Mix (Applied Biosystems, Foster City, California, USA) and 0.8 µl of 20 × SNP Genotyping Assay and adjusted to a final volume of 20 µl. PCR was carried out on an ABI Prism 7000 real-time PCR instrument (Applied Biosystems, Foster City, CA). The protocol was performed according to the instructions of the manufacturer and the results were analyzed using Sequence Detection System (SDS) Software V. 1.2.3. (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

The mucosal biopsies were compared between CD and UC. Additionally, comparisons were made between biopsies from inflamed and non-inflamed mucosa, between *NOD2* mutated and *NOD2* wildtype patients and between patients receiving TNF-α blocking therapy, and patients without this therapy. Statistical analysis was performed using the GraphPad Prism 4 Software (La Jolla, CA, USA). Parameters were analyzed by Student's *t* test after normal distribution was ensured. *p* values <0.05 were considered significant (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Results

Patient characteristics

The patient characteristics of the CD patients are listed in Table 2. The disease activity in CD patients was measured via the CDAI [49]. The mean CDAI was 186 (38 –332). Three patients received a therapy with infliximab/adalimumab and two patients received a therapy with azathioprine/6-mercaptopurine.

Table 2 Patient characteristics of the CD patients

Number of patients with CD	26
Sex	11 male:15 female
Age mean	41 (22–67)
Patients with AZA/6-MP	2
Patients with IFX/ADA	3
CDAI mean	185.65 (38–332)
Number of patients with *NOD2* mutations	5

The patient characteristics of the UC patients are listed in Table 3. The disease activity in the UC group was measured via the Mayo score [50]. The mean Mayo score was 4.28 (0–8). Two patients received therapy with infliximab/adalimumab.

NOD2 genotyping

Twenty-four of the 26 patients with CD were genotyped for the three major *NOD2* mutations (SNP 8, SNP 12, and SNP 13). Two patients did not give informed consent for genotyping. Five of 24 patients (20.8 %) were positive for one mutation, which is close to the described percentage in the literature [51]. The frequency of *NOD2* mutations was not examined in UC patients because mutations in the *NOD2* gene do not seem to be associated with the development of UC [52].

Analysis of the abundance of seven different bacterial strains as representatives of different phyla

In order to assess the role of the above mentioned bacterial strains as potential representatives of their corresponding phylum (see Table 1), we examined their abundance in different clinical situations (CD vs. UC, inflamed vs. non-inflamed mucosa, presence of *NOD2* mutations, and use of TNF-alpha blocking therapy).

Differences of the intestinal bacterial composition in CD compared to UC were investigated (Fig. 1). The biopsies were taken from inflamed and non-inflamed appearing mucosa. The abundance of seven different bacterial strains was analyzed quantitatively by real-time PCR. In CD, there was a significant decrease in the abundance of *B. fragilis* ($p=0.0089$) and *B. bifidum* ($p=0.024$) compared to biopsies from UC patients. There was no significant difference for *E. coli*, *P. melaninogenica*, *C. coccoides*, *C. difficile*, and *F. prausnitzii*.

Biopsies from macroscopically inflamed and non-inflamed colonic mucosa were compared in CD patients (Fig. 2). In biopsies from macroscopically inflamed mucosa of CD patients, a significant decrease in the abundance of *B. fragilis* ($p=0.0243$) and of *C. coccoides* ($p=0.0013$) was observed, whereas the abundance of *E. coli* was increased ($p=0.0194$).

Table 3 Patient characteristics of the UC patients

Number of patients with UC	7
Sex	4 male:3 female
Age mean	55 (43–75)
Patients with AZA/6-MP	0
Patients with IFX/ADA	2
Mayo score mean	4.28 (0–8)

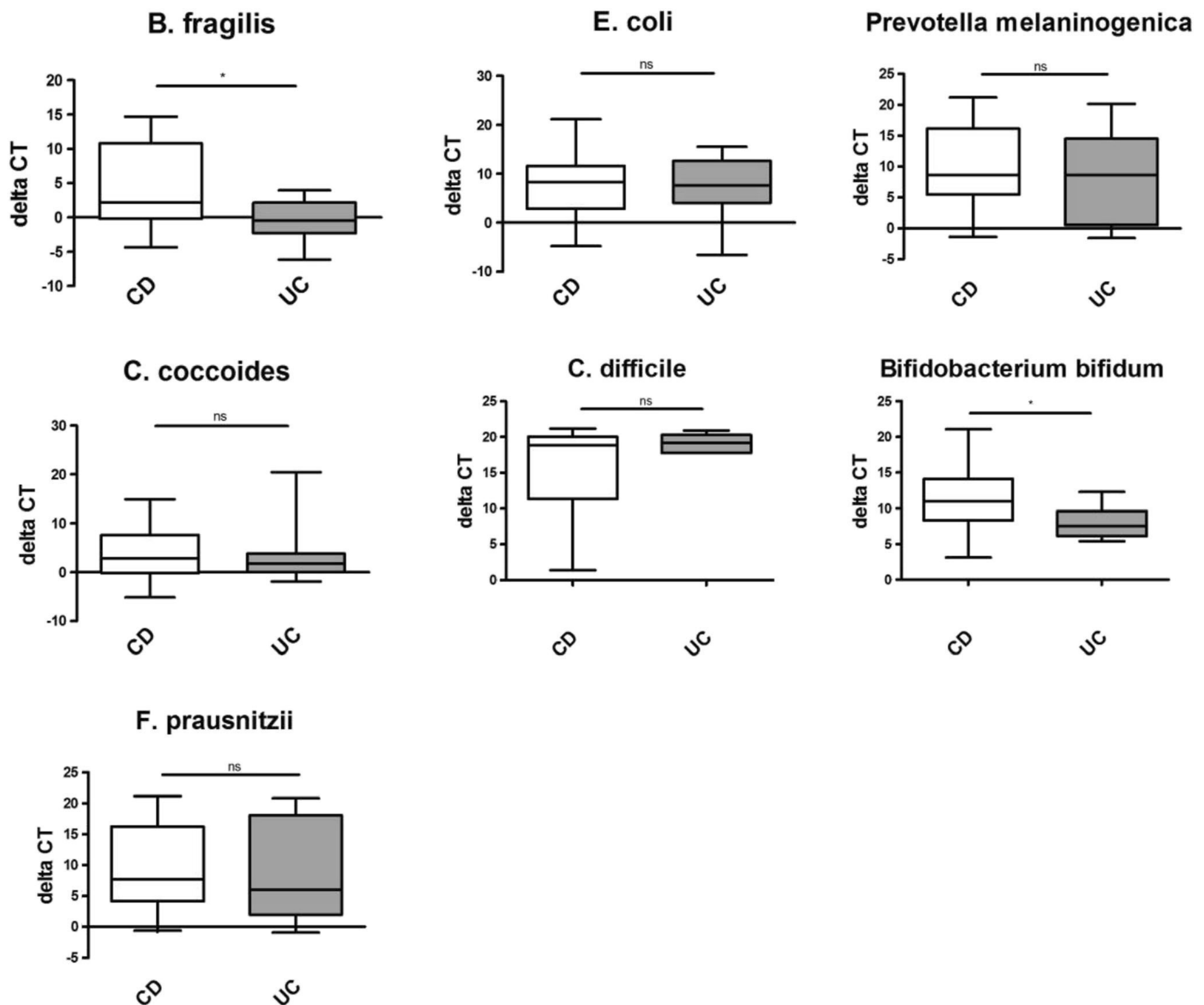

Fig. 1 Mucosa-associated bacterial composition in biopsies from patients with CD and UC. Biopsies from patients with CD ($n = 26$) and ulcerative colitis (UC) ($n = 7$) were analyzed for the abundance of seven different bacterial strains. Delta CT values were compared. In CD, there was a significant decrease in the abundance of *B. fragilis* and *B. bifidum*. Student's *t* test was used for statistical analysis (*$p < 0.05$)

There was no significant difference for *P. melaninogenica*, *C. difficile*, *B. bifidum*, and *F. prausnitzii*. A separate analysis of UC patients was not performed due to the small number of patients.

Biopsy samples from inflamed and non-inflamed mucosa from patients with CD were compared according to the *NOD2* status of the patients (*NOD2* mutated vs wildtype, Fig. 3). In biopsies from *NOD2* mutated CD patients, there was a significant increase in *E. coli* ($p = 0.0014$) and in *C. difficile* ($p = 0.0007$) compared to biopsies from *NOD2* wildtype CD patients. There was no significant difference for *B. fragilis*, *P. melaninogenica*, *C. coccoides*, *B. bifidum*, and *F. prausnitzii*.

Biopsies from inflamed and non-inflamed mucosa from CD and UC patients receiving either TNF-α blocking therapy (infliximab or adalimumab) or not were compared (Fig. 4). In patients receiving TNF-α blocking therapy, there was a significant higher abundance of *F. prausnitzii* ($p = 0.0176$) than in those who did not reiceive TNF-a blocking medication. There was no significant difference for *B. fragilis*, *E. coli*, *P. melaninogenica*, *C. coccoides*, *C. difficile*, and *B. bifidum*.

Discussion

The present study addresses the question how inflammation and the mucosa-associated bacterial composition interact with each other and how this may be influenced by pharmacological intervention.

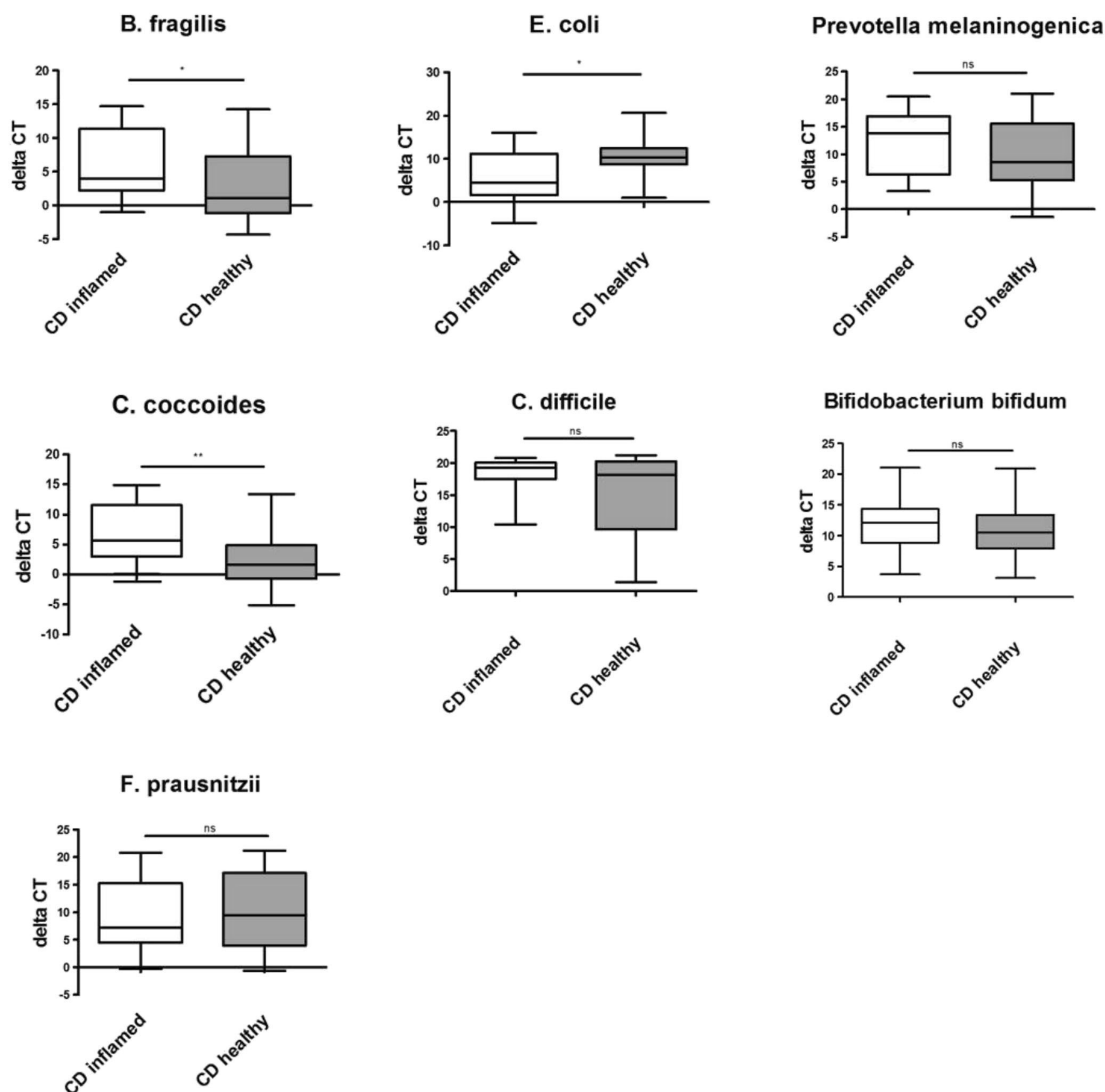

Fig. 2 Mucosa-associated bacterial composition in macroscopically inflamed and non-inflamed mucosal biopsies from CD patients. Biopsies from inflamed and non-inflamed colonic mucosa of patients with x disease (CD) were obtained and the abundance of seven different bacterial strains was analyzed. Delta CT values were compared. In macroscopically inflamed biopsies from CD patients, there was a significant decrease in *B. fragilis*, in *C. coccoides* and an increase in *E. coli* compared to non-inflamed biopsies. Student's *t* test was used for statistical analysis (*$p < 0.05$, **$p < 0.01$)

In the present study, seven different bacterial strains were analyzed, because they were regarded as important representatives of the most important bacterial phyla in the human gut. *B. fragilis* and *P. melaninogenica* for Bacteroidetes, *B. bifidum* for Acinetobacteria, *C. coccoides* for Firmicutes, and *E. coli* for Proteobacteriae. *C. difficile* belongs to the phylum of Firmicutes but may have a strain specific biology as it may cause *C. difficile* associated enteritis. *F. prausnitzii* also belongs to the phylum of Firmicutes but appears to have strain specific role in intestinal health in general and in IBD in particular. While these tested seven strains quantitatively comprise only a small amount of the mucosa-associated bacterial composition, changes in each strain may reflect changes of the corresponding phylum. It is possible though that the interaction between host and gut microbiota does not occur at the level of bacterial phyla but either at specific strains, such as

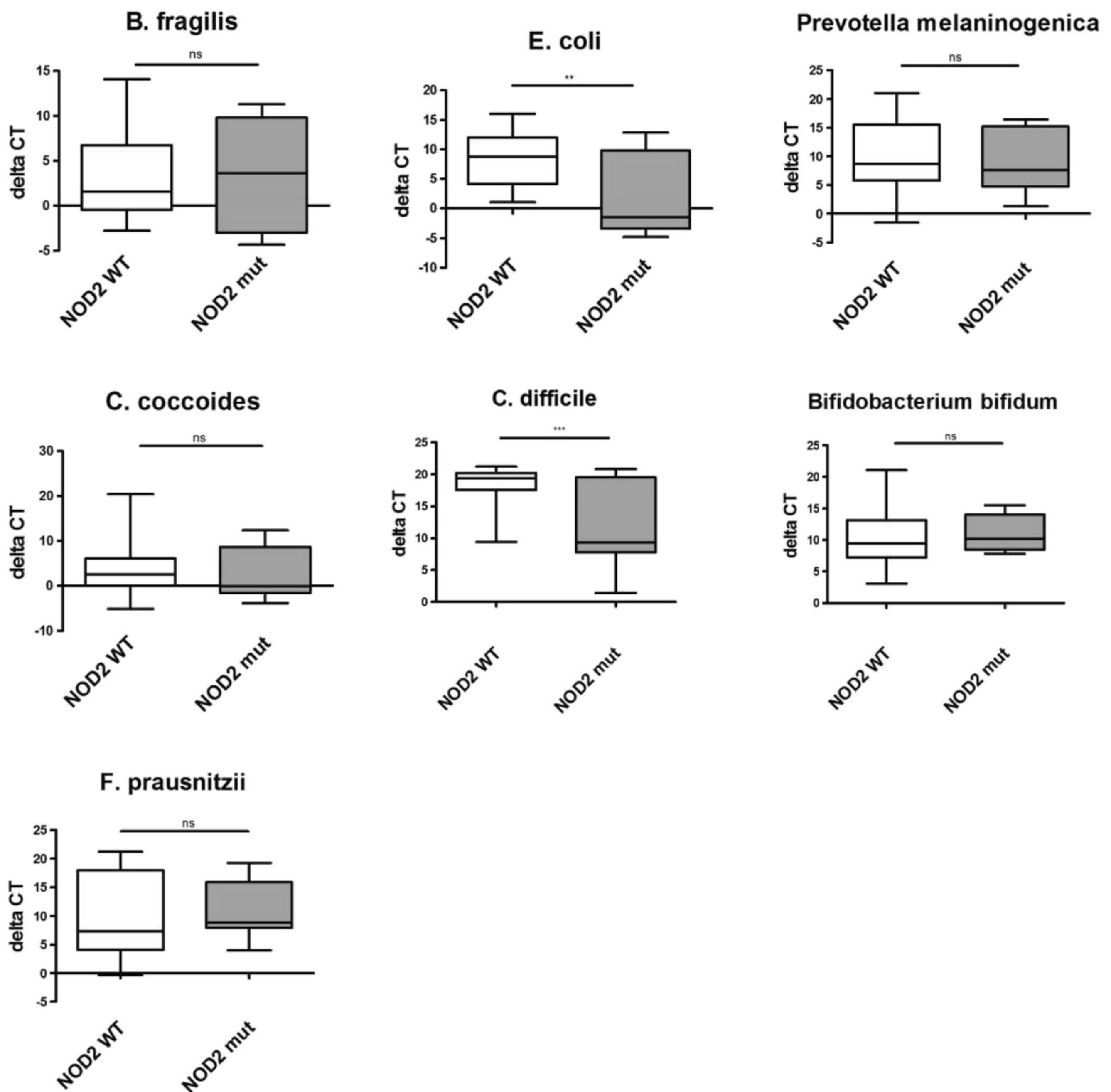

Fig. 3 Mucosa-associated bacterial composition in biopsies from *NOD2* mutated and *NOD2* wildtype CD patients. Biopsy samples from inflamed and non-inflamed patients with CD were compared according to the *NOD2* genotype (*NOD2* mutated vs. *NOD2* wildtype). Seven different bacterial strains were analyzed. Delta CT values were compared. In biopsies from *NOD2* mutated CD patients ($n = 5$), there was a significant increase in *E. coli* and in *C. difficile* compared to biopsies from *NOD2* wildtype patients ($n = 19$). Student's t test was used for statistical analysis (*$p < 0.05$; **$p < 0.01$)

Faecalibacterium prausnitzii or at a more metabolic-functional level. The potential beneficial or disadvantageous role of a single strain on the intestinal immune system can only be uncovered using a distinct mouse model and metabolic-functional characteristics may be addressed using metagenomic analysis, both of which are beyond the scope of this study. On the other hand, it is rather conceivable that the genetic composition of the host and pharmacologic intervention of the immune response lead to changes in bacterial phyla more than individual bacterial strains or metabolically active bacterial groups.

To address a possible interaction of inflammation and the local mucosa-associated bacterial composition, inflamed and non-inflamed mucosa were compared. *B. fragilis* and *C. coccoides* as representatives of Bacteroidetes and Firmicutes had a reduced relative abundance. Interestingly,

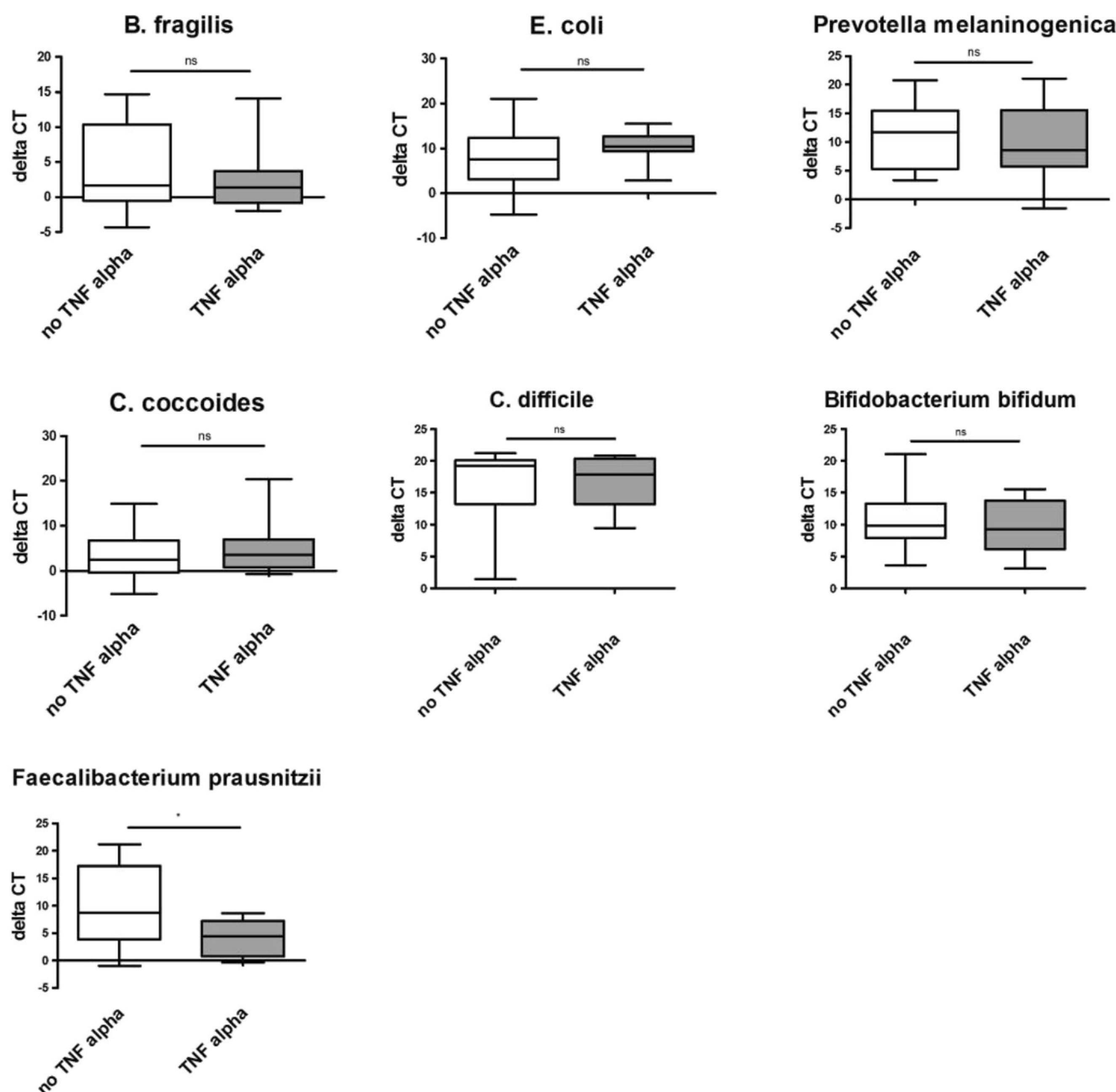

Fig. 4 Mucosa-associated bacterial composition in colonic mucosa from patients receiving either TNF-α blocking therapy or not. Biopsy samples from inflamed and non-inflamed mucosa from CD and UC patients were stratified into receiving TNF-α blocking therapy (infliximab or adalimumab) or not. Seven different bacterial strains were analyzed. Delta CT values were compared. In patients receiving TNF-α blocking therapy ($n = 5$), there was a significant increase in *F. prausnitzii* compared to patients without TNF-α blocking therapy ($n = 28$). Student's *t* test was used for statistical analysis ($*p < 0.05$)

in biopsies from macroscopically inflamed mucosa, there was also found a higher prevalence of *E. coli*, possibly according to our results in the *NOD2* mutated biopsies. We can also speculate that the reduced number of *C. coccoides* (phylum Firmicutes) goes in hand with the study of Sokol et al. [53], where the *C. leptum* and *C. coccoides* groups were also less represented in active CD. In contrast to these results, we rather found an increased amount of *B. fragilis* in healthy compared to inflamed appearing mucosa.

The intestinal microbial composition apparently plays an important role in the development and the clinical course of IBD. Specific changes in the gut microbiota may put individuals at an increased risk of developing IBD [54]. In addition to the intestinal microbiota, genetic factors also play an important role in the pathogenesis of CD, e.g., mutations in the *NOD2* gene [10, 11, 51, 55]. However, whether mutations in the *NOD2* gene and the intestinal microbiota are linked together in the development of IBD or act independently is still

not completely understood. Rehman et al. demonstrated that mutations in the *NOD2* gene (SNP13) lead to an altered microbial composition in CD patients, e.g., an increased amount of Bacteroidetes and Firmicutes in biopsies and fecal samples [28]. To further study this possible association, the present study addressed the relation between genetic risk factors and alterations in the gut microbiota. In biopsy samples from patients with *NOD2* mutations, we found a significant increase of *E. coli* compared to biopsies from wildtype patients. These findings suggest that it may be the host that triggers or allows an increased abundance of *E. coli* in CD. In contrast to the work of Rehman et al., no difference was found in the representatives of the phyla of Bacteroidetes and Firmicutes. It remains elusive if this is due to the fact that in the abovementioned study the very specific group of patients homozygous for SNP13 were analyzed. It was shown that adherent-invasive *E. coli* (AIEC) are highly present in ileal mucosa of CD patients [9]. Additionally, in a recently published study, it was shown that macrophages from CD patients cannot control AIEC replication which leads to a disordered cytokine secretion profile [56]. If the higher abundance of *E. coli* in the *NOD2* mutated patients in our patient cohort is associated with these studies could be proven by further studies utilizing AIEC specific primers.

Maybe the most interesting finding was that a TNF-alpha blockade was associated with an increased abundance of *F. prausnitzii*. *F. prausnitzii* seems to be an important sensor and indicator of human health [36]. Different diseases, such as IBD [33, 57], IBS [58], and colorectal cancer [59] have been found to be associated with a decreased abundance of *F. prausnitzii*. The present study showed that patients receiving TNF-α blocking therapy had a significantly higher abundance of *F. prausnitzii*. This might go in hand with a study by Rajca et al., which showed that CD patients had lower counts of Firmicutes and *F. prausnitzii* compared to healthy controls [60]. Additionally, low rates of *F. prausnitzii* were an independent predictor of a relapse of CD. An effective medical therapy with a TNF-α blocker might therefore increase the abundance of *F. prausnitzii*. As the patients enrolled in this study receiving a TNF-α blocker were evaluated for a secondary failure, not a primary failure of this drug. One can speculate that the initial response to this therapy regiment could possibly alter the abundance of *F. prausnitzii*, and therefore, the measurement of this specific strain could possibly be a target of assessing the response of treatment.

Differences were also found in the mucosa between CD and UC patients: in UC patients there was a significant higher abundance of *B. fragilis* and *B. bifidum*.

There are some limitations within this study. Although the study group consisted of a substantial number of patients, a greater number, especially regarding the cohort of UC patients, would strengthen the conclusions drawn from the results. The seven investigated strains compromise only a small proportion of the known mucosa-associated bacterial strains. However, these strains in general are regarded as important representatives of their corresponding phylum. Additionally, a similar approach was already used in other studies [42, 61]. As a further limitation, a healthy cohort would have been an optimal reference for our IBD patients. But performing a colonoscopy in young, age-matched non-IBD probands when the microbial composition in healthy individuals has already been extensively studied [62, 63] is ethically debatable. This study provides insights into the mucosa-associated bacterial composition in IBD patients in certain clinical situations. The analyzed bacteria could serve as targets for a directed and cost-effective surveillance in IBD patients. In future, more precise analysis might result in a therapeutic approach applying certain bacterial strains in defined clinical situations.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Mutations in the *NOD2* gene are associated with a specific phenotype and lower anti-tumor necrosis factor trough levels in Crohn's disease

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Objective: Nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene mutations are known to be an important risk factor in the pathogenesis of Crohn's disease (CD). Specific disease phenotypes are associated with the presence of *NOD2* gene mutation. One treatment option is to use an anti-tumor necrosis factor (TNF)-α agent. Therapeutic drug monitoring (TDM) is usually performed in cases of a loss of response. Our aim was to explore whether *NOD2* gene mutations have an effect on the disease phenotype, vitamin D levels, and on TDM in CD patients.

Methods: This was a retrospective genotype–phenotype association study on *NOD2* gene mutations in 161 patients with CD.

Results: Altogether 55 (34.2%) patients carried at least one mutant allele of *NOD2*. *NOD2* gene mutations were associated with ileocecal disease, ileocecal resection, stricturing and perianal disease, and patients with *NOD2* gene mutation had significantly less frequent colonic disease and received an ostomy less frequently. TDM in patients with *NOD2* gene mutation showed more frequent anti-TNF trough levels in the subtherapeutic range and lower anti-TNF trough levels than in *NOD2* wild-type (WT) patients.

Conclusions: CD patients with *NOD2* gene mutation have a specific clinical phenotype and they may require higher doses of anti-TNF agents to achieve sufficient anti-TNF trough levels. They may therefore benefit from a proactive TDM than a reactive approach. This could be another step in the direction of personalized medicine.

KEYWORDS

anti-TNF trough level, Crohn disease, drug monitoring, inflammatory bowel diseases, *NOD2*

1 | INTRODUCTION

Inflammatory bowel diseases (IBD) are disorders of the alimentary tract and consist mainly of two entities, Crohn's disease (CD) and ulcerative colitis.[1] Although the pathogenesis of IBD remains unknown, studies have shown that genetic and environmental factors lead to an inappropriate activation of the intestinal immune system. This process may be triggered by the intestinal microbiota in genetically predisposed individuals.[1,2] Mutations in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene are associated with the development and clinical course of CD, e.g., ileal disease.[3–5] *NOD2* is an intercellular pattern recognition receptor that senses muramyl dipeptide,[6] a known fragment of peptidoglycan. However, peptidoglycan on its own may also lead to the activation of *NOD2*,[7] but this process depends on a toll-like receptor 2 co-stimulatory signal.[8]

Abbreviations: ADA, adalimumab; AZA, azathioprine; CD, Crohn's disease; CI, confidence interval; CDAI, Crohn's disease activity index; 25[OH]D, 25-hydroxyvitamin D; IBD, inflammatory bowel diseases; IFX, infliximab; OR, odds ratio; *NOD2*, nucleotide-binding oligomerization domain-containing protein 2; SNP, single nucleotide polymorphism; TDM, therapeutic drug monitoring; TNF, tumor necrosis factor; WT, wild type

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Although tumor necrosis factor (TNF)-α inhibitors (infliximab [IFX], adalimumab [ADA], golimumab and certolizumab pegol) are a mainstay in the therapy of IBD, they are not equally effective in all patients.[9,10] On the one hand, a considerable percentage of patients do not respond to this treatment, on the other hand, secondary loss of response is frequent.[11,12] In patients with loss of response to anti-TNF agents the analysis of anti-TNF trough levels and anti-drug antibodies can establish a line of action.[13,14] While traditionally, therapeutic drug monitoring (TDM) is performed reactively in case of a clinical loss of response (i.e., reactive TDM), proactive TDM is not associated with remission but with fewer flares than reactive TDM.[15] A recent recommendation proposed by the American Gastroenterological Association has suggested reactive TDM but did not recommend routine proactive therapeutic drug monitoring.[16] However, there are no predictive markers for the response rate to an anti-TNF treatment in IBD, which may be of clinical value for the treating physician.

Our hypothesis was that the presence of a mutation in the *NOD2* gene might have an impact on the clinical outcome in patients with CD. Therefore, we performed a genotype–phenotype correlation in a well-characterized patient cohort in a single north German tertiary center aiming to explore whether mutations in the *NOD2* gene have an effect on the disease phenotype, vitamin D levels, and, in particular, on TDM in patients with CD.

2 | MATERIALS AND METHODS

2.1 | Patients

From October 2015 until September 2017, 161 patients with CD from the Department of Gastroenterology of Rostock University Medical Center (Rostock, Germany) were included in the study. A diagnosis of CD was made based on the clinical, endoscopic, histological and radiological results of the patients. The following data were collected: clinical characteristics (age both at diagnosis and at the start of the study, gender, disease location, disease behavior, disease activity, immunosuppressive or immunomodulatory medications (azathioprine [AZA] and TNF-α inhibitor), previous history of surgery (i.e., colectomy) and 25-hydroxyvitamin D (25[OH]D) levels. To exclude the potential effect of vitamin D substitution, only vitamin D values without substitution were included in the study. Disease activity was assessed using the Crohn's disease activity index (CDAI)[17] and the Harvey–Bradshaw index (HBI).[18] CD was stratified via the Montreal classification.[19] All patients with CD under therapy with TNF-α inhibitor, where TDM was performed from October 2015 until September 2017, were included in the study. The study was approved by the Local Ethics Board of the University of Rostock (A-2015-0042). We obtained written informed consent from all participants prior to their enrollment.

2.2 | *NOD2* genotyping

NOD2 genotyping was performed as previously described.[20] Briefly, we isolated DNA from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All patients were genotyped for three major mutations in the *NOD2* gene (single nucleotide polymorphism [SNP] 8: R702W, NCBI reference SNP ID rs2066844; SNP12: G908R, NCBI reference SNP ID rs2066845; and SNP13: 1007fs, NCBI reference SNP ID rs2066847). The specific regions of the *NOD2* gene were amplified by polymerase chain reaction (PCR) with *Taq* PCR Master Mix Kit (Qiagen); the primers used and the PCR conditions are summarized in the Supporting Information Table S1. After Sanger sequencing (Seqlab, Göttingen, Germany), all data were analyzed with Chromas software version 2.6 (Technelysium, Brisbane, Queensland, Australia).

2.3 | Measurement of drug concentrations and anti-drug antibodies

We performed reactive TDM, defined as the clinical suspicion of primary or secondary loss of response and afterwards, confirmation of this action, in our cohort. The quantification of IFX and ADA was performed with two specific enzyme immunoassays (IDKmonitor Infliximab and IDKmonitor Adalimumab drug level, ELISA; Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions. In brief, the therapeutic antibody was bound to the specific monoclonal anti-IFX or anti-ADA antibodies, which were coated on a plate. After washing, peroxidase-labeled antibodies were added and the conversion of tetramethylbenzidine was monitored via a photometer. There was a direct proportion between the intensity of the yellow color and the IFX or ADA concentration. Furthermore, total IFX and ADA anti-drug antibodies were detected using the IDKmonitor infliximab or adalimumab total ADA ELISA (Immundiagnostik). The anti-drug antibodies were separated from the therapeutic antibody during the sample preparation to minimize interference from therapeutic antibodies in the serum of the patients. In the assay peroxidase conjugate (peroxidase-labeled antibody), biotinylated therapeutic antibodies replaced the unmarked therapeutic antibodies. The marked antibodies formed a complex with the anti-drug antibody binding via biotin to the streptavidin-coated plate. The conversion of tetramethylbenzidine was monitored photometrically. Subtherapeutic trough levels were defined as <3.0 μg/mL for IFX[15] and <4.9 μg/mL for ADA,[21] respectively.

2.4 | Statistical analysis

Data were collected and recorded in a Microsoft Access database (Microsoft, Redmond, WA, USA) and analyzed by using the SPSS Statistics software version 22.0 (IBM, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD) or standard error of mean (SEM), respectively, whereas categorical variables were expressed as numbers and percentages or frequencies.

To assess whether a mutation in the *NOD2* gene (yes *vs* no) has an effect on binary clinical characteristics, the Fisher's exact test was performed. The analyses of age, disease activity (HBI and CDAI), and vitamin D level in the mutated *vs* wild-type (WT) *NOD2* groups was performed with the Student's *t*-test in case of normally distributed variables; if not, we used the Mann–Whitney *U* test instead. Normality of the variables was examined with the Kolmogorov–Smirnov test. The mean vitamin D levels per patient were compared in the *NOD2*

mutated and *NOD2* WT patients. To exclude an influence of the seasonal differences in vitamin D levels we compared mean vitamin D levels in winter (from October to March) and summer (from April to September).

For the statistical analysis of TDM, different TDM (subtherapeutic *vs* therapeutic) were compared between the *NOD2* groups using the Fisher's exact test. The analysis of absolute trough levels was performed with a two-sided *t*-test or Mann–Whitney *U* test.

A logistic regression model was applied to assess the associations between binary *NOD2* mutations and several categorical clinical characteristics at the same time. First, odds ratio (OR) and the respective 95% confidence interval (CI) were calculated for univariate models to select appropriate candidates (with a cut-off value with a *P* value of 0.20), which were subsequently tested in a multivariate model. The resulting adjusted OR (OR_{adj}) with their respective *P* values and CI are recorded. *P* value less than 0.05 was regarded as statistically significant.

3 | RESULTS

In total, 161 patients with CD were included in the study, among them 55 (34.2%) had at least one mutation in the *NOD2* gene. The characteristics of the patients are shown in Table 1.

3.1 | Clinical characteristics associated with the presence of a *NOD2* mutation

Mutations in the *NOD2* gene were found to be associated with ileocolonic disease (*NOD2* mutated *vs NOD2* WT: 44 [80.0%] *vs* 67 [63.2%], *P* = 0.032). They appeared to be protective for colonic disease (*NOD2* mutated *vs NOD2* WT: 4 [7.3%] *vs* 29 [27.4%], *P* = 0.003). Stricturing disease was more common in patients with a *NOD2* mutation than in those with *NOD2* WT (*NOD2* mutated *vs NOD2* WT: 33 [60.0%] *vs* 43 [40.6%], *P* = 0.021). Perianal disease was more frequent in patients with a *NOD2* gene mutation compared with those with *NOD2* WT, although the difference was not statistically significant (19 [34.5%] *vs* 21 [19.8%], *P* = 0.054). In addition, patients with a *NOD2* mutation were more likely to undergo ileocecal resection than patients with WT *NOD2* (22 [40.0%] *vs* 23 [21.7%], *P* = 0.017). While the SNP was usually not associated with specific clinical conditions, we found a high significance for SNP13 and the presence of perianal disease (*NOD2* mutated *vs NOD2* WT: 11 *vs* 29, *P* = 0.028). Other clinical factors, such as age, the use of disease-specific drugs, loss of response and disease activity did not differ between the two groups. In addition, no significantly different vitamin D levels were found in the two groups, either in summer or in winter.

To analyze further a possible association between the presence of a mutation in the *NOD2* gene and patient's different clinical characteristics a logistic regression analysis was performed (Table 2). We found that the presence of a mutation in the *NOD2* gene was associated with a more than doubled risk for perianal disease (OR_{adj} 2.460, 95% CI 1.120–5.380, *P* = 0.024) and ileal/ileocecal resection (OR_{adj} 2.110, 95% CI 1.040–4.280, *P* = 0.039), as well as with a lower risk of receiving an ostomy (OR_{adj} 0.270, 95% CI

TABLE 1 Clinical characteristics of all patients

	NOD2 variant (*N* = 55)	*NOD2* wild-type (*N* = 106)	*P* value
Genotype (*n*)			
Heterozygous$^{-/+}$	40		
SNP8	18		
SNP12	8		
SNP13	14		
Compound heterozygous$^{+/+}$	8		
Homozygous$^{+/+}$	7		
Female gender (*n*, %)	28 (50.9)	66 (62.3)	0.181
Age, years (mean ± SD)			
At study start (October 2015)	41.6 ± 15.2	42.3 ± 14.4	0.845
At diagnosis	30.7 ± 13.0	31.1 ± 13.5	0.815
Disease location (*n*, %)			
L1 (ileal)	7 (12.7)	10 (9.4)	0.591
L2 (colonic)	4 (7.3)	29 (27.4)	**0.003**
L3 (ileocolonic)	44 (80.0)	67 (63.2)	0.032
L4 (isolated upper GI disease)	7 (12.7)	18 (17.0)	0.647
Disease behavior (*n*, %)			
B1 (non-stricturing/ non-penetrating)	15 (27.3)	45 (42.4)	**0.062**
B2 (stricturing)	33 (60.0)	43 (40.6)	**0.021**
B3 (penetrating)	7 (12.7)	18 (17.0)	0.647
P (perianal)	19 (34.5)	21 (19.8)	**0.054**
Drugs (*n*, %)			
Azathioprine	42 (76.4)	75 (70.8)	0.450
TNF-α inhibitors	33 (60.0)	69 (65.1)	0.605
Vitamin D, nmol/L (mean ± SEM)			
Summer (April to September)	56.0 ± 26.5	51.6 ± 25.5	0.685
Winter (October to March)	54.2 ± 29.4	53.9 ± 25.7	0.959
Surgery (*n*, %)			
Small intestine	19 (34.5)	26 (24.5)	0.181
Ileocecal resection	22 (40.0)	23 (21.7)	**0.017**
Colonic resection	20 (36.4)	31 (29.2)	0.376
Stoma (*n*)	5	19	0.165
Ileostoma	2	8	
Colostoma	2	8	
Combined (Ileostoma/ colostoma)	1	3	
Loss of response (*n*, %)	10 (18.2)	18 (17.0)	0.830
Primary (<6 months)	3 (30.0)	2 (11.1)	0.315
Secondary (≥6 months)	7 (70.0)	16 (88.9)	0.227
Disease activity (mean ± SD)			
Crohn's disease activity index	103.9 ± 50.4	94.2 ± 43.2	0.959
Harvey–Bradshaw index	5.62 ± 3.0	5.2 ± 2.7	0.101

NOD2, nucleotide-binding oligomerization domain-containing protein 2; GI, gastrointestinal; SD, standard deviation; SEM, standard error of mean; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

TABLE 2 The potential association of different clinical characteristics with the presence of a mutation in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene was assessed with logistic regression analysis

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR_{adj} (95% CI)	*P* value
Perianal disease (yes *vs* not[a])	2.140 (1.030–4.450)	**0.042**	2.460 (1.120–5.380)	**0.024**
Ileal/ileocolonic resection (yes *vs* not[a])	1.930 (0.997–3.750)	**0.051**	2.110 (1.040–4.280)	**0.039**
Ostomy (yes *vs* not[a])	0.458 (0.161–1.300)	0.143	0.270 (0.088–0.831)	**0.022**
Gender (female *vs* male[a])	0.629 (0.325–1.210)	0.167	0.606 (0.302–1.220)	0.158
Colonic resection (yes *vs* not[a])	1.320 (0.664–2.630)	0.427		
Anti-TNF-α treatment (yes *vs* not[a])	0.804 (0.411–1.570)	0.525		
Age (≥40 years *vs* <40 years[a])	0.855 (0.445–1.650)	0.639		
Vitamin D deficiency		0.829		
Insufficiency *vs* normal[a]	0.681 (0.255–1.820)	0.442		
Deficiency *vs* normal[a]	0.864 (0.318–2.350)	0.775		
Severe deficiency *vs* normal[a]	1.040 (0.227–0.473)	0.963		
Vitamin D substitution (yes *vs* not[a])	1.050 (0.541–2.020)	0.895		

Bold text indicates significance.
[a] Represents reference.
CI, confidence interval; OR, odds ratio; OR_{adj}, odds ratio adjusted; TNF, tumor necrosis factor.

0.088–0.831, *P* = 0.022). By using this analysis we combined all colonic resections that included parts of the ileum as opposed to segmental colonic resections (not including the ileum) to discriminate further between these procedures and could not find an association of colonic resections with *NOD2* mutation. Other conditions, e.g., gender, the use of TNF-α inhibitor, age, vitamin D deficiency and vitamin D substitution were also not associated with the presence of *NOD2* gene mutation.

3.2 | Trough levels of anti-TNF agents

Altogether 29 patients undergoing TNF-α inhibitor therapy received 55 TDM. Twenty-one (72.4%) of these 29 patients were *NOD2* WT, while the other 8 (27.6%) had at least one mutation in the *NOD2* gene (SNP8 [*n* = 4], SNP12 [*n* = 2], SNP13 [*n* = 4]). We performed 39 TDM on patients with WT *NOD2* (26 treated with IFX and 13 with ADA, with a mean TDM of 1.9 per patient) and 16 TDM on patients with mutated *NOD2* (6 treated with IFX and 10 with ADA, with a mean TDM of 2 per patient). Other clinical characteristics of the patients with TDM are depicted in Table 3. The reason for TDM was primary loss of response (*NOD2* WT *vs* *NOD2* mutated: 4 *vs* 29), secondary loss of response (*NOD2* WT *vs* *NOD2* mutated: 20 *vs* 9) or to assess an action that had been undertaken (*NOD2* WT *vs* *NOD2* mutated: 15 *vs* 5). The reason for the TDM was independent of the presence of a possible mutation in the *NOD2* gene (*P* = 0.830).

As showed in Table 1, the age between patients with *NOD2* WT and *NOD2* mutated was not significantly different, either at the start of the study or at the diagnosis of the disease. However, TDM in patients with a mutation in the *NOD2* gene was performed at a significantly younger age (*P* < 0.001) and a shorter duration of the disease than patients with *NOD2* WT (*P* = 0.003). However, when the TDM of the *NOD2* mutated and the *NOD2* WT groups was compared, other clinical characteristics (e.g., disease location and disease behavior) did not show a statistically significant difference.

Patients undergoing TDM with a mutation in the *NOD2* gene had a significantly higher probability of subtherapeutic anti-TNF trough levels than patients with *NOD2* WT (*NOD2* WT: TDM subtherapeutic [*n* = 17], TDM therapeutic [*n* = 22]; *NOD2* mutated: TDM subtherapeutic [*n* = 13], TDM therapeutic [*n* = 3]; *P* = 0.016; Figure 1). In

TABLE 3 Clinical characteristics of the patients undergoing therapeutic drug monitoring (TDM)

	NOD2 variant	*NOD2* Wild-type	*P* value
Genotype (*n*)	8	21	
Heterozygous$^{-/+}$	6		
SNP8	4		
SNP12	2		
SNP13	4		
Compound heterozygous$^{+/+}$	2		
Homozygous$^{+/+}$	0		
Gender			
Female/male	5/3	13/8	1.0
TDM (*n*)	16	39	NS
With IFX	6	26	NS
With ADA	10	13	NS
Age, years (mean ± SD)			
At diagnosis	22.6 ± 10.3	25.1 ± 8.9	0.354
At TDM	27.9 ± 11.1	36.2 ± 10.7	**<0.001**
Disease duration until TDM	6.5 ± 3.7	12.2 ± 4.7	**0.003**

Bold text indicates significance. ADA, adalimumab; IFX, infliximab; *NOD2*, nucleotide-binding oligomerization domain-containing protein 2; NS, not significant; SNP, single nucleotide polymorphism.

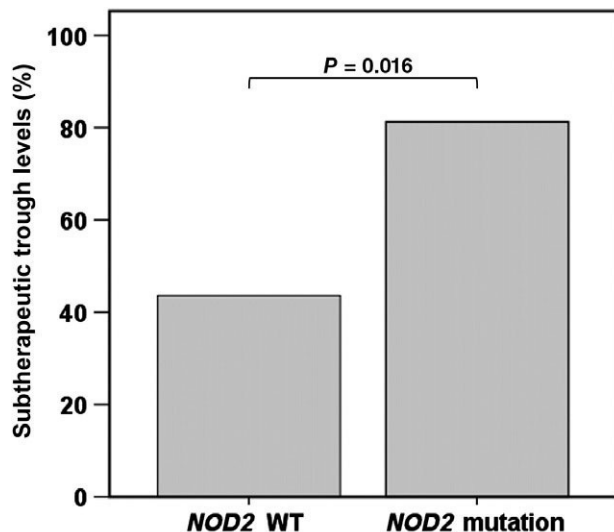

FIGURE 1 Mutation in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene was associated with lower infliximab (IFX) and adalimumab (ADA) trough levels. Patients with *NOD2* gene mutation had significantly lower (A) IFX and (B) ADA levels than those with *NOD2* wild-type (WT). Subtherapeutic trough levels were defined as <3.0 μg/mL for IFX and <4.9 μg/mL for ADA.

addition to the qualitative presence of subtherapeutic anti-TNF trough levels we further analyzed the numerical anti-TNF trough concentration in patients with a mutation in the *NOD2* gene. Patients undergoing TDM with *NOD2* gene mutation displayed significantly lower levels of IFX (*NOD2* WT [n = 26] *vs NOD2* mutated [n = 6]: [3.2 ± 2.7] μg/mL *vs* [1.1 ± 1.3] μg/mL, P = 0.038; Figure 2A) and ADA (*NOD2* WT [n = 13] *vs NOD2* mutated [n = 10]: [9.5 ± 4.5] μg/mL *vs* [4.5 ± 5.9] μg/mL, P = 0.033; Figure 2B) compared with patients with *NOD2* WT undergoing TDM.

3.3 | Anti-drug antibodies

We then investigated whether the lower anti-TNF trough levels were associated with the presence of anti-drug antibodies and compared anti-drug antibodies in patients with *NOD2* WT and those with *NOD2* gene mutation. There was no significant difference in the detection of anti-drug antibodies between the two groups (*NOD2* WT, no anti-drug antibodies in 27 TDM, positive anti-drug antibodies in 12 TDM; *NOD2* mutated, no anti-drug antibodies in 12 TDM, positive anti-drug antibodies in four TDM; P = 0.754). In addition, the absolute anti-drug antibody concentrations were comparable between patients with *NOD2* WT and *NOD2* mutated in IFX (*NOD2* WT [n = 22] *vs NOD2* mutated [n = 6]: [38.1 ± 111.1] μg/mL *vs* [115.1 ± 179.3] μg/mL, P = 0.428) and ADA (*NOD2* WT [n = 10] *vs NOD2* mutated [n = 6]: [3.9 ± 4.2] μg/mL *vs* [40.7 ± 71.9] μg/mL, P = 1.000).

3.4 | Effect of AZA on TDM

The SONIC study has shown that the concomitant use of AZA has an effect on the IFX trough levels.[22] Therefore, we examined whether lower anti-TNF trough levels in patients with *NOD2* mutations were dependent on concomitant therapy with AZA. When TDM from patients treated with AZA were excluded from the analysis, TDM in patients with a *NOD2* gene mutation had a significantly higher probability of anti-TNF trough levels in the subtherapeutic range (*NOD2* WT: 10 TDM subtherapeutic, 14 TDM therapeutic; *NOD2* mutated: 13 TDM subtherapeutic, three TDM therapeutic; P = 0.022). Thus, a concomitant therapy with AZA had no effect on the TDM levels.

4 | DISCUSSION

The discovery of *NOD2* as a genetic risk factor for CD has made a substantial contribution to our understanding of the pathogenesis of CD.[3,4] In this study we performed a genotype–phenotype correlation in a single-center north German tertiary IBD center. The present study demonstrated three major results. First, we found that mutations in the a mutation in the *NOD2* gene were associated with ileocecal disease, ileocecal resection, stricturing disease behavior, and perianal disease. Second, patients with *NOD2* gene mutations had a significantly

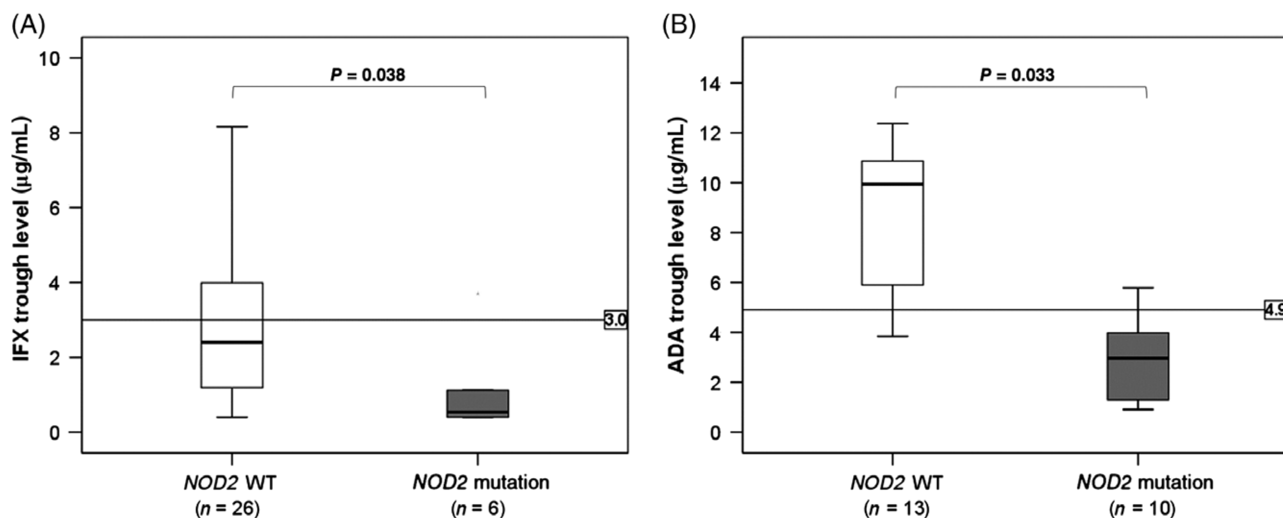

FIGURE 2 Mutation in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene was associated with a higher prevalence of subtherapeutic anti-tumor necrosis factor (TNF) trough levels of (A) infliximab (IFX) and (B) adalimumab (ADA). Subtherapeutic trough levels were defined as <3.0 μg/mL for IFX and <4.9 μg/mL for ADA. WT, wild type.

lower probability of colonic CD or a stoma. Third, the presence of a mutation in the *NOD2* gene mutation was associated with a higher probability of subtherapeutic and numerically lower anti-TNF trough levels, but not anti-drug antibodies.

In previous studies mutations in the *NOD2* gene were associated with a younger age at diagnosis, ileal involvement and ileocecal resection,[5] the latter of the two was also shown in our study. Mutations in the *NOD2* gene were also found to be associated with stricturing and perianal disease.[23,24] Underscoring these results, in our cohort the presence of *NOD2* gene mutations (SNP8, SNP12, and SNP13) was also more prevalent in patients with stricturing and perianal disease. Interestingly, the age at disease onset of patients with and without *NOD2* mutation was not significantly different. However, in terms of the loss of response when TDM was performed, patients with a mutation in the *NOD2* gene were significantly younger than those with *NOD2* WT. The possible association between a loss of response and the presence of a *NOD2* gene mutation should be further investigated in larger CD patient cohorts.

The low rate of mutations in the *NOD2* gene in patients with colonic disease has been described previously in Cantó *et al*.'s study.[25] In addition, in our cohort the presence of a *NOD2* gene mutation was significantly associated with a lower risk of a stoma. Since this effect was found in patients with an ileostoma and in those with a colostoma, the lower frequency of colonic CD in patients with *NOD2* mutations cannot explain this result. In a previous study we showed that *NOD2* was associated with the development of intestinal failure in the absence of CD.[26] For the authors it remains unclear why patients with a *NOD2* gene mutation have a significantly lower risk of receiving an ostomy.

The measurement of anti-TNF trough levels can influence the therapeutic approach, especially in the case of a loss of response.[14] In our study we found that *NOD2* gene mutation was associated with a higher prevalence of subtherapeutic anti-TNF trough levels. In addition, *NOD2* gene mutations were associated with significantly lower trough levels of IFX and ADA. This effect did not depend on co-medication with AZA. Moreover, the presence of anti-drug antibodies was not significantly different between the two groups. The reason for which TDM was undertaken was not statistically different between the *NOD2* WT and *NOD2* mutated cohort. Two hypotheses can be postulated for this result. First, NOD2 has an effect on the intestinal barrier function.[27,28] A study in patients with severe ulcerative colitis by Brandse *et al*. showed, that IFX can be lost through the feces, a mechanism that is associated with a loss of response.[29] While our cohort contained patients with CD, with only a small proportion of colonic disease localization, especially in patients with a *NOD2* gene mutation, it remains unknown whether anti-TNF agents can also be lost in the ileocecal region or small bowel in CD; a question that might be addressed in further studies. However, it may be speculated that a *NOD2*-induced barrier dysfunction may also be responsible for lower anti-TNF trough levels. Second, a *NOD2* gene mutation could lead to higher basal TNF-α levels with consecutively (after treated with an anti-TNF agent) lower anti-TNF trough levels. In a monocytic cell line, an overexpression of *NOD2* reduced the effects of anti-TNF agents.[30] In agreement with our results, a study by Juanola *et al*. showed that a mutation in the *NOD2* gene was associated with a loss of response to the treatment with anti-TNF agents.[31] On the other hand, in different studies the presence of a mutation in the *NOD2* gene did not have an effect on response to treatment with an anti-TNF agent.[32,33] However, based on our data we cannot elucidate the mechanisms by which the presence of a *NOD2* gene mutation may lead to lower anti-TNF trough levels.

A limitation of our study was that the number of TDM performed was relatively low. However, our data suggested that there was a strong association between the presence of *NOD2* gene mutation and lower anti-TNF trough levels, since there was a noticeable difference between the anti-TNF trough levels in IFX and ADA between patients with *NOD2* WT and *NOD2* mutated. This effect is of specific clinical relevance, since the presence of a *NOD2* gene mutation may not only predispose patients to ileocolonic disease, ileocecal resection and perianal disease, but also to lower anti-TNF trough levels. Patients with a mutation in the *NOD2* gene may therefore require higher doses of anti-TNF agents in order to obtain sufficient levels of these drugs, or a more intense TDM strategy. Therefore, one can argue that patients with a mutation in the *NOD2* gene might particularly benefit from proactive therapeutic drug monitoring rather than reactive TDM. This would be another important step in the direction of personalized medicine in IBD and the results should be further investigated in a larger, prospective, controlled clinical trial.

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CONFLICTS OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Original article

Vitamin D administration leads to a shift of the intestinal bacterial composition in Crohn's disease patients, but not in healthy controls

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OBJECTIVE: Dysbiosis is a common feature in the pathogenesis of inflammatory bowel diseases (IBD). Environmental factors, such as vitamin D deficiency, seem to play a role in the intestinal inflammation of IBD. The aim of this study was to investigate whether vitamin D administration has an impact on the bacterial composition in Crohn's disease (CD) compared to healthy controls (HC).

METHODS: A prospective, longitudinal, controlled interventional analysis was conducted in seven patients with CD in clinical remission and 10 HC to investigate the effect of orally administrated vitamin D on the intestinal bacterial composition using 16S ribosomal RNA gene amplicon sequencing. Clinical parameters were assessed.

RESULTS: In contrast to HC, microbial communities of CD patients changed significantly during early vitamin D administration. However, a further increase in vitamin D level was associated with a reversal of this effect and additionally with a decrease in the bacterial richness in the CD microbiome. Specific species with a high abundancy were found during vitamin D administration in CD, but not in HC; the abundancy of *Alistipes*, *Barnesiella*, unclassified Porphyromonadaceae (both Actinobacteria), *Roseburia*, *Anaerotruncus*, *Subdoligranulum* and an unclassified Ruminococaceae (all Firmicutes) increased significantly after 1-week vitamin D administration in CD.

CONCLUSIONS: Vitamin D has a specific influence on the bacterial communities in CD, but not in HC. Administration of vitamin D may have a positive effect in CD by modulating the intestinal bacterial composition and also by increasing the abundance of potential beneficial bacterial strains.

KEY WORDS: Crohn disease, inflammatory bowel diseases, microbiota, vitamin D.

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INTRODUCTION

Inflammatory bowel diseases (IBD) consist of Crohn's disease (CD) and ulcerative colitis (UC) which are chronic inflammatory diseases of the alimentary tract.[1] While the pathogenesis is still not completely understood, an important part of the disease is known to be inappropriate activation of the

mucosal immune system caused by intestinal microbiota in patients with a genetical risk profile.[1–4] Mutations in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene, encoding for the NOD2 receptor, are major risk factors for the development of CD.[5–8] In addition, environmental factors, such as vitamin D deficiency, seem to play a role in the pathogenesis of IBD as well.[8,9] Vitamin D is commonly known as an important regulator of calcium and phosphate metabolism and is therefore essential for bone health.[10,11] However, there is increasing evidence that vitamin D also plays an important role as a regulator of the innate and adaptive immune system.[11–13] In a murine colitis model, the application of 1,25-dihydroxyvitamin D_3 (1,25 $[OH]_2D_3$) was associated with reduced mucosal injury.[14] Vitamin D deficiency is highly prevalent in IBD patients.[15,16] Interestingly, stimulation with vitamin D increased the expression of the NOD2 receptor in primary monocytic and epithelial cells, linking the innate immune system with vitamin D.[17] Vitamin D was shown to have an effect on the dendritic and monocyte-derived macrophages cell function.[19–21] From a clinical point of view, the administration of tumor necrosis factor (TNF)-alpha inhibitor is associated with higher vitamin D levels in IBD.[18] On the other hand, in CD patients where infliximab was initiated, lower vitamin D levels were associated with a higher rate of clinical remission at week 14.[22]

Even now, it is still not clear whether vitamin D administration has a beneficial effect on the disease course in IBD. In a prospective study, infliximab treatment had a positive effect on bone metabolism.[23] Along the same line in different studies in CD patients, an inverse association between vitamin D levels and intestinal inflammation was found.[24,25] In several interventional studies, vitamin D administration appeared to have beneficial effects on the clinical disease activity and C-reactive protein (CRP) values in IBD patients.[26–29]

However, whether vitamin D also has an influence on the intestinal bacterial composition is still not known. In murine models, vitamin D and the vitamin D receptor (VDR) are important regulators of intestinal bacterial composition.[30–32] In a recent genome-wide association study (GWAS), mutations in the *VDR* gene were associated with different intestinal microbial profiles.[33]

To address the hypothesis, whether administration of vitamin D has an effect on the intestinal microbial communities in CD, we performed a controlled prospective and longitudinal analysis in CD patients in clinical remission and healthy controls (HC).

MATERIAL AND METHODS

Study design

The study was approved by the Institutional Review Board of the University Medical Center Rostock (A 2016-0109). The study was registered in the German Clinical Trials Register (Registration number DRKS00013485). Written informed consent was obtained from each participant prior to their enrollment.

Seven patients with ileocolonic CD (Montreal classification[34]: L3) and vitamin D deficiency (25[OH]D <75 nmol/L) were recruited from the Outpatient Clinic of Rostock University Medical Center (Rostock, Germany). The patients were in clinical remission and did not have a change of their CD-specific therapy during the previous 6 months before they were enrolled in this study. HC with vitamin D deficiency and no history of IBD were recruited from the Rostock Medical School. In both groups, oral vitamin D administration was given with cholecalciferol (MIBE GmbH Arzneimittel, Brehna, Germany) 20 000 IU daily from day 1 until day 3, then every other day for a total of 4 weeks. In this study, a target vitamin D level was set to be between 100 and 150 nmol/L. Serum 25-hydroxyvitamin D (25[OH]D) levels were measured weekly (before administration = week 0). In both groups, 300 000 IU vitamin D were administered per patient over the course of the study. In CD patients, calprotectin levels were measured at week 0 and week 4. Fresh stool samples were collected weekly (weeks 0, 1, 2, 3 and 4) for analysis of the intestinal bacterial microbiota. The disease activity in CD patients was assessed using the Crohn's disease activity index (CDAI);[35] other clinical parameters, including the localization of the disease, medical therapy, duration of the disease, were recorded. Clinical characteristics of all the participants are shown in Table 1.

DNA extraction

DNA was isolated from whole blood with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

NOD2 genotyping

Regarding the *NOD2* genotyping we focused on the three major mutations of the *NOD2* gene (SNP

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Table 1. Clinical characteristics of the Crohn's disease (CD) patients and health controls (HC)

Characteristics	CD group (*N* = 7)	HC group (*N* = 10)
Age, years (mean ± SD)	44.9 ± 12.4	24.8 ± 3.1
Sex (M/F), *n*	4/3	7/3
Localization of CD (Montreal classification)	L3	-
NOD2 mutations (CD), *n*	2 (SNP8 heterozygous)	-
CD treated with TNF-α inhibitor (infliximab/ adalimumab), *n*	5	-
Total vitamin D administrated (IU)	300 000	-
CDAI (mean ± SD)		-
Week 0	81.6 ± 43.0	
Week 4	57.3 ± 36.2	-
Stool calprotectin, mg/kg (mean ± SD)		
Week 0	297.8 ± 613.0	-
Week 4	178.6 ± 305.6	-

CDAI, Crohn's disease activity index; F, female; M, male; NOD2, nucleotide-binding oligomerization domain-containing protein 2; SD, standard deviation; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

8, R702W, National Center for Biotechnology Information [NCBI] reference single nucleotide polymorphism [SNP] ID: rs2066844; SNP 12, G908R, NCBI reference SNP ID: rs2066845; and SNP 13, 1007 fs, NCBI reference SNP ID: rs2066847). For the amplification of the regions of the *NOD2* gene, the *Taq* PCR Master Mix Kit (Qiagen) and primers/polymerase chain reaction (PCR) conditions as specified in Table S1 were employed. Additionally, the PCR products were subjected to Sanger sequencing (Seqlab, Göttingen, Germany). The resulting data were analyzed using with the Chromas 2.6 (Technelysium Pty. Ltd., Brisbance, Australia).

Preparation of 16S rRNA gene sequencing libraries, sequencing run and data analysis

The isolated DNA was amplified with the bacterial 16S ribosomal RNA (rRNA) gene primers Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC).[36] The amplicon PCR, the index PCR, a quantity and a quality control and the sequencing of the individual libraries as a pool in one Illumina MiSeq run was performed as described in a previous study.[37] The raw sequences of the study were deposited at the Short Sequence Archive (SRA) under the accession number 'PRJEB21819'. For our data analysis, the resulting sequences were assembled using the program QIIME 1.9.1[38] with the 'joins paired-end Illumina reads' function with default settings to merge forward and reverse sequences with an overlap of at least 30 bp. We discharged sequences without overlap. After converting 'fastq' to 'fasta' using the 'convert_fastaqual_fastq' function, we used the SILVA NGS pipeline for the resulting sequences, using default settings.[39] This pipeline aligns the reads to a database with the SINA aligner.[40] With this program, problematic reads such as PCR artefacts (including potential chimeras) and non-ribosomal reads are filtered out and consecutively discarded. The reads are then quality filtered using the following settings: reads less than 50 aligned nucleotides and reads with more than 2% of ambiguities, 2% of homopolymers or low alignment quality, defined by a 40 alignment score reported by SINA. After the alignment, the sequences were dereplicated by clustering by a 98% sequence identity to each other using CD-HIT.[41] The longest read in each cluster was Basic Local Alignment Search Tool (BLAST) searched against SILVA SSU Ref 128 to classify the sequences. The resulting classification of the reference sequence of each cluster was mapped to all the members of the cluster as well as their replicates. The sequences which have an average BLAST alignment coverage and alignment identity of less than 93% were considered as unclassified and we defined them as the virtual taxonomical group 'No Relative'.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation or medians and interquartiles, whereas categorical variables were expressed as numbers and percentages. The disease activity, measured by CDAI and the calprotectin value in the CD cohort from week 0 and week 4, was compared using a paired *t*-test.

Operational taxonomic unit (OTU) counts based on genus level were rarefied to 3500 reads per sample using the single_rarefraction.py script implemented in QIIME. To compare the dominant taxa in the different time points (week 0 until week 4) during vitamin D administration, the occurrence of the 22 most abundant OTUs was visualized in a heatmap using Explicet.[42] This program was also used in a rarefaction-based analysis, here with bootstrapping for richness. We visualized the differences in the bacterial community composition through non-metric

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multidimensional scaling (NMDS) plots using Bray–Curtis dissimilarity indices based on a genus rank classification. We used the software package PAST[43] for non-parametric multivariate analysis of variance (PERMANOVA) to analyze the differences between OTU compositions and a Tukey's pairwise test to calculate significant differences between the number of OTUs between the patient samples. A linear discriminant analysis (LDA) effect size (LEfSe) analysis[44] was performed to determine bacterial groups which are significantly different between the samples using the 'one against all' strategy for multi-class analysis. The program LEfSe uses a non-parametric test that couples standard tests for statistical significance with additional tests encoding biological consistency and effect relevance. $P < 0.05$ was regarded as statistical significance.

RESULTS

Effect of vitamin D administration on clinical parameters in CD and HC

Vitamin D administration led to a significant increase in 25(OH)D levels in CD and HC (CD: week 0 [39.7 ± 23.0 nmol/L] *vs* week 4 [121.4 ± 43.2 nmol/L], $P < 0.001$; HC: week 0 [29.6 ± 6.3 nmol/L] *vs* week 4 [143.0 ± 25.2 nmol/L], $P < 0.001$; Fig. 1). The vitamin D levels was not significantly different between CD and HC at each time point from week 0 to week 4. We identified two of the seven patients as having a mutation in the *NOD2* gene (SNP 8, heterozygous; Table 1) but these did not show a specific response to the vitamin D treatment (data not shown).

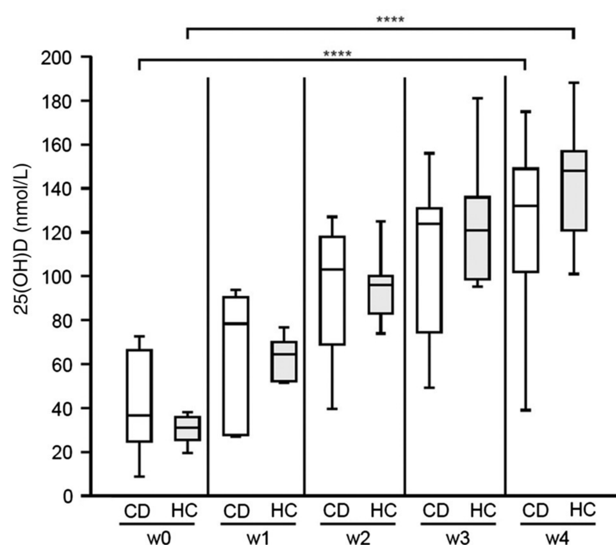

Figure 1. The level of 25-hydroxyvitamin D (25[OH]D) in Crohn's disease (CD) patients and healthy controls (HC) from week 0 (w0) to week 4 (w4). The administration of vitamin D increases the 25(OH)D levels in CD and HC significantly. However, the increase in 25(OH)D levels does not differ between CD and HC at different time points from week 0 to week 4. Values are expressed as medians and interquartile ranges. The largest data points is <1.5 times the box height ("upper-inner fence") as shown with short horizontal lines and similarly for that below the box. ****$P < 0.001$ by Turky's pairwise test.

In CD, vitamin D administration was associated with a non-significant decline of the CDAI and the calprotectin levels between week 0 and week 4 (CDAI: week 0 [81.6 ± 43] *vs* week 4 [57.3 ± 36.2], Fig. 2a; calprotectin: week 0 [297.8 ± 613.0 mg/kg] *vs* week 4 [178.6 ± 305.6 mg/kg], Fig. 2b). One patient showed a strong decline of the calprotectin level under vitamin D administration (week 0 [1685 mg/kg] *vs* week 4 [793 mg/kg]) while staying in clinical remission for the whole study period.

Vitamin D administration was associated with a temporal shift of the intestinal microbiota in CD, but not in HC

Bacteroidetes and *Clostridia* were among the most abundant phyla/classes in the bacterial community analysis (Fig. S1). This could be observed at all time points (week 0 to week 4) in CD as well as in HC. However, to assess the effect of vitamin D administration on different microbial communities, we visualized changes in the bacterial composition on the bacterial genus levels using NMDS in HC (Fig. 3a) and CD (Fig. 3b). In the HC group, there was no significant difference between week 0 and week 4 as well as between the different time points of vitamin D administration. In the CD group, we observed a shift of the bacterial composition from week 0 to week 1, which reversed in weeks 2, 3 and 4 (Table S2). The shift from week 0 to week 1 was not significant, which might be attributed to the fact that the bacterial composition of two patients at week 0 clustered within the bacterial communities of week 1. The bacterial community at week 1 differed significantly from those at week 2, week 3 and week 4 in all patients during vitamin D administration (week 1 *vs* week 2, $P = 0.007$; week 1 *vs* week 3, $P = 0.01$; week 1 *vs* week 4, $P = 0.011$). In contrast, the bacterial community at week 0 did not differ significantly from those at weeks 2, 3 and 4 (all $P > 0.05$). The use of TNF-α inhibitor, the disease activity (CDAI) and the presence of a mutation of the *NOD2* gene did not have a

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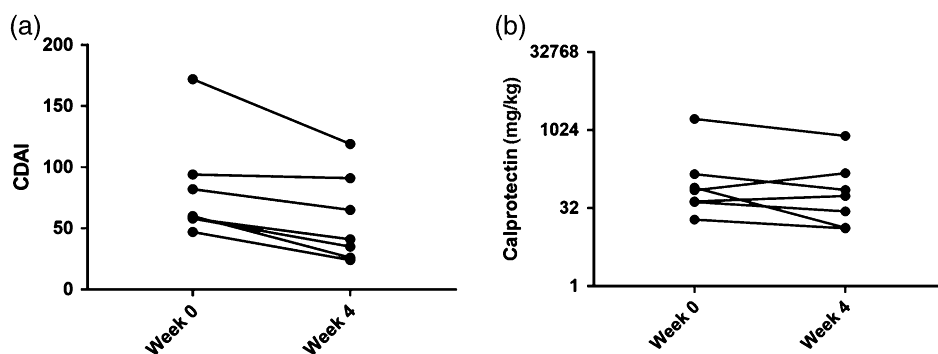

Figure 2. Crohn's disease activity index (CDAI) and calprotectin during vitamin D administration in Crohn's disease patients. Vitamin D administration leads to a non-significant decrease in (a) the CDAI and (b) the calprotectin level. One patient shows a strong decline of the CDAI and the calprotectin level. Logarithmic scale in (b).

significant effect on the bacterial communities during vitamin D administration.

Vitamin D administration was associated with specific abundant bacteria in CD, but not in HC

To further assess if vitamin D has an impact on the abundance of specific strains in CD and HC, we characterized alterations in the bacterial genera using LEfSe.[44] While in CD different specific abundant bacteria before and at different time points during vitamin D administration were found, the analysis of the HC cohort did not detect any abundant strains. The results of the CD group are shown in a heatmap (Fig. 4). The bacteria were stratified into five groups to differentiate the response to the vitamin D administration (highest abundance at weeks 0 to 4). Before vitamin D administration (week 0) the typical bacteria (significantly more abundant based on LEfSe analysis) were *Sutterella* (Betaproteobacteria), next to *Bifidobacterium* (Actinobacteria) and an unclassified lineage of the *Lachnospiracea.* After 1 week of vitamin D administration (week 1), the typical bacteria shifted toward an *Alistipes* (Bacteroidetes)-dominated bacterial community. *Barnesiella* and unclassified Porphyromonadaceae (both Actinobacteria), as well as *Roseburia*, *Anaerotruncus*, *Subdoligranulum* and an unclassified Ruminococaceae (all Firmicutes) were also highly prevalent at this time point. After 2 weeks of vitamin D administration (week 2), the Bacteroidetes became less prominent and Firmicutes, especially *Faecalibacterium*, *Veillonella*, and *Blautia*, *Fusicatenibacter* and *Intestinibacter* became the typical part of the bacterial community composition. In the third week of vitamin D administration, *Parabacteroides* (*Bacteroides*) were mainly abundant throughout the study but were significantly less abundant in weeks 1–2. Other indicator OTUs were *Lachnospira* (Firmicutes), *Coprobacter* (*Bacteroides*) and *Parasutterella* (Betaproteobacteria). At week 4, *Lactobacillus* and *Megasphera* (both Firmicutes) were significantly enriched. However, both had numerically a relatively low abundance.

Vitamin D administration was associated with a decrease in the bacterial taxa in CD, but not in HC

In previous studies, a reduction of the bacterial diversity was found in CD.[45,46] To address the question if vitamin D administration also has an effect on the diversity in CD and HC, we analyzed the number of bacterial taxa at the different time points (week 0 until week 4). There was no significant difference between CD and HC in the number of bacterial taxa before vitamin D administration ($P > 0.05$, Tukey's test). While the number of bacterial taxa in HC did not change significantly (HC: week 0 *vs* week 4, $P > 0.05$; Tukey's test), the bacterial taxa in CD decreased significantly during vitamin D administration (CD: week 0 *vs* week 4, $P = 0.001$; Tukey's test). Additionally, the number of bacterial taxa was significantly lower in CD compared to HC at weeks 3 and 4 of vitamin D administration (CD *vs* HC, week 3: $P = 0.007$, week 4: $P = 0.0001$; Tukey's test; Fig. 5).

DISCUSSION

Dysbiosis is an important feature in the pathogenesis of IBD.[45–48] Environmental factors such as vitamin D deficiency also seem to play a role in the development and the clinical disease course of IBD. Here we have investigated whether there is an interaction of vitamin D and intestinal microbiota in CD because vitamin D has been shown to have an influence on intestinal inflammation and therefore possibly on microbial communities as well. Vitamin D has been

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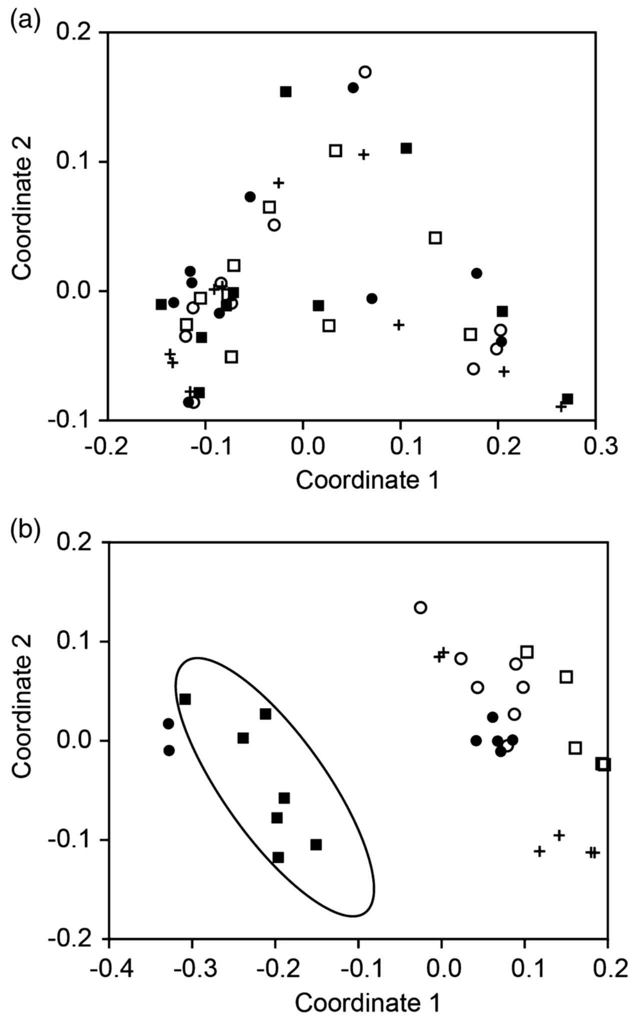

Figure 3. Vitamin D administration leads to a temporal shift of the bacterial communities in Crohn's diseae (CD), but not in healthy controls (HC). Non-metric multidimensional scaling plot (NMDS) based on Bray–Curtis dissimilarity of the bacterial communities from (a) HC and (b) CD at different time points during vitamin D administration (NMDS 0.092) without CD patient no. 6 on week 4 and CD patient no. 1 on week 3. While there is a shift of the bacterial composition in the CD group from week 0 to week 1, no such effect is found in HC. (●) Week 0, (■) Week 1, (+) Week 2, (□) Week 3, (○) Week 4.

shown to ameliorate intestinal inflammation, but it is not clear whether this is a direct effect on the inflammatory response and the intestinal microbiota is only secondarily altered or whether this is a direct effect on the intestinal microbiota. Therefore, vitamin D was given to HC and CD patients who were in clinical remission to analyze the microbial composition in both groups. Vitamin D administration was associated with a significant shift of the intestinal bacterial communities in CD patients at week 1. While the bacterial composition in HC was not significantly affected by vitamin D administration, we observed temporal changes in the bacterial communities during vitamin D administration in CD patients (Fig. 3). Vitamin D has therefore a strong effect on the microbial composition in CD, but not in HC, suggesting an important role in the pathogenesis of IBD. The strongest effect was observed at week 1 of vitamin D administration. However, the increase in 25(OH)D levels over three additional weeks caused again a shift in the bacterial community that was more similar to the initial bacterial community at week 0. There may be alternative explanations for the temporal nature of the effect of vitamin D. First, the effect of vitamin D on the bacterial communities is in itself only temporal and the microbiota revert after 2 weeks. Second, there may be an optimal 25 (OH)D level ('vitamin D window') which resembles the vitamin D levels at week 1 (64.6 ± 29.8 nmol/L) and a further increase in vitamin D levels might therefore cause reversal of effect on the bacterial composition.

Another important finding of our study is the change in the bacterial diversity during vitamin D administration (Fig. 5). Studies found a lower bacterial species diversity in CD patients compared to HC.[49,50] However, in our study, the number of bacterial taxa was not significantly different between HC and CD before the administration of vitamin D. This might be attributed to the fact that our patients were in stable clinical remission. After 2 weeks of vitamin D administration, the number of bacterial taxa declined in CD patients but did not change in HC. In general, a higher diversity is thought to be associated with beneficial effects for the host. Our results suggest that an increased vitamin D concentration causes a loss of OTUs that are potentially beneficial and supports the 'vitamin D window' hypothesis. However, it can be speculated that week 4 is too early to see a long-term effect of vitamin D administration on the composition of the bacterial communities. Further, prospective studies are needed to test the presence of an optimal vitamin D range in IBD.

The mechanisms of how vitamin D administration leads to a shift of the bacterial communities in CD remains speculative. Several studies have shown a correlation between vitamin D status, the mucosal immune system and the microbiota in IBD.[51,52] Mutations in *VDR* are risk factors for the development of IBD[8] and vitamin D can activate the *NOD2* pathway.[17] A recent study by Wang *et al*. showed that

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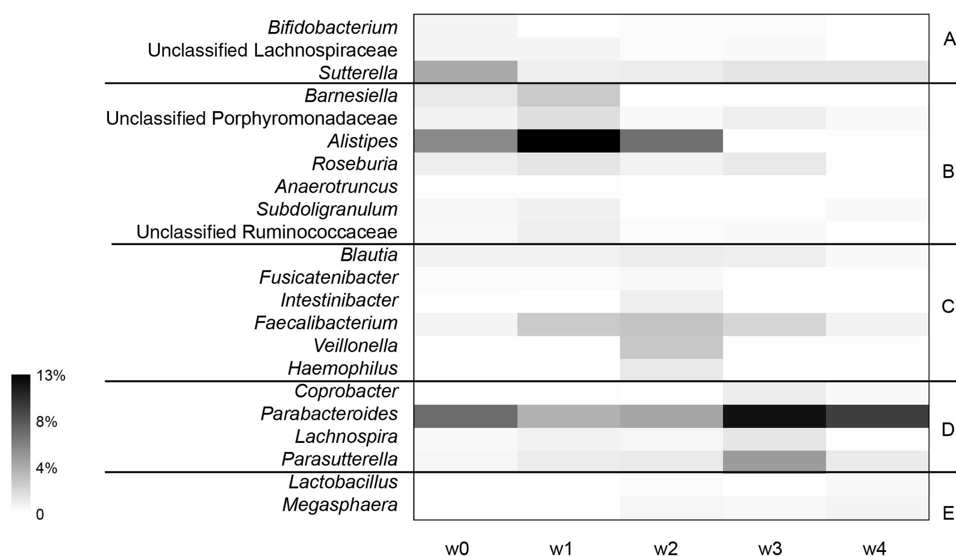

Figure 4. Heatmap of the bacterial communities during vitamin D administration in the Crohn's disease (CD) cohort. Group A shows the bacteria with a high abundance in week 0 (w0), group B in week 1 (w1), group C in week 2 (w2), group D in week 3 (w3) and group E in week 4 (w4). This figure shows the abundant species in the CD group.

variations in *VDR* had an influence on gut microbiota.[33]

For the treatment of IBD, the role of probiotics remains controversial. While in UC probiotics may have a positive effect in specific, well-defined clinical situations, the efficacy of probiotics in CD remains uncertain.[53,54] In the present analysis we have identified specific bacterial species which show an increased abundance during vitamin D administration. In addition to many other abundant strains, we observed a prominent change in the abundancy of *Alistipes* and *Parabacteroides* during vitamin D administration in CD but not in HC. Both species appear to be of special importance in the pathogenesis of IBD. In contrast to the increased abundance of *Alistipes*, *Parabacteroides* showed a decreased abundance during vitamin D administration in the first and second week of the study but an increase at week 3 and week 4. In murine dextran sulphate sodium (DSS)-induced colitis, *Alistipes finegoldii* was protective against colitis.[55] Additionally, in a study using *VDR*-knockout mice, *Alistipes* was depleted in cecal stool.[31] Other studies have proposed an important role for *Parabacteroides* in the pathogenesis of intestinal inflammation and found a decrease in *Parabacteroides* at inflamed compared to non-inflamed sites of the intestine.[56,57] Additionally, oral administration of *Parabacteroides distasonis* led to decreased severity in a murine model of DSS colitis.[58] Interestingly, in our analysis the abundance of *Parabacteroides* decreased in the first 2 weeks. As a consequence, *Alistipes* and *Parabacteroides* might therefore have beneficial effects on the host and vitamin D administration may possibly induce the growth of these species. In addition to these highly abundant species, several other OTUs were found to be significantly increased during vitamin D administration in the first week. These

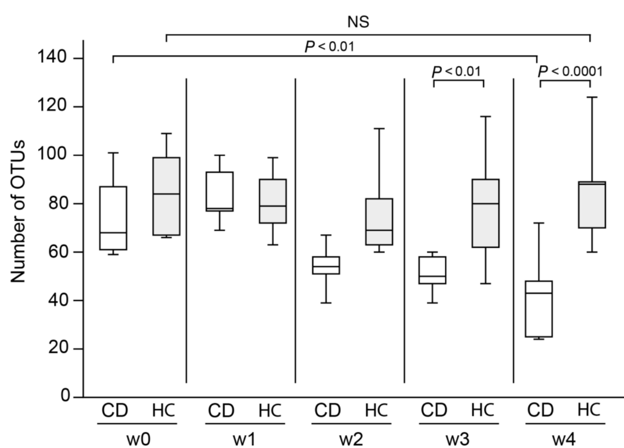

Figure 5. Number of bacterial taxa during vitamin D administration for 4 weeks in patients with Crohn's disease (CD) and healthy controls (HC). While the number of bacterial taxa, shown in operational taxonomic units (OTUs) at week 0 (w0) is not different between CD and HC, it decreases significantly in CD compared to HC during vitamin D administration at week 3 (w3) and week 4 (w4). Values are expressed as medians and interquartile ranges. The largest data points is <1.5 times the box height ("upper-inner fence") as shown with short horizontal lines and similarly for that below the box. NS, not significant.

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included *Roseburia*, of which a decrease of *Roseburia hominis* has been associated with a higher disease activity in UC.[59] Absence of *Roseburia* before colectomy in UC was associated with a higher risk of pouchitis in UC.[60] *Faecalibacterium prausnitzii*, which showed high abundance from week 1 to week 3, is a well-known butyrate-producing strain which is thought to have anti-inflammatory properties.[61–63] A specific microbial community containing *Barnesiella*, also showing a high abundance at week 1, had beneficial effects on the intestinal microbial composition.[64] We hypothesize from our data that the appearance of several beneficial bacterial strains during vitamin D administration has a protective effect on the disease course in CD. Vitamin D might therefore enhance the probiotic capacity of these strains via an increased abundance. In line with that, vitamin D was required for a positive probiotic effect in a murine colitis model.[65] A combination of probiotics and vitamin D may have a synergistic effect on the disease activity in CD. Further studies will be needed to test this hypothesis.

One aspect of criticism in this study is that the control group was significantly younger than the CD group due to technical reasons, which might also have an effect on the intestinal microbial composition. Although we found a highly significant change of the bacterial communities in CD but not in HC, this effect could also be influenced by the age of the participants in this study. In further studies, age-matched control groups might clarify this aspect.

As a conclusion, the administration of vitamin D has a specific impact on the bacterial profile in CD, shown by the shift of the bacterial composition, the different highly abundant and potentially beneficial bacterial strains and the reduced diversity during vitamin D administration. In contrast to CD, in the HC group, no specific effects of vitamin D administration have been detected. This is the first controlled prospective interventional analysis which shows a specific effect of vitamin D administration on the microbial communities in CD, but not in HC. Therefore, vitamin D administration may be an important additional therapeutic intervention in the management of CD.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1. Stack bar graphs of the bacterial community composition on phyla/class level at week 0 (w0), week1 (w1), week 2 (w2), week 3 (w3) and week 4 (w4) in Crohn's disease (CD) and healthy controls (HC).

Table S1. Primer and polymerase chain reaction (PCR) conditions for *NOD2*-genotyping

Table S2. Statistical analysis of the bacterial composition at the different weeks in Crohn's disease (CD) using a Bonferoni corrected PERMANOVA.

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Mucosa-attached bacterial community in Crohn's disease coheres with the clinical disease activity index

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Summary

In inflammatory bowel diseases (IBD), microbial communities often become imbalanced suggesting abnormal microbial-gut interactions. In this study, we analysed the mucosa-attached gut microbiota from 26 Crohn's disease (CD) patients using 16S rRNA gene amplicon sequencing. The samples were stratified according to their disease activity (Crohn's disease activity index, CDAI). The different disease activity categories had a comparable bacterial richness. Bacterial communities of patients in remission and intermediate CDAI (0–220) were relatively similar and dominated by the genus *Bacteroides* (>40%). The bacterial composition of patients assigned to a high CDAI category was dominated by *Pelomonas* (25%) *and Flavobacterium* (13%) but had a low relative abundance of *Bacteroidetes* (4%). This indicates the presence of specific abundant bacterial taxa at different CDAI levels. In addition, bacterial communities were also significantly influenced when a tumour necrosis factor (TNF)-α inhibitor was applied or by the local mucosal inflammation level. As a consequence, a shift of the microbial composition may also indicate a change of the disease activity in CD patients.

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Importance

The intestinal microbiota plays a major role in the development and the clinical course of IBD. The microbial composition of IBD patients in contrast to healthy controls has already been extensively studied. Here, we show for the first time that there is a clear coherence between changes in the mucosa-attached bacterial community in CD and the disease activity (measured in CDAI). Additionally, we were able to demonstrate that there is coherence of the bacterial community with the use of a TNF-α inhibitor and the local mucosal inflammation status. These results lead to a better understanding of the mucosa-attached microbial communities in CD and could potentially result in the development of novel therapeutic strategies.

Introduction

Inflammatory bowel diseases (IBD) mainly consist of Crohn's disease (CD) and ulcerative colitis (UC). The etiology of these disease entities is still not completely understood. However, chronic activation of the intestinal immune system caused by the microbiota in a genetically predisposed host might play an important role (Sartor, 2008; Abraham and Cho, 2009; Frank *et al.*, 2011). Different studies reported alterations in the intestinal microbiota of IBD patients compared to healthy controls (Frank *et al.*, 2007; 2011; Willing *et al.*, 2010). The intestinal microbiota therefore might play a role in the pathogenesis of IBD (Manichanh *et al.*, 2012). For example, it was shown that patients with CD have a decreased proportion of so-called beneficial bacteria, like *Bifidobacteria* and *Lactobacilli* and an increased proportion of potentially pathogenic bacteria like *Escherichia coli* (Favier *et al.*, 1997; Darfeuille-Michaud *et al.*, 1998; Ott *et al.*, 2004; Backhed *et al.*, 2005; Frank *et al.*, 2007; Sartor, 2008; Neish, 2009; Willing *et al.*, 2009; Arumugam *et al.*, 2011; Chassaing and Darfeuille-Michaud, 2011; Martinez-Medina and Garcia-Gil, 2014). However, in another study an increased proportion of *Bifidobacterium* and the *Lactobacillus* group was found in IBD patients compared to healthy controls (Wang *et al.*, 2014). In active IBD patients *Faecalibacterium prausnitzii* is significantly decreased compared to healthy controls (Swidsinski *et al.*, 2008; Sokol *et al.*, 2009).

Another study showed a decrease in *Clostridium family* in active UC and both active and inactive CD as well as an increase of *Bacteroides* in CD (Andoh *et al*., 2011). IBD patients have a depletion of commensal bacteria (Frank *et al*., 2007) and their microbial community is less diverse (Ott *et al*., 2004; Dicksved *et al*., 2008; Nishikawa *et al*., 2009).

Mutations in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene are a risk factor for the development of CD (Hampe *et al*., 2001; Hugot *et al*., 2001; Ogura *et al*., 2001). *NOD2* was shown to be important for the regulation of commensal microbiota in the murine intestine (Petnicki-Ocwieja *et al*., 2009). *NOD2*-mediated microbial imbalance also increases the risk of colitis and colitis-associated carcinogenesis in a murine model (Couturier-Maillard *et al*., 2013). Different studies have also linked certain CD associated mutations in the *NOD2* gene with decreased expression of antimicrobial peptides from Paneth cells in patients with ileal CD (Zasloff, 2002; Wehkamp *et al*., 2005). However, whether these changes in the microbial community are cause or consequence for the development of IBD is still under debate.

To further improve our understanding of how the gut microbiome contributes to the development of CD, we analysed the mucosa-attached intestinal microbiota from biopsies obtained during routine colonoscopies of CD patients using high resolution 16S rRNA gene amplicon sequencing. In contrast to previous studies, we analysed the samples with respect to the disease activity, as assessed by the Crohn's disease activity index (CDAI) in CD (Best *et al*., 1976). Our results show coherence of the bacterial community with the different CDAI categories, the use of a tumour necrosis factor (TNF)-α inhibitor and the local mucosal inflammation.

Impact of the disease activity in CD to the bacterial community

Colonoscopy of 26 CD patients was performed for a clinical indication and biopsies were taken from macroscopically healthy and if present also from inflamed mucosa (Table 1 and Table S1). A total of 54 biopsy samples were PCR amplified and sequenced using Illumina MiSeq that resulted in 17.5 million reads (80% of the sequences and index reads with a Q-score $\geq$30). The sequences were assembled using QIIME (Caporaso *et al*., 2011) and investigated by the SILVA NGS pipeline (Quast *et al*., 2013). SILVA_NGS quality control and clustering resulted in 5 101 365 assembled sequences that were assigned to 558 operational taxonomic units (OTUs) on the bacterial genera level and used for the further analysis.

Table 1. Patient characteristics.

	Crohn's disease (CD)
Number of patients	26
Sex	11 male: 15 female
Age mean	41 (22–67)
Patients with AZA/6-MP	2
Patients with IFX/ADA	3
CDAI 0–150	$n = 18$
CDAI 151–220	$n = 18$
CDAI 221–450	$n = 18$
CDAI mean	185.6 (38–332)
Number of biopsies	54
Number of biopsies from macroscopically inflamed mucosa	17
Number of biopsies from macroscopically healthy mucosa	37
Number of patients with *NOD2* mutations	4

26 CD patients were recruited from the University Medical Center Rostock, Germany and in total 54 biopsies analysed. Additionally, an ethylenediaminetetraacetic acid blood sample was drawn for analysis of mutations in the *NOD2* gene. The disease activity was assessed using the CDAI (Best *et al.*, 1976) and stratified into three categories: remission (CDAI < 150); moderate (CDAI 150–220) and highly active disease (CDAI 220–450). Three patients were treated with a TNF-α inhibitor (Infliximab, IFX/Adalimumab, ADA). Twenty-four of twenty-six patients were genotyped for the three major *NOD2* mutations (SNP 8, SNP 12 and SNP 13). Two patients did not give informed consent for genotyping. Four of twenty-four patients (16.7%) were positive for at least one mutation in the *NOD2* gene. The study was approved by the ethic board of the University of Rostock (A 2012-0121). Written informed consent was obtained from each participant prior to enrollment.

AZA, Azathioprine; 6-MP, 6-mercaptopurine; IFX, infliximab; ADA, adalimumab; CDAI, Crohn's disease activity index; NOD2, nucleotide-binding oligomerization domain-containing protein 2.

We characterized the disease activity of our cohort using the CDAI (Best *et al*., 1976) and made a stratification into mildly active (CDAI 0–150), moderately active (CDAI 151–220) and severely active disease (CDAI 221–450). In our cohort, we found no significant difference in the number of bacterial taxa in the different disease activity categories (Kruskal–Wallis test $p > 0.05$) (Fig. 1). Previous studies found a lower microbial diversity in IBD patients compared to healthy controls (Ott *et al*., 2004; Manichanh *et al*., 2006; Frank *et al*., 2007; Willing *et al*., 2010; Tong *et al*., 2013). However, these studies compared healthy mucosal biopsies to inflamed, whereas our study rather focused on the impact of the microbial composition in different clinical situations.

The dominant bacterial phyla in the samples covering almost half of the bacterial population samples were *Bacteroidetes* (46%) followed by *Firmicutes* (15%) and the classes *Betaproteobacteria* (18%) and *Gammaproteobacteria* (14%) (Fig. 2). *Verrucomicrobia*, *Actinobacteria* and *Deinococcus-Thermus* were only present in single samples.

In contrast to previous studies (Eckburg *et al*., 2005; Frank *et al*., 2007), we found *Bacteroidetes* to be

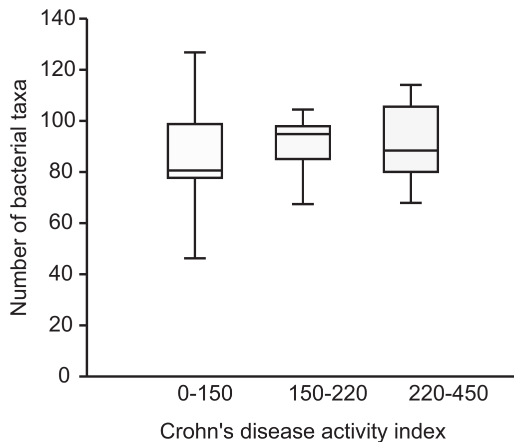

Fig. 1. Boxplots showing the number of bacterial taxa in Crohn's disease in the different disease activity categories.The biopsy specimens were collected in ATL buffer (Qiagen, Hilden, Germany) and frozen at −80°C immediately. Tissue samples were homogenized with the Tissue Disruptor (Qiagen, Hilden, Germany) and DNA isolation was performed with the DNA stool extraction kit (Qiagen, Hilden, Germany). Samples were PCR amplified and processed according to the Illumina protocol using a 500 cycle V2 chemistry kit on an Illumina MiSeq machine. We used the QIIME (Caporaso *et al*., 2011) 'joins paired-end Illumina reads' function with default settings to merge forward and reverse sequence with an overlap of at least 20 bp whereas sequences without overlap were discharged. The resulting assembled sequences were analysed using Silva_NGS (SILVA release version 115) with default settings (ambiguity and homopolymers 2%, OTU clustering 98%, min seq. quality 30%, min length 150 bp, min align. Identity 50%). For richness estimations, we used Explicet (Robertson *et al*., 2013), which performed a rarefaction-based analysis through bootstrapping.

dominant over *Firmicutes*. However, the study of Frank *et al*. (2007) in IBD also showed a reduction of *Firmicutes* and an expansion of *Proteobacteria* in line with our findings. Moreover, DNA extraction can bias the recovery of *Bacteroidetes* strongly (Momozawa *et al*., 2011). For example, the DNA stool kit, which was also used in this study, leads to a lower yield of *Firmicutes* compared to other extraction methods (Wu *et al*., 2010).

The samples between the CDAI categories 0–150 and 151–220 were relatively similar. However, a major difference was found between the mucosa-attached microbial communities of the CDAI group 221–450 compared to the other CDAI groups. An exception was patient P10 in the CDAI category 221–450. The mucosa-attached bacterial community of this specific patient was similar to the CDAI category 0–150. Interestingly, this patient received a TNF blocker. Other exceptions were patients P6, P19 (both category 0–150) and P21 (category 151–220). Their bacterial communities were more similar to the CDAI category 221–450.

To assess the differences in the microbial communities according to the disease activity, we visualized the dissimilarities of the bacterial composition on bacterial genus levels using non-metric multidimensional scaling (NMDS) (Fig. 3). Bacterial communities clearly separated on the first coordinate, depending on whether they were obtained from patients with a low or high CDAI. In addition we tested the differences of the mucosa-attached bacterial communities in the different CDAI categories, in a one-way PERMANOVA test using Bray–Curtis dissimilarity index. The bacterial community composition between the disease activity (CDAI) categories was significantly different ($p < 0.01$). This result suggests coherence between the bacterial composition and the disease activity, indicating that the bacterial composition in a state of remission differs significantly compared to the bacterial composition in highly active CD.

Specific abundant bacteria in the different disease activity groups

From a clinical point of view it would be of major interest to identify specific bacterial strains which are representatives of a certain disease activity group. In order to elucidate this question, we further characterized alterations in the bacterial communities using LEfSe (Segata *et al*., 2011). Since the separation between CDAI categories was highly significant (Table S2), we arranged the data according to the CDAI categories. LEfSe first compares the data using a non-parametric test (Kruskal–Wallis with alpha = 0.05). The results are then compared between the categories with a Wilcoxon rank test (alpha = 0.05). Subsequently, significant different features are compared with linear discriminant analysis (logarithmic score 2.0) to assign OTUs with significant higher abundance in a CDAI category.

Among the high abundant bacteria (> 0.1% in average) identified in the biopsies from patients in clinical remission were *Anaerostipes* (*Clostridia*) and an uncultured *Coriobacteriaceae* (*Actinobacteria*). *Bifidobacteria* were also significantly enriched but low in abundance. In the category CDAI 151–220, we detected a relatively high number of *Lachnospiraceae* (*Firmicutes*) among which were *Roseburia*, *Blautia*, *Pseudobutyrivibrio* and other 'uncultured Lachnospiraceae'. In addition, we found other *Firmicutes* (*Megasphera*, *Flavonifractor*, *Veillonella*, 'uncultured Erysipelotrichaeae') and *Haemophilus* (*Gammaproteobacteria*). However, the most abundant bacterium in this group was assigned to the genus *Bacteroides* (*Bacteroidetes*) which had very high abundances in some samples. The highest CDAI category was dominated by *Burkholderiales* (*Betaproteobacteria)* belonging to the genera *Pelobacter*, *Paucibacter*, *Aquabacterium*, *Acitovorax* and *Ralstonia*. Among the highly abundant bacteria were also *Flavobacterium*, *Brevundimonas* and *Sphingomonas*. Among the *Lachnospiraceae* we detected only *Coprococcus*.

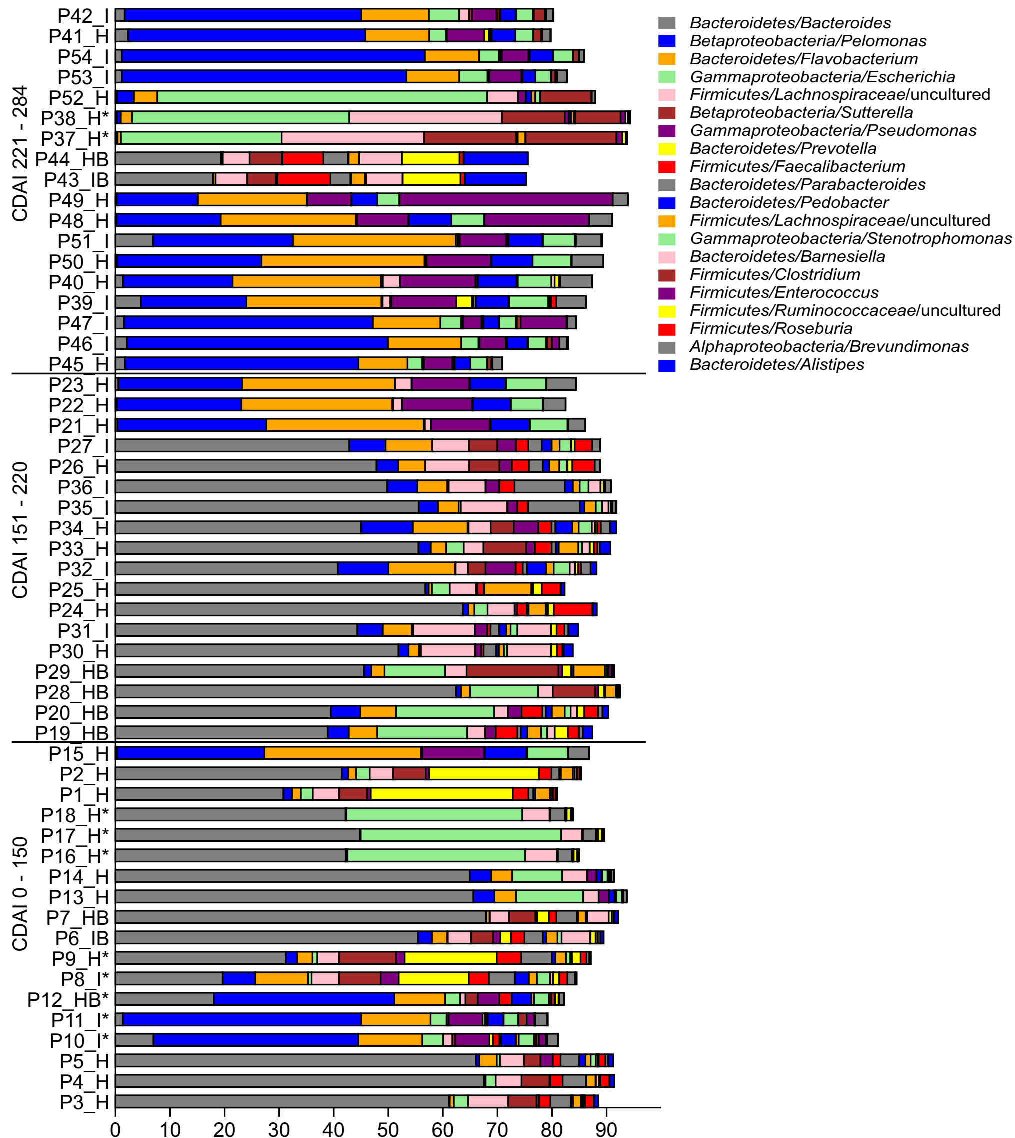

Fig. 2. Bacterial composition in patients with CD. The disease activity was assessed via CDAI. H = healthy; I = inflamed; B = Application of a tumour necrosis factor -α (TNF-α blocker; * *Nucleotide-binding oligomerization domain-containing protein 2* (*NOD2*) gene mutation.

Since IBD can be in remission and also flare, the bacteria in the highest CDAI category could potentially serve as predictors for a more severe disease course and therefore serve as bacterial markers for upcoming disease flares or may even be pathophysiologically related to the disease activity. As a consequence, one can hypothesize that restoring the microbial community in highly active IBD patients back to a 'non-inflamed' microbial community could also lead to a reduction of the disease activity. However, it is still not completely understood if the changes in the microbial community lead to the inflammation in IBD or if the mucosal inflammation triggers the changes in the microbial composition.

In our cohort, the abundance of *F. prausnitzii* (*Firmicutes*) did not change significantly in the different CDAI categories. A diminished abundance of *F. prausnitzii* has been associated with IBD (Swidsinski *et al.*, 2002; Sokol *et al.*, 2008; 2009). *F. prausnitzii* seems to have anti-inflammatory capacities *in vitro* as well as and *in vivo* (Sokol *et al.*, 2008). In our study the bacterial genera, *Blautia* and *Roseburia*, had a high abundance in the mildly active CD cohort (CDAI 151–220). Both, *Roseburia hominis* and *F. prausnitzii* are butyrate-producing strains (Machiels *et al.*, 2014), which might play an important role in the regulation of Treg cell homeostasis in the colon (Smith *et al.*, 2013). In a study with UC patients (Machiels *et al.*, 2014), a decrease of *R. hominis*

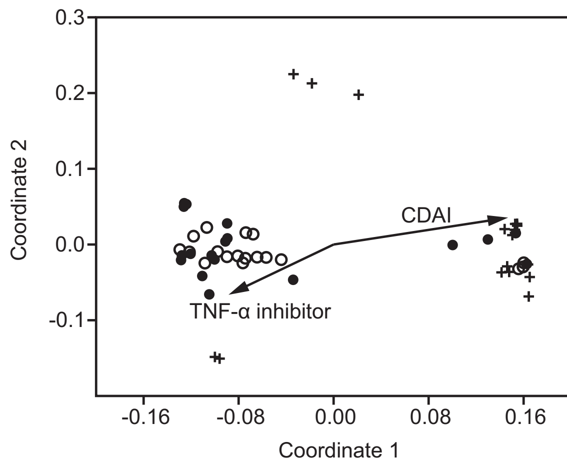

Fig. 3. NMDS-based on Bray–Curtis dissimilarity of the mucosa-attached bacterial community from CD patients (NMDS stress 0.11).The plot covers a total of 54 probes from CD patients whereas (●) represent biopsies from patients with a CDAI of 0–150, (○) represent biopsies with a CDAI of 151–220 and (+) represent biopsies with a CDAI 221–450. The vectors for CDAI and TNF-α inhibitor were added post hoc and represent the correlation coefficients between these factors and the NMDS scores. Variations in the bacterial community structure were characterized using NMDS with Bray–Curtis dissimilarity using the PAST software package version 3.08 (Hammer *et al*., 2001).

and *F. prausnitzii* was inversely correlated with the disease activity. Willing *et al.* (2010) found a decrease of *Roseburia* in ileal CD compared to healthy controls. Further, another study described an association of a diminished abundance of *Blautia* and *Roseburia* in patients with UC before colectomy with an increased risk of pouchitis after ileal pouch-anal pouch anastomosis (Machiels *et al*., 2015). It remains speculative if these bacteria have anti-inflammatory capacities and are therefore more prevalent in the mildly active CD group because of the inflammation and potentially could also be beneficial in patients with a highly active disease. A murine model would be needed in order to test this hypothesis.

Coherence between different clinical characteristics and the mucosa-attached bacterial communities

In addition to the disease activity (CDAI), we measured a possible coherence between the mucosa-attached microbial communities and the use of a TNF-α inhibitor, the inflammation status of the biopsy and the presence of a mutation in the *NOD2* gene. Again, the differences of the mucosa-attached bacterial communities compared to the above mentioned parameters were tested using a one-way PERMANOVA.

Tumour necrosis factor-α

The parallel direction and length of the vectors for the CDAI and the use of a TNF-α inhibitor to the first coordinate indicate a strong opposing impact of these factors to the separation of the bacterial communities. In a prospective study, dysbiosis, defined as a reduction in *Bacteroides*, *F. prausnitzii* and *Clostridium coccoides*, was associated with relapse after discontinuation of infliximab therapy (Rajca *et al*., 2014). In other studies with CD and UC patients in remission and subsequent exacerbations of their disease, the use of thiopurines was associated with a specific microbial composition and diversity of the fecal samples (Wills *et al*., 2014). In our study, patients with CD showed a significantly different bacterial community composition when TNF-α inhibitors were applied ($p = 0.02$). Moreover, the opposite direction of the disease activity (CDAI) and the use of a TNF-α inhibitor in the NMDS plot (Fig. 3) suggest a positive effect of this treatment on the bacterial community composition. One can hypothesize that this specific therapy could reverse the microbiota to the typical composition of remission. However, the low sample size in the group of patients treated with a TNF-α inhibitor makes it difficult to draw firm conclusions.

Inflammation status

The natural course of IBD includes poorly predictable phases of activity and remission (Baumgart, 2009; Cosnes *et al*., 2011). If the composition of the gut microbiota is associated with intestinal inflammation, a better understanding of the imbalanced microbiota in the inflamed lesions and the macroscopically healthy parts of the gut could also result in better surveillance methods or even new therapeutic approaches, e.g. a specific probiotic therapy. In our cohort, the bacterial communities detected in macroscopically healthy and macroscopically inflamed mucosa were small but significant ($p = 0.03$). Our results therefore suggest that the impact of the disease activity (CDAI) on the mucosa-attached bacterial community is stronger than the local inflammation status.

NOD2 gene

A study by Rehman *et al.* (2011) showed that *NOD2* plays an important role in the intestinal microbial composition and that certain mutations are associated with a distinct intestinal microbial profile suggesting that the genetic composition of the host modifies its microbiome. In our study, there was a non-significant trend between the presence of mutations in the *NOD2* gene and the bacterial composition ($p = 0.12$). There are different potential explanations for this result. In contrast to the study of Rehman *et al.* (2011), which had a very distinct group of patients homozygous for SNP13, our analysis also included patients with SNP8 and SNP12, which potentially do not have the same clinical impact on the

pathogenesis of CD compared to SNP13 (Hampe *et al.*, 2001; Hugot *et al.*, 2001). Another potential explanation for this result is that the *NOD2* receptor seems to play a more pronounced role in the ileum of patients with CD (Wehkamp *et al.*, 2004; 2005) whereas we used only biopsies from colonic mucosa. Additionally, this non-significant trend may also be attributed to the small absolute number of *NOD2* mutated patients in our cohort and their uneven distribution to the different categories. To further elucidate the impact of *NOD2* on the inflammation and the bacterial community, a higher number of patients with *NOD2* mutations may be needed and also non-IBD patients with *NOD2* mutations should be screened towards their bacterial composition. However, such a study cohort will be difficult to obtain.

This is the first study which describes a clear coherence between changes in the bacterial community in CD and the disease activity (CDAI). In summary, we find a correlation of the mucosa-attached bacterial community with the disease activity. In future, these results might also be used as a diagnostic tool in defining the disease's course and could also serve as targets in evolving new treatment strategies in IBD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Detailed Patient characteristics.

Table S2. Abundant (>0.1%) bacterial genera with a significantly increased abundance at different disease activity levels in Crohn's disease.

File S1. Experimental Procedures: *NOD2*-genotyping.

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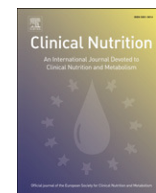

Original article

NOD2 mutations are associated with the development of intestinal failure in the absence of Crohn's disease

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SUMMARY

Background & aims: Short bowel syndrome (SBS) and intestinal failure (IF) are multi-factorial conditions which in adults result from extensive intestinal resection. NOD2 is an intracellular pattern recognition receptor associated with CD. An unexpected high frequency of NOD2 mutations has been found in patients undergoing intestinal transplantation (35%). The role of NOD2 in a cohort with SBS/IF not specifically requiring intestinal transplantation has not been studied yet.
Methods: The course of 85 patients with non-malignant SBS/IF was characterized. The major NOD2 mutations, as well as ATG16L1 and IL23R were determined. The allele frequencies were compared to the published frequencies of CD patients and controls.
Results: In non-CD patients (72%) allele frequencies of NOD2 mutations were statistically more frequent than in controls (14% vs 6%, $p = 0.006$). In CD patients (28%) allele frequencies were not different between SBS and controls (29% vs 22%, $p = 0.23$). NOD2 mutations were neither associated with parameters potentially heralding the need for transplantation nor with an earlier time to the indication for intestinal transplantation.
Conclusions: NOD2 mutations are associated with the development of SBS/IF in the absence of CD, but not with specific complications. NOD2 mutations may increase the risk for more extensive intestinal resection or may impair intestinal adaptation.
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1. Introduction

Short bowel syndrome is a chronic condition secondary to extensive resection of small intestine. The most frequent underlying non-malignant diseases in adults are Crohn's disease, mesenteric ischemia, ileus or (post) surgical complications, trauma and desmoid tumors of the mesentery. Treatment consists of either long term special diet plus medical treatment including high dose anti-motility agents, bile acid binders and electrolyte as well as micronutrient substitutions or home parenteral nutrition (HPN). Intestinal transplantation becomes a treatment option, if HPN fails because of intestinal failure associated liver disease (IFALD), thrombosis of central veins used for catheter insertion or frequent line related sepsis.[1,2]

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is an intracellular pattern recognition receptor that senses muramyl dipeptide and peptidoglykan from bacterial cell walls and subsequently activates NFkB.[3] Mutations in the NOD2 gene have been identified as risk factors for the development of Crohn's disease (CD).[4–6] In a healthy British/German cohort the allele frequency of the NOD2 mutations, R702W, G908R and 3020insC, also known as SNP8, SNP 12 and SNP 13, were 3.5, 0.6 and 2.1%,[7] conferring an increased risk for Crohn's disease of 2–4 fold in the heterozygous state and of up to 17 fold in the homozygous or compound heterozygous state.[8] Besides NOD2, other risk alleles for Crohn's disease are described in the literature, for example autophagy-related protein 16 L1 (ATG16L1) or mutations in the Interleukin-23 receptor (IL-23R).[9,10]

Abbreviations: SBS, short bowel syndrome; IF, intestinal failure; IFALD, intestinal failure associated liver disease; CD, Crohn's disease; HPN, home parenteral nutrition.

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Acute cellular rejection after intestinal transplantation shares some histological and more so macroscopic-endoscopic features with acute Crohn's disease. This has led Fishbein to study the frequency of NOD2 mutations in intestinal and multivisceral transplant recipients.[11] 12 of 34 intestinal failure patients undergoing transplantation carried at least one such mutation. Interestingly only three of these patients had CD, two of which had a NOD2 mutation. In their study, which addressed the post-transplantation outcome, NOD2 mutations were a risk factor for rejection and were accompanied by decreased secretion of defensins, which are stimulated by NOD2 signaling. These findings were not replicated in the study by Janse, who found no difference in NOD2 mutations between intestinal transplant recipients and donors.[12] On the other hand, the study by Ningappa, which was focused but not limited to children, detected a strong association of the frame shift mutation (3020insC/SNP13) with combined liver and intestinal failure requiring combined transplantation.[13]

Both Fishbein and Ningappa have pointed out, that their cohorts represent intestinal failure patients with the need for transplantation, and that a study in patients without or prior to the need for transplantation would shed light on why NOD2 mutations appear to be related to complicated intestinal failure and a problematic posttransplant course.[11,13] Therefore we have studied NOD2 mutations and the clinical course of 85 adult patients in our intestinal failure program, which includes long term conservative management (oral and parenteral nutrition), rehabilitative surgery and both intestinal and combined transplantation. Here we report, that in non-CD patients NOD2 mutations are associated with the development of IF but not specifically with the need for intestinal or multivisceral transplantation. Pathophysiologically, NOD2 mutations may impair the physical and/or immunological intestinal barrier function and thereby increase the risk for perioperative complications leading to more extensive resection and/or compromise intestinal adaptation.

2. Materials and methods

The study was approved by the Ethics committee of the university hospital of the University of Tübingen (022/2011BO2). All patients gave written informed consent (separately for the study and for the genotyping).

2.1. Patient cohort

All patients were Caucasian adults of northern European ancestry and fulfilled the definition of short bowel syndrome or intestinal failure.[14] 16 patients had never been on long term (>4 weeks) HPN, but required intensive dietary measures to maintain oral compensation (special diet, >5 meals per day, high dose antidiarrheal medication); these patients are referred to as short bowel syndrome (SBS). All other patients (69 of 85) fulfilled the definition of intestinal failure requiring HPN.[14] Of these 9 were weaned from HPN. The beginning of SBS/IF was defined as the time point when specific nutritional intervention became necessary for the first time. Index surgery was defined as the surgical intervention, which led to the need for specific nutritional intervention. 2 patients had intestinal failure that occurred at childhood (age 0–10). 2 patients had an underlying malignancy that had lead to intestinal failure. They did not receive antineoplastic therapy but were only treated to maintain their nutritional status. Both died 1673 and 460 days after intestinal failure had ensued. None of the patients received or had received growth hormone, epidermal growth factor, teduglutide, somatostatin/octreotide or a dipeptidylpeptidase-IV-inhibitor. Data were analyzed as of May 31st 2011.

2.2. Definition of HPN related complications and the need for intestinal or multivisceral transplantation

Central vein thrombosis was detected by Doppler, contrast enhanced CT scan or contrast enhanced MRI, which were initiated upon clinical suspicion or prior to insertion of a new central catheter. All other data were extracted from clinical files and from personal interview.

The indication for intestinal transplantation was defined as the failure of HPN because of impending loss of venous access (thrombosis of 2 or more central veins) or the development of IFALD as judged by a persistently elevated bilirubin of >2 mg/dl despite attempts to optimize the composition of the parenteral nutrition. Need for multivisceral transplantation including the liver was defined, if the bilirubin was persistently elevated above 4 mg/dl. Recurrent sepsis per se was not defined as an indication.

2.3. Mutation detection

The three CD-associated mutations in the NOD2 gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844 and SNP 12; G908R, NCBI reference SNP ID: rs2066845 and 3020insC, SNP 13; 1007fs, NCBI reference SNP ID: rs2066847) were detected in genomic DNA extracted from whole blood as described by Fishbein et al. with minor modifications.[11] Briefly DNA was extracted from whole blood collected in EDTA-anticoagulated tubes using the QIAamp DNA Mini kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The Taqman MGB biallelic discrimination assay was applied using the two pre-made assays c_ _ 11717468_20 and c_ _11717466_20 for the R702W and the G908R point mutations in NOD2 as well as a custom developed assay for the 1007 frame shift mutation (forward primer, GTCCAATAACTGCATCACCTACCT; reverse primer, CAGACTTCCAGGATGGTGTCATTC and VIC-labeled probe, CAGGCCCCTTGAAAG; FAM-labeled probe, CAGGCCCTTGAAAG).

The ATG16L1 SNP (rs 2241880) and IL23R SNP (rs1004189) were detected using the two pre-made biallelic discrimination assays c_ _9095577_20 for ATG16L1 and c_ _1272321_10 for IL23R (Applied Biosystems).

Briefly, 50 ng of genomic DNA was mixed with 10 μl of 2 × TaqMan Universal PCR Master Mix No AmpErase UNG and 1 μl of 20 × SNP Genotyping Assay in a final volume of 20 μl, and PCR was carried out on an ABI Prism 7000 Real Time PCR instrument (Applied Biosystems, Foster City, CA). Thermal cycling conditions were: 95 °C/10 min followed by 40 cycles of 92 °C/15 s and 60 °C/60 s. Detection of fluorescent signal was performed according to the recommended protocols for the ABI Prism 7000 Real Time PCR machine (Applied Biosystems, Foster City, California, USA), and the results were analyzed by the associated Sequence Detection System (SDS) Software V. 1.2.3. (Applied Biosystems).

2.4. Statistical analysis

Statistical analysis was performed using JMP 9 (SAS, Cary, NC). Allele frequencies of NOD2 mutations were calculated by dividing the sum of mutated alleles by the sum of chromosomes analyzed. Differences in the proportions were tested using the chi-squared test. Continuous data such as the rate of both line related sepsis and thrombosis of central veins were compared using the unpaired two-sided *t* test. Residual intestinal length and duration of HPN were positively skew; they were therefore log-transformed prior to using the *t*-test. These data are presented as geometric means and 95% lower and upper confidence intervals. Survival rates were estimated by the Kaplan–Meier method, and the log rank test was used to test the difference between survival curves. Frequencies of

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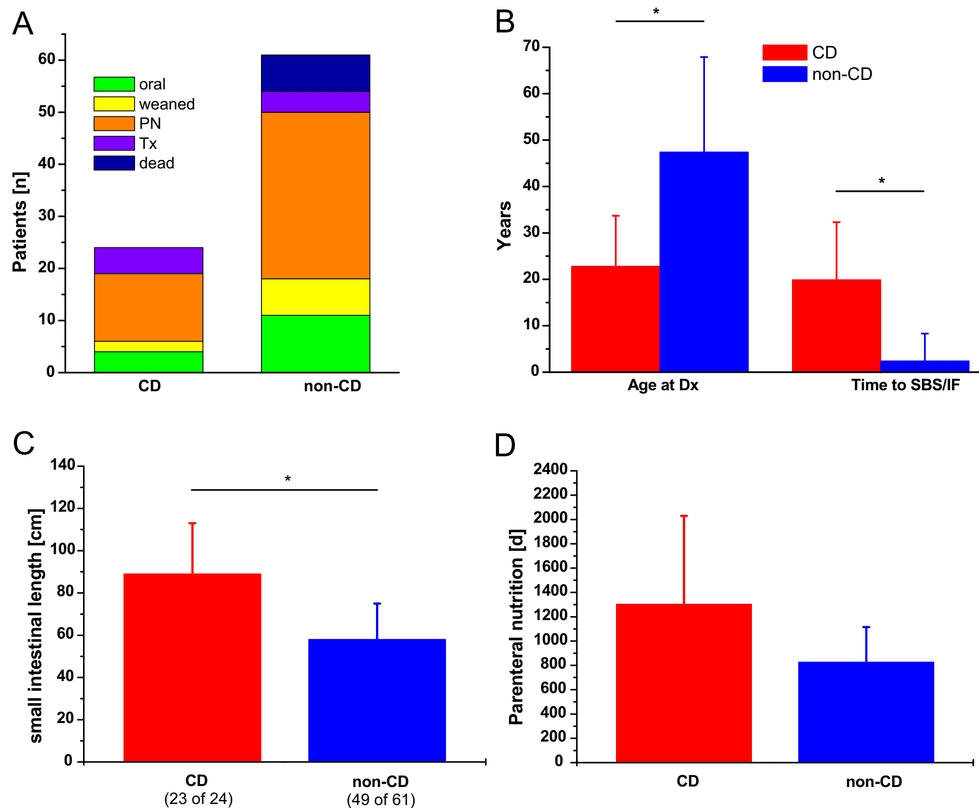

Fig. 1. Patient cohort. Patients with CD as the underlying etiology of SBS/IF are compared to other etiologies (non-CD). A: Current status (two CD patients who lost their graft and went back to PN are included in the Tx group, see text for details). B: Age at diagnosis and time to development of SBS/IF. C: Residual small intestinal length. D: Duration of parenteral support (included are all patients with parenteral support, see text for details).

line related sepsis and thrombosis of central veins were compared using the student's *t*-test.

3. Results

3.1. Study cohort

Figure 1 summarizes the cohort of 85 patients. Given the known association of CD with NOD2 mutations and given the fact that in the adult population CD is among the most frequent causes of SBS and IF,[1,2] patients with a history of CD were analyzed separately. The median follow up since the diagnosis of SBS/IF was 1354 days (95% CI 2012–3421 days) with no significant difference between CD and non-CD patients. Figure 1A shows the current status of CD and non-CD patients. 15 patients never received long term HPN and are therefore referred to as orally compensated SBS. 9 patients were weaned from PN after a median of 471 days (range 183–1197 days). Among the transplanted patients two with CD lost their graft. Patients suffering from CD were significantly younger at their diagnosis of CD than those with another etiology (22.8 ± 10.9 vs. 47.1 ± 20.6 years; $p < 0.0001$). In CD patients it took 19.9 ± 12.4 years after their initial diagnosis of CD until they developed SBS/IF; this reflects recurrent resection(s). None of the CD patients had a gastroenterostomy and none had a bypassed segment. In comparison, non-CD patients were not only significantly older at diagnosis but also their SBS/IF was mostly established in one operation and thus it took only 2.3 ± 5.7 years after their initial diagnosis to develop IF (Fig. 1B). Residual small intestinal length was significantly shorter in non-CD patients than in CD patients (Fig. 1C). In addition in the non-CD group ultra short bowel with less than 20 cm small intestine was more frequent (13 of 49 non-CD vs. 1 of 23 CD patients with known residual small intestinal length, $p < 0.001$). The duration of parenteral support was 895.5 days, not significantly different between CD and non-CD patients (Fig. 1D).

3.2. Survival and need for transplantation in CD and non-CD patients receiving PN

Figure 2 shows overall survival (panel A) and survival without transplantation (panel B) of CD and non-CD patients with IF. The analysis was limited to the 69 patients receiving PN (20 CD and 49 non-CD) because they had IF and were thus theoretical candidates for intestinal transplantation if their PN would have failed. Patients who only transiently received PN were included because death (due to PN related complications) occurred in some patients during the time period when others, who were eventually weaned, still received PN.

Death was limited to the non-CD group and mostly occurred during the first 3 years of PN ($p = 0.03$ compared to the CD group by Log-rank test, Fig. 2A). 5 patients were transplanted in the CD group and 4 in the non-CD group. A trend towards an early (up to about 1500 days of HPN) worse prognosis of IF in non-CD patients became also evident, when these transplantations, which were intended to prevent death, were included in the survival analysis (Fig. 2B).[f]

[f] The two patients who lost their grafts and went back to PN (see Table 2) are depicted as transplanted in Fig. 2B. This trend is not reflected in the statistical analysis because the statistical analysis addressed the entire time frame of up to 6500 and 8300 days respectively.

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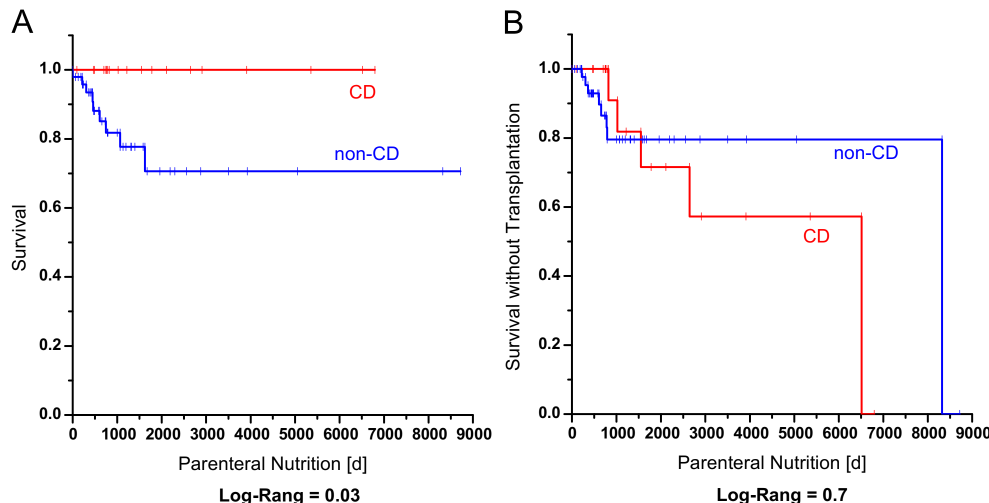

Fig. 2. Survival and transplantation of CD and non-CD patients. A: Overall survival. B: Survival without transplantation, i.e. combination of spontaneous survival and transplantation (to prevent death) as undesirable outcomes of if. $n = 69$.

Taken together the data of Fig. 2 confirm the better early (up to about 5 years) outcome of CD patients on HPN compared to non-CD patients,[15] but they also indicate that the need for intestinal or multivisceral transplantation does occur in some CD patients with IF and long standing HPN.

3.3. NOD2 mutations are unusually frequent in intestinal failure

Because of the association of three NOD2 mutations (R702W, G908R and 3020insC) with CD as well as with IF requiring transplantation[11,13] we tested our cohort of SBS/IF for the allele frequency of these mutations. 70 of 85 patients were genotyped (24 CD and 46 non-CD). 3 patients died before genotyping was initiated and thus no DNA and no approval for typing could be obtained. 12 patients did not give informed consent for genotyping. Taking into account the comparably small numbers there were no discernable differences in the clinical course between patients that were genotyped and those who were not. Allele frequencies of NOD2 mutations of the CD patients with SBS/IF were compared to published frequencies of Caucasian CD patients of north European ancestry and allele frequencies of NOD2 mutations of the non-CD patients were compared to the published frequencies of healthy Caucasian controls[7] (Table 1). In non-CD patients with SBS/IF the allele frequency of NOD2 mutations was significantly higher than in healthy controls (14% vs. 6%, $p = 0.006$). In CD patients with SBS/IF the NOD2 allele frequency was somewhat higher than in the CD control cohort, but the difference was not significant (29% vs. 22%, $p = 0.23$). It was not possible to formally stratify the CD patients into ileal, ileocolonic and colonic disease, because many of these patients had been operated before that classification became widely used or operations were performed for secondary complications not attributable to either of these disease manifestations. But given that all the CD patients had undergone significant resection of their small intestine it can be assumed that they all had either ileal or ileocolonic disease. In this subgroup of CD the allele frequency of NOD2 mutations is higher than in the entire CD population (19.7% and 26.9%,[7]). Thus there is probably no or only a minor difference between CD patients with or without SBS/IF with regard to NOD2 mutations. All together 22 of 70 genotyped patients (31%) carried al least one NOD2 mutation.

Table 2 summarizes the etiology of SBS/IF of the non-CD patients and their NOD2 mutational status in order to correlate NOD2 mutations to a specific etiology. There was a non-significant trend for a higher allele frequency in the ischemia group compared to the other non-CD etiologies (20% vs 8.3%, $p = 0.095$).

3.4. NOD2 mutations in patients with an indication for intestinal transplantation

Table 3 summarizes the 16 of 69 patients with IF and an indication for intestinal, combined or multivisceral transplantation

Table 1
Allele frequencies NOD2 mutations in SBS/IF patients 70 of 85 SBS/IF patients were genotyped (24 CD and 46 non-CD). Frequencies of NOD2 mutations of the CD patients with SBS/IF were compared by X^2 test to published frequencies of Caucasian CD patients of north European ancestry and allele frequencies of NOD2 mutations of the non-CD patients were compared to the published allele frequencies of healthy Caucasian controls.[6] 31% of all genotyped patients carried at least one NOD2 mutation (50% of CD and 22% of non-CD patients).

Etiology	Allele frequencies of NOD2 mutations (wt/hetero/homo or compound-hetero)		*p*
	SBS/IF	Control	
CD (24 of 24 genotyped)	29% (12/10/2)	22% (442/191/55)	$p = 0.006$ by X^2 test
Non-CD (46 of 61 genotyped)	14% (36/7/3)	6% (256/34/0)	$P = 0.23$ by X^2 test

Table 2
Etiologies of short bowel syndrome/intestinal failure in the study cohort and NOD2 mutational status. In the non-CD patients with ischemia as the underlying etiology the NOD2 allele frequency appears to be higher than in the other etiologies of non-CD patients, but the difference is not statistically significant ($p = 0.095$).

Etiology	*n*	NOD2 wt	NOD-2 mutation (hetero/homo or compound)	Unknown
CD	24	12	12 (10/2)	0
Non-CD	61	36	10 (7/3)	15
Ischemia	29	15	7 5/2	7
Surgical complication	10	8	1 1/0	1
Ileus	6	4	0	2
Trauma	4	3	0	1
Other	12	6	2 1/1	4
All	85	48	22 (17/5)	15

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Table 3
Transplantation status of patients with if all 70 patients with HPN (61 permanent and 9 weaned) were analyzed for the need for intestinal or combined transplantation.

Status for Tx	N	CD/non-CD	n	Indication of Tx	Type of Tx	Outcome
Transplanted	9	CD	5	3 IFALD	1 Multivisceral 1 combined liver and intestine 1 isolated intestine	3 Full oral autonomy
				1 Central vein thrombosis	1 Isolated intestine	Initially, full oral autonomy chronic graft failure after 3.5 y graft enterectomy, back on HPN by catheter in VCI
				1 Recurrent sepsis	1 Isolated intestine plus kidney	Acute rejection graft enterectomy after 3 months, back on HPN, multiple complications
		Non-CD	4	2 IFALD	2 Multivisceral	2 Full oral autonomy
				2 Central vein thrombosis	2 Isolated intestine	2 Full oral autonomy
Waiting list	2	CD	1	1 Central vein thrombosis		
		Non-CD	1	1 Central vein thrombosis		
Died on waiting list	2	CD	0			
		Non-CD	2	2 IFALD		
Candidacy but contraindication	3	CD	2	1 Central vein thrombosis1 IFALD		
		Non-CD	1	1 IFALD		1 Dead secondary to IFALD

based on HPN failure. Overall IFALD was the most frequent indication, followed by central vein thrombosis (≥2 central veins) and recurrent sepsis. Of the 9 transplantations performed 5 were for IFALD. 2 patients died on the waiting list as a result of IFALD, both early after the start of the transplantation program in 2006. The NOD2 mutational status was available in 13 of these 16 patients. 5 of the 13 patients (38%) carried at least one NOD2 mutation. This number remarkably resembles the 31% patients with at least one NOD2 mutation in the entire cohort. Also, the time until individual patients developed an indication for intestinal or multivisceral transplantation was not different whether they carried a NOD2 mutation or not (NOD2 wild type 1188 days, 95% CI 245–4869 days and NOD2 mutation 1025, 95% CI 744–10409 days; $p = 0.34$ by Log-rank test).

Because of the low absolute number of transplanted patients in our cohort we tested whether clinical parameters that may herald the need for transplantation were associated with the carriage of NOD2 mutations. To this end persistently elevated levels of bilirubin (>1 mg/dl), the frequency of central line related sepsis and the frequency of thrombosis of central veins were analyzed, but no such association became evident (Table 4).

3.5. SNPs in ATG16L1 and IL23R are not associated with SBS/IF

In order to test whether the association of NOD2 mutations with SBS/IF was specific, we tested single nucleotide polymorphisms (SNPs) of two other CD susceptibility genes, ATG16L1 (rs 2241880) and IL23R (rs1004189). The association of ATG16L1 and NOD2 is still controversial,[9,16] while IL23R and NOD2 are thought to act independently in the pathophysiology of CD. There was a non-significant trend in the ATG16L1 rs2241880 SNP towards higher risk allele frequencies in the CD and non-CD SBS/IF cohorts (SBS/IF CD 70.4% vs CD control 58.1%, $p = 0.21$ and SBS/IF non-CD 53.8% vs healthy control 51.3%, $p = 0.70$). The risk allele frequencies of the IL23R SNP (rs1004189) did not differ from those reported in the literature for CD patients and non-CD healthy controls (SBS/IF CD 38.6% vs CD control 38.1%, $p = 0.99$ and SBS/IF non-CD 21.2% vs healthy control 29.6%, $p = 0.27$).[9,10]

Table 4
Markers potentially heralding the need for intestinal transplantation A persistently elevated bilirubin (>1 mg/dl), the frequency of line related sepsis and the frequency of thrombosis of central veins (each per 1000 catheter days) were taken as parameters potentially heralding the need for transplantation.

	NOD2 wild type	NOD2 mutated	Test	p
Persistently elevated bilirubin (>1 mg/dl) [n]	5 of 37	3 of 19	Fisher's exact test	1.00
Line related sepsis/1000 catheter days	1.62	1.66	Student's t-test	0.96
Thrombosis of central veins/1000 catheter days	0.37	0.27	Student's t-test	0.65

4. Discussion

Two groups have reported unexpected high frequencies of NOD2 mutations (about 30%) in IF patients who undergo intestinal or multivisceral transplantation because of failing HPN.[11,13] The question of the current study was, whether NOD2 mutations are associated with SBS/IF in general or specifically with a complicated course requiring transplantation. The data indicate that NOD2 mutations are associated with the development of intestinal failure in the absence of Crohn's disease but not specifically with the development for the need for transplantation.

Prior to transplantation NOD2 mutations may in principle affect the clinical course in three ways: (a) they may put the individual at an increased risk in the context of the index surgery leading to an increased loss of intestinal surface, (b) they may inhibit adaptation of the residual bowel or (c) they may be related to the development of specific complications resulting in the need for transplantation. In order to address these different potential mechanisms we genotyped our SBS and IF patients and characterized their clinical course.

Several aspects indicate that the current study addressed a valid patient cohort: (a) A long enough observational period was covered to include a subset of patients who develop or have developed the need for transplantation. In a recent European prospective survey 28.6% of adult HPN patients with IF had died or had been transplanted after 5 years.[2] In our cohort it was 23% (16/69) of patients after a median follow up of 2.45 years (895.5 days). (b) 9 patients were weaned of parenteral support mostly as a result of reconstructive surgery putting unused intestinal segments in continuity. These patients were included in the IF group because they were at risk, although for a shorter period of time, to develop IF or HPN related complications. Again these data match the 12.8% patients weaned in the European survey.[2] (c) The need for transplantation was clearly defined as failure of HPN even if contraindications existed that precluded transplantation, i.e. candidacy for transplantation.[17] Recurrent sepsis per se was not defined as an indication for transplantation because in our experience this can usually be managed by medical treatment and change of the central line if indicated.[18] This approach to transplantation has also been

validated in the European survey.[2] (d) The cohort was stratified for CD and non-CD as the underlying etiology because of the known increased frequency of NOD2 mutations in CD patients.[7] For the non-CD patients it included a variety of different and typical underlying etiologies.[1] Thus, given the unknown association of NOD2 mutations with specific diseases other than CD, there was no bias towards specific etiologies.

With regard to the role of NOD2 mutations our cohort includes 29% CD patients who in most series of adult intestinal failure comprise about one third of patients requiring parenteral support[2] but who are more rarely transplanted[1] and who made up only a small minority in the three studies addressing the role of NOD2 mutations in patients requiring intestinal or multivisceral transplantation.[11–13]

In non-CD patients with IF we found a statistically significant increased allele frequency of NOD2 mutations compared to healthy controls of European ancestry. Furthermore in the limited number of patients with an indication for transplantation (both CD and non-CD) the rate of NOD2 mutations (5 of 13 patients with a known NOD2 mutational status; 38%) appeared similar to the entire cohort (22 of 70 patients with a known NOD2 mutational status; 31%). Taken together these data suggest that NOD2 mutations are associated with the development of SBS/IF after abdominal surgery in non-CD patients, but not specifically with the development of an indication for intestinal or combined/multivisceral transplantation. This is further supported by the lack of an association of the NOD2 mutational status with the time until the development for an indication of transplantation or with clinical markers potentially heralding the need for. While this study was under review similar findings were reported from an independent cohort.[19]

While NOD2 mutations apparently favor the development of SBS/IF in non-CD patients, two other CD-related polymorphisms in ATG16L1, which is part of the autophagy pathway, and in IL23R, which is part of the adaptive immune system, were not associated with the development of SBS/IF. This could be due to several reasons: a) The risk allele frequency of ATG16L1 is much higher than that of the NOD2 mutations. Thus, while there was a trend for a higher than expected allele frequency, a true association of the ATG16L1 polymorphism with the development of SBS/IF may have remained undetected in the presence of this high background. b) Along the autophagy pathway ATG16L1 acts down-stream of NOD2.[20,21] Thus any NOD2 related pathway may diverge from the CD pathway without the involvement of ATG16L1. c) In the pathogenesis of CD, the IL23R polymorphism is thought to act independent of NOD2. Thus the finding that the IL23R polymorphism was unrelated to the development of SBS/IF indirectly supports the specificity of the NOD2 association.

Our data confirm the notion, that NOD2 mutations affect the pretransplant course of intestinal failure[11,13] but extend this finding to indicate that they are not associated with the specific development for the need for transplantation. Thus NOD2 mutations may affect the development of SBS/IF at two steps: They may either pose the individual at an increased risk for complications in the context of abdominal surgery or they may negatively affect the process of adaptation. NOD2 is mainly expressed in Paneth cells in the small intestine and in antigen presenting cells (monocytes, macrophages and dendritic cells). It is a cytosolic pattern recognition receptor for muramyl dipeptide (MDP) and peptidoglykan (PGN), which are components of gram-positive and gram-negative bacteria.[3] The exact physiological role of NOD2 and the pathophysiology resulting from its characteristic mutations are still incompletely understood. At least five different hypotheses are currently debated: 1) NOD2 is part of an inhibitory system, which blocks the activation of NF-kappa-B after stimulation of TLR2. As a consequence, NOD2 mutations lead to an exaggerated immune response.[22] 2) An intact NOD2 signal leads to a polarization of the adaptive immune response towards a Th2 type response, while a defective NOD2 signal leads to an excessive Th1 response.[23] 3) NOD2 mutations lead to a defective production of alpha-defensins in Paneth cells[24] thus favoring a change in the luminal microbiota in the ileum. 4) Mutant NOD2 inhibits the expression of the anti-inflammatory cytokine interleukin 10 (IL-10) by suppressing the activity of heterogenous nuclear ribonucleoprotein A1.[25] 5) NOD2 mutations lead to a defect in the autophagy pathway resulting in impaired bacterial handling and antigen presentation in dendritic cells.[20,21] In humans, NOD2 mutations are also linked to an increased mortality in the setting of sepsis, bone marrow transplantation and spontaneous bacterial peritonitis, suggesting a critical impairment of the intestinal barrier function and the handling of subsequent translocation of bacteria or components of the bacterial wall.[26–28] Thus patients carrying a NOD2 mutation in these cohorts and IF patients without CD in our cohort may share with CD patients a defect in their innate immunity and their intestinalv barrier function with a higher risk for bacterial translocation.

Adaptation after extensive intestinal resection is a multifactorial but incompletely understood process[29] and at present there is no defined role for NOD2 or NOD2 mutations in it. Nevertheless physical barrier function is a prerequisite for vectorial transport.[30] To our knowledge the effect of NOD2 mutations on the physical intestinal barrier function has not been addressed experimentally but some evidence points to a possible role. IL-10 is required for stabilization of the deranged intestinal barrier function in the setting of total parenteral nutrition.[31,32] Furthermore IL-10 production depends on an intact NOD2 function at least under some experimental conditions.[25,33] Thus NOD2 mutations may lead to a reduced IL-10 production and thus to a reduced epithelial barrier function, which in turn short circuits transport and inhibits functional adaptation.

In summary we present direct evidence that NOD2 mutations are associated with the development of SBS/IF after intestinal resection. Furthermore indirect evidence suggests that this is linked to reduced physical and/or immune mediated intestinal barrier function resulting in more extensive resection and/or impaired capacity for intestinal adaptation of nutrient, electrolyte and water absorption. If a much larger and very well characterized cohort would be available to study there might be an approach to relate the clinical course to the NOD2 mutational status and draw conclusions regarding the role of NOD2 in the pathophysiology of SBS/IF. Unfortunately neither such a cohort nor a scientific consortium is available at present. Thus, elucidation of the role of NOD2 mutations in the pathophysiology of SBS/IF will require an animal study.

Ethics

The study protocol was approved by the Ethics Committee of the University of Tübingen (022/2011BO2). All patients gave their informed consent before starting the study.

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Author contribution

N. Schneider, H. Schäffler and J. Rainer gathered the data and analyzed them in the data base. J. Reiner and G. Lamprecht programmed the data base and recruited patients for the study. C-J.

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Hsieh performed the mutational analysis. G. Lamprecht and H. Schäffler designed the study and wrote the manuscript. G. Blumenstock performed the statistical analysis. A. Königsrainer, S. Nadalin and M. Witte performed the transplantations and non-transplantation surgeries and recruited patients for the study.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

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CASE REPORT Open Access

Two patients with intestinal failure requiring home parenteral nutrition, a *NOD2* mutation and tuberculous lymphadenitis

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Abstract

Background: Mutations in the *NOD2* gene are a significant risk factor to acquire intestinal failure requiring home parenteral nutrition. Tuberculous lymphadenitis is the main manifestation of extrapulmonary tuberculosis. Defects in the innate immunity, including *NOD2* mutations, may increase the risk for acquiring infections caused by *M. tuberculosis*. An association of intestinal failure, mutations in the *NOD2* gene and tuberculous lymphadenitis has not been described before.

Case presentation: We report of two patients with intestinal failure secondary to mesenteric ischemia. Both patients presented with fever and weight loss while receiving long term home parenteral nutrition. Both of them were found to have mutations in the *NOD2* gene. Catheter related infections were ruled out. FDG-PET-CT scans initially obtained in search for another infectious focus that would explain the symptoms unexpectedly showed high FDG uptake in mediastinal lymph nodes. Direct or indirect evidence proved or was highly suggestive for tuberculous lymphadenitis. Intravenous tuberculostatic therapy was started and led to a reversal of symptoms and to resolution of the lesions by FDG-PET-CT.

Conclusion: Mutations in the *NOD2* gene may put patients both at an increased risk for acquiring *M. tuberculosis* infections as well as at an increased risk of intestinal failure after extensive intestinal resection. Thus we suggest to specifically include reactivated and opportunistic infections in the differential diagnosis of suspected catheter related infection in patients with intestinal failure who carry mutations in their *NOD2* gene.

Keywords: NOD2, Intestinal failure, Tuberculous lymphadenitis, Catheter related blood stream infection

Background

Tuberculous lymphadenitis is the most frequent site of extrapulmonary tuberculosis. About 20% of all TBC cases in the US are extrapulmonary. From this group, about 40% are tuberculous lymphadentis [1]. Detection of *M. tuberculosis* is mainly via the innate immune system by extracellular or intracellular pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain receptors (NOD) [2-4]. Mutations in specific TLR genes were found to be associated with susceptibility to TBC [5,6].

Clinical relevance of mutations in the *NOD2* gene arises from their association with Crohn's disease [7-10]. In 2001, a link between mutations in the *NOD2* gene and Crohn's disease was first established independently by two different groups [11,12]. However, the exact function of *NOD2* is still under debate [13]. Besides Crohn's disease, other disease entities seem to be related to mutations in the *NOD2* gene like GvHD [14-16], acute septicemia [17], spontaneous bacterial peritonitis in liver cirrhosis [18,19] and worsened outcome after intestinal transplantation [20]. Mutations in the *NOD2* gene and an increased susceptibility for infectious diseases have been reported in the literature [21]. *NOD2* is also thought to be an important receptor in recognizing *M. tuberculosis*, because on the one hand both the receptor and the pathogen are intracellular and on the other hand the cell wall of *M. tuberculosis* contains peptidoglycan, which is one of the ligands of *NOD2* [22]. However, the role of *NOD2* in tuberculous lymphadenitis has not been

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studied yet. To this end a recent study has described a new SNP in the *NOD2* gene as a possible risk factor for pulmonary tuberculosis in the Chinese Han population [23]. In another study it was reported that genes in the *NOD2* signaling pathway are associated with susceptibility to infections with *Mycobacterium leprae* in China [24].

Short bowel syndrome (SBS) and intestinal failure requiring long term home parenteral nutrition (HPN) are rare heterogeneous clinical conditions in which extensive parts of the intestine have been removed surgically. The main causes of short bowel syndrome in adults are Crohn's disease, intestinal ischemia, volvulus, ileus, desmoid tumors and trauma [25]. Recently we have described an increased frequency of *NOD2* mutations in SBS patients without underlying Crohn's disease [26]. Infections associated with intestinal failure requiring home parenteral nutrition are mainly catheter-related [27,28].

Here we describe two individuals with short bowel syndrome, mutations in the *NOD2* gene and tuberculous lymphadenitis. This is a clinically important finding because in a HPN patient intermittent fever, the key symptom of tuberculous lymphadenitis, usually indicates catheter related blood stream infection. To our best knowledge, this is the first case report linking these clinical entities together.

Materials and methods

Genotyping of patients was performed as part of a larger study in a cohort of short bowel patients, which was approved by the Ethics Committee of the University of Tuebingen (022/2011BO2). The patients gave written informed consent. The three major mutations in the *NOD2* gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844 and SNP 12; G908R, NCBI reference SNP ID: rs2066845 and 3020insC, SNP 13; 1007 fs, NCBI reference SNP ID: rs2066847) were detected in genomic DNA extracted from whole blood as described previously [26].

Direct Nucleic Acid Amplification Test (NAAT) to detect *M. tuberculosis* complex DNA was performed using the ProbeTec ET DTB (DTB) (Becton-Dickinson). For identification of *Mycobacterium tuberculosis* complex species GenoType® MTBC (Hain) was used according to manufacturer's instructions. As gold standard culture techniques using 2 solid and BACTEC™ *MGIT*™ 960 liquid broth were applied. For direct susceptibility testing we used the BACTEC™ *MGIT*™ 960. As interferon-gamma-release assay we used the *QuantiFERON*-TB® Gold In-Tube test (Cellestis) according to manufacturer's instructions.

Case presentation

Patient 1

Patient 1 is a 44 year-old Caucasian woman with intestinal failure. In 2008, she required surgical resection of most of her small intestine (except for 70 cm of proximal jejunum) and the right colon resulting in a duodenotransversostomy due to acute occlusion of her superior mesenteric artery. Total parenteral nutrition was initiated at the University of Tübingen intestinal failure outpatient clinic. The initial clinical course was dominated by numerous infectious complications, e.g. recurrent line infections (05/2009, 04/2010, 02/2012), a liver abscess and an episode of acute cholecystitis in the absence of cholelithiasis, which was interpreted as another ischemic episode in the splanchnic circulation (Figure 1).

Despite an extensive workup (including ultrasound and CT-scan) the etiology of the ischemic events could not be determined. The diagnosis of Takayasu Arteriitis was entertained because of diminished/absent peripheral pulses but vasculitis was neither found by histology in the resected specimens nor by PET-CT in the large vessels. Workup for a coagulation disorder revealed a heterozygous prothrombin mutation (G20210A). An antiphospholipid syndrome was ruled out and the JAK2-mutation was also not detected. A HIV test was negative.

Because of the severity of two acute episodes of arterial occlusion the patient was maintained on low dose steroids (Prednisolone 5 mg) and received long term anticoagulation with enoxaparin (Clexane) and later fondaparinux (Arixtra). Under this regimen, the patient did not develop another episode of intestinal or systemic arterial ischemia.

Genetically she was found to be heterozygous for the 1007 fs *NOD2* mutation. In 2010, after two years of successful parenteral nutrition without any infectious complications the patient started to lose weight and had intermittent episodes of fever. The laboratory values showed an increased CRP value (30.5 mg/dl) and anemia (6.7 g/dl). A catheter-related infection was excluded by repeated blood cultures. Another FDG-PET-CT was obtained addressing again the question of a large vessel vasculitis. Unexpectedly it showed for the first time an intensive uptake of the FDG tracer in three lymph nodes in the mediastinum. An interferon-γ-release assay was positive (Quantiferon®). A transbronchial biopsy of one of the lymph nodes revealed necrotic material and a granuloma by histology, highly suggestive for tuberculous lymphadenitis. *M. tuberculosis*-DNA was detected by PCR and cultures obtained from the lymph node also grew *M. tuberculosis*. Intravenous tuberculostatic therapy with Ethambutol, Isoniazid and Rifampicin was given for 4 months followed by Isoniazid and Rifampicin for another 8 months. The follow-up FDG-PET-CT after 7 months of therapy showed a significant size reduction and decreased FDG uptake of the lymph nodes in the mediastinum. The patient stayed on prophylactic therapy with Isoniazid until today, because she continued low dose steroids.

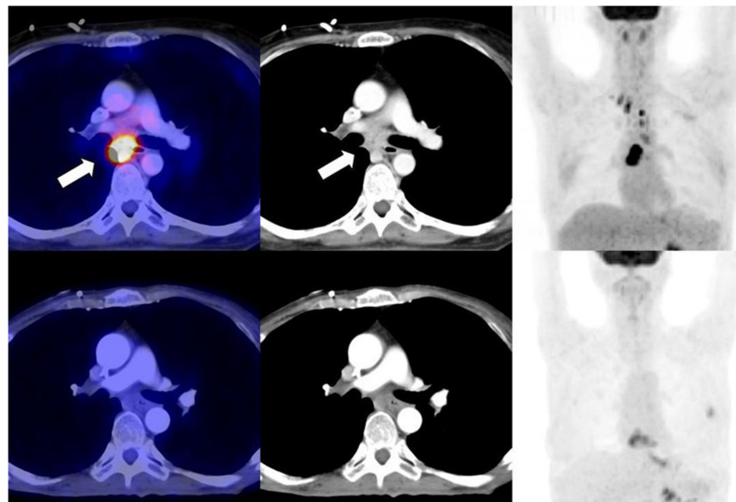

Figure 1 Patient 1. Upper row - before tuberculostatic therapy: High FDG uptake in an infracarinal lymph node (left panel: fusion images of PET/CT) correlating with central necrosis in the contrast enhanced CT (middle panel). Coronal MIP with high FDG uptake in several mediastinal lymph nodes (right panel). Lower row - 8 month follow up: No FDG uptake (left panel: PET/CT) and significant size reduction of the visualized lymph nodes (middle panel: ceCT). No pathological FDG uptake in the mediastinal lymph nodes on coronal MIP of PET (right panel).

Patient 2

Patient 2 is a 77 year-old Caucasian woman who had an infarction of the small intestine and right colon due to atherosclerotic occlusion of the superior mesenteric artery in November 2003. She required extensive resection of her small bowel, which resulted in a jejunotransversostomy with 20 cm of proximal jejunum. Total parenteral nutrition was started and was managed at the University of Tübingen intestinal failure outpatient clinic. In 2010 she developed fever, weight loss and night sweats. An elevated ESR and LDH were found. Repeated attempts to verify catheter related blood stream infection including numerous blood cultures, several rounds of empiric antibiotic therapy and an empiric exchange of the catheter had no sustained effect on these symptoms. A FDG-PET-CT scan was performed addressing a potential infectious focus other than the catheter. Unexpectedly it revealed PET-positive lymphadenopathy in the cervical region and in the mediastinum. An interferon-γ-release assay was negative (Quantiferon®). A bronchoscopic biopsy of the suspicious lymph node revealed granulomatous necrotizing lymphadenitis, but acid fast bacilli could not be stained. A specific pathogen could not be cultured and eubacterial PCR as well as PCR for *M. tuberculosis* were negative. The family history revealed that several relatives had suffered from tuberculosis. Based on the sum of indirect evidence the diagnosis of tuberculous lymphadenitis was made. Tuberculostatic therapy (Isoniazid, Rifampicin, Ethambutol and Levofloxacin) was applied intravenously for 6 months. The patient soon felt better and gained weight. Fever and anemia resolved, and the LDH and the ESR returned to normal values. Regression of the enlarged and hypermetabolic lymph nodes was verified by another PET-CT-scan obtained 7 months after the initiation of specific therapy. One remaining small mediastinal lymph node was regarded as non-specific, (Figure 2).

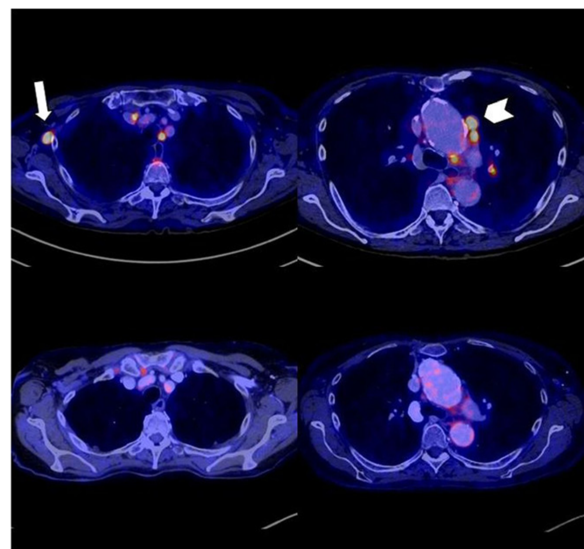

Figure 2 Patient 2. Upper row - before tuberculostatic therapy: high FDG uptake in several mediastinal and one right axillary (arrow) lymph nodes. Additionally, further lymph nodes with high FDG uptake, especially in the left paraaortal (arrow head) region. Lower row - 7 month follow up after initiation of tuberculostatic therapy: No FDG uptake in the right axillary lymph nodes and significant regression of FDG uptake of the paraaortal lymph nodes after therapy. All PET positive lymph nodes had a morphological correlate and showed a significant size reduction after therapy in contrast enhanced CT (not shown).

The patient was found to be heterozygous for the R702W mutation in the *NOD2* gene.

Conclusion

In patients with intestinal failure on HPN the occurrence of low grade fewer, night sweats, declining performance status, low albumin and sometimes increased bilirubin usually prompts the diagnosis of a line related infection. This is because about 30% of line related infections in this cohort do not present with typical symptoms of high grade fever and rigors upon start of a new infusion but rather with those atypical symptoms [28]. In addition line related infections are the most frequent complication in HPN patients occurring with a frequency between two episodes per year and one episode every three years [28]. Nevertheless the diagnosis of line related infection could not be firmly established in either of these patients and empiric therapy was not successful. Instead tuberculous lymphadenitis was diagnosed and successfully treated. Predisposition for acquiring or reactivating tuberculosis may have been facilitated by the fact that patients with SBS receiving long term HPN have an impaired immune response [29]. Furthermore patient 1 was receiving steroids over a prolonged period of time system. It must also be noted that patient 2 may have had an atypical mycobacterial infection. On the other hand, tuberculosis, especially tuberculous lymphadenitis, has not been recognized as a specific problem in intestinal failure patients on HPN yet. So how may these conditions be related other than by chance?

Both patients carried a mutation in the NOD2 gene (patient 1: 1007 fs, patient 2: R702W). *NOD2* is an intracellular pattern recognition receptor which recognizes muramyl dipeptide (MDP) as part of peptidoglycan of the bacterial cell wall [30,31]. The cumulative incidence (homozygous, heterozygous and compound heterozygous) of mutations in the 3 major *NOD2* SNPs (R702W, G908R and 1007 fs) is 13.6% in a cohort of healthy controls [7]. Recently we and others have reported an increased frequency of NOD2 mutations in SBS patients [26,32]. At present it is not clear, whether a defect in NOD2 signaling leads to an altered response to operative stress ultimately resulting in a SBS or whether intestinal adaptation to the SBS situation is diminished resulting in long term HPN [26].

M. tuberculosis is mainly recognized via PRR like TLRs and NOD2. Certain mutations in the TLR genes, e.g. TLR1 and TLR6 are associated with an increased risk of acquiring *M. tuberculosis* [5,6]. In one study, host cells after exposure to *M. tuberculosis* were sensing the microbe-associated molecular pattern (MAMP) using independent PRRs like NOD2 and TLR. The study showed, that these receptors were non-redundant and interacted with each other synergistically [33]. In addition monocytes from patients homozygous for the 1007 fs mutation (3020insC) show diminished TNF and IL-10 cytokine response after stimulation with *M. tuberculosis* compared to heterozygous or homozygous wild-type controls. A cohort study in 377 African Americans with tuberculosis found that certain SNPs in the NOD2 gene were associated with either resistance or susceptibility to tuberculosis [34]. Nevertheless NOD2 mutations have not been firmly established as a risk factor for tuberculosis and several studies argue against such a correlation [35,36].

Thus, NOD2 mutations may be the common risk factor for both patients to develop intestinal failure requiring HPN and to acquire or reactivate tuberculosis, in these two cases tuberculous lymphadenitis. From a clinical point of view these two cases highlight the importance to search for alternative infectious complications other than line related blood stream infection in patients with SBS on HPN, including tuberculosis.

Consent

Written informed consent was obtained from both patients for publication of this Case report and any accompanying images. A copy of the written consent from both patients is available for review by the Editor of this journal.

Abbreviations

ESR: Erythrocyte sedimentation rate; FDG-PET: Fluordeoxyglucose positron emission tomography; GvHD: Graft-versus-host-disease; HPN: Home parenteral nutrition; IL: Interleukin; JAK2: Janus kinase 2; LDH: Lactate dehydrogenase; MAMP: Microbe-associated molecular pattern; NAAT: Nucleic Acid Amplification Test; NOD: Nucleotide-binding oligomerization domain receptors; PCR: Polymerase chain reaction; PRR: Pattern recognition receptor; SBS: Short bowel syndrome; SNP: Single nucleotide polymorphisms; TBC: Tuberculosis; TLR: Toll-like receptor; TNF: Tumor necrosis factor.

Competing interests

All authors declare that they have no competing interests.

Authors' contributions

GL and HS gathered the information about the two patients. CH performed the mutational analysis. MT and SF collected the data from the FDG PET CT scans and analyzed them. JSF completed the microbiological part in Material and Methods. GL and HS wrote the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

NOD2- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients

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Author contributions: Schäffler H, Rohde S and Jaster R designed the study; Huth A, Schäffler H and Lamprecht G took responsibility for patient care and follow-up; Rohde M, Rohde S, Hollborn H, Jaster R and Koczan D (microarray studies) performed the experiments; Gittel N, Huth A and Schäffler H collected the samples and performed the clinical characterization of the patients; Glass Ä performed the biostatistics; all authors analyzed the data; and Schäffler H and Jaster R wrote the manuscript.

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Abstract

AIM

To investigate disease-specific gene expression profiles of peripheral blood mononuclear cells (PBMCs) from Crohn's disease (CD) patients in clinical remission.

METHODS
Patients with CD in clinical remission or with very low disease activity according to the Crohn's disease activity index were genotyped regarding nucleotide-binding oligomerization domain 2 (*NOD2*), and PBMCs from wild-type (WT)-*NOD2* patients, patients with homozygous or heterozygous *NOD2* mutations and healthy donors were isolated for further analysis. The cells were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS) for defined periods of time before RNA was isolated and subjected to microarray analysis using Clariom S assays and quantitative real-time PCR. *NOD2*- and disease-specific gene expression profiles were evaluated with repeated measure ANOVA by a general linear model.

RESULTS
Employing microarray assays, a total of 267 genes were identified that were significantly up- or downregulated in PBMCs of WT-*NOD2* patients, compared to healthy donors after challenge with vitamin D and/or a combination of LPS and PGN (P < 0.05; threshold: ≥ 2-fold change). For further analysis by real-time PCR, genes with known impact on inflammation and immunity were selected that fulfilled predefined expression criteria. In a larger cohort of patients and controls, a disease-associated expression pattern, with higher transcript levels in vitamin D-treated PBMCs from patients, was observed for three of these genes, *CLEC5A* (P < 0.030), *lysozyme* (*LYZ*; P < 0.047) and *TREM1* (P < 0.023). Six genes were found to be expressed in a *NOD2*-dependent manner (*CD101*, P < 0.002; *CLEC5A*, P < 0.020; *CXCL5*, P < 0.009; *IL-24*, P < 0.044; *ITGB2*, P < 0.041; *LYZ*, P < 0.042). Interestingly, the highest transcript levels were observed in patients with heterozygous *NOD2* mutations.

CONCLUSION
Our data identify *CLEC5A* and *LYZ* as CD- and *NOD2*-associated genes of PBMCs and encourage further studies on their pathomechanistic roles.

Key words: Peripheral blood mononuclear cells; Gene expression; *NOD2*; *Lysozyme*; Crohn's disease; *CLEC5A*

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Core tip: Peripheral blood mononuclear cells (PBMCs) are a useful tool to study peculiarities of the immune response in the context of Crohn's disease (CD). Here, we investigated whether PBMCs from patients with CD, even at the stage of clinical remission, exhibit altered gene expression profiles after challenge with pathogen-associated molecular patterns and vitamin D. For *TREM1*, *lysozyme* and *CLEC5A*, disease-associated expression patterns, with higher transcript levels in patient-derived PBMCs, were observed. The two latter genes, along with four other transcripts, also showed *NOD2*-dependent expression profiles. *TREM1* and *CLEC5A* may act with *NOD2* in a regulatory network with a pathophysiological role in CD.

Schäffler H, Rohde M, Rohde S, Huth A, Gittel N, Hollborn H, Koczan D, Glass Ä, Lamprecht G, Jaster R. *NOD2*- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients. *World J Gastroenterol* 2018; 24(11): 1196-1205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1196.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1196

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic intestinal disorders and mainly consist of the two entities Crohn's disease (CD) and ulcerative colitis (UC)[1,2]. The clinical course of IBD is characterized by intermittent periods of relapses and remission, which are unpredictable in clinical practice. The pathogenesis of IBD is multifactorial, including genetic and environmental factors, and involves an inappropriate activation of the mucosal immune system, which is triggered by the intestinal microbiota in genetically predisposed individuals[1-5]. In Caucasian populations, nucleotide-binding oligomerization domain 2 (*NOD2*) has emerged as one of the main susceptibility genes for CD[6-8]. NOD2 is an intracellular pattern recognition receptor sensing muramyl dipeptide (MDP)[9,10], a fragment of peptidoglycan (PGN), but also PGN by itself[11,12] and, upon ligand binding, induces activation of the transcription factor NF-κB[13]. However, NOD2 activation via PGN is dependent on a TLR2 co-stimulatory signal[14].

In addition, the environment, *e.g.*, vitamin D deficiency, also affects the development and clinical course of IBD[15-17]. Vitamin D deficiency has a high prevalence in IBD patients[17,18]. We have recently shown that clinical factors, *e.g.*, the use of tumor necrosis factor (TNF)-α inhibitor, are associated with significant changes in vitamin D levels[19]. Vitamin D was originally mainly implicated in bone health, regulating calcium and phosphate metabolism[20,21], but recent evidence has shown that vitamin D also profoundly impacts the innate and adaptive immune system[22-24]. Underscoring its role in the pathogenesis of CD, vitamin D was shown to be an inductor of *NOD2* gene expression[25]. Using peripheral blood mononuclear cells (PBMCs) and dendritic cells, Dionne *et al*[26] showed that 1, 25-vitamin D acts as a modulator of the innate immune system. However, little is known about the effects of vitamin D and the presence of *NOD2* mutations on different gene expression levels in CD. The aim of our study was therefore to further characterize different gene expression profiles in CD patients and healthy controls correlating to *NOD2* mutation status and vitamin D pretreatment. We identified different genes associated with the presence of CD and mutations in the *NOD2*

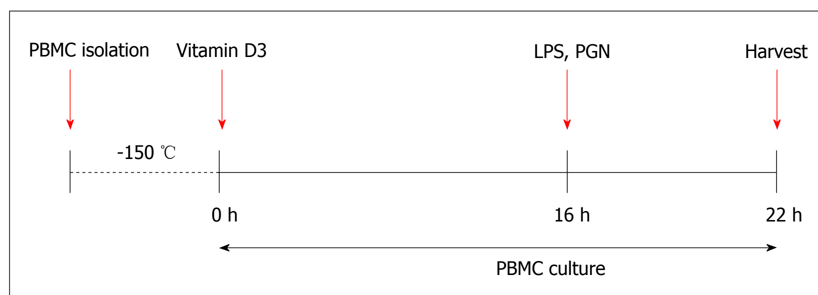

Figure 1 Protocol of peripheral blood mononuclear cells treatment.

gene. Follow-up studies on these genes may provide novel insights into the pathogenesis of CD and could contribute to the establishment of biomarkers to better predict the clinical course of the disease.

MATERIALS AND METHODS

Patients and controls

Sixteen patients with CD were recruited from the Rostock University Medical Center. The disease activity was determined via the Crohn's disease activity index (CDAI)[27]. Furthermore, all patients were classified according to the Montreal classification[28], and age, gender and disease-specific medication were recorded. Six healthy volunteers without immune-mediated gastrointestinal or other autoimmune disorders served as controls. EDTA blood samples were drawn from all participants for genotyping studies and isolation of PBMCs. Plasma levels of vitamin D and C-reactive protein (CRP) were determined using routine laboratory methods.

The study was approved by the ethics board of the University of Rostock (A-2015-0042). Written informed consent was obtained from each participant prior to enrollment.

Isolation, culture and treatment of PBMCs

PBMCs were isolated from EDTA venous blood using density-gradient centrifugation over Pancoll (PAN-Biotech, Aidenbach, Germany). Immediately after isolation, PBMCs were resuspended in cryopreservation medium [fetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO)] and stored at -150 ℃ until required. After thawing, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (all reagents from Biochrom/Merck, Berlin, Germany), and exposed to 1α,25-dihydroxyvitamin D3 (Santa Cruz Biotechnology, Dallas, TX, United States) at 40 nmol/L as indicated. After an incubation period of 20 h at 37 ℃ in a 5% CO_2 humidified atmosphere, lipopolysaccharide (LPS; 1 μg/mL; Sigma-Aldrich, Deisenhofen, Germany) and peptidoglycan (PGN; 10 μg/mL; Sigma-Aldrich) were added to the cells as indicated, and incubation continued for another 6 h (Figure 1). Subsequently, the cells were lysed in RTL Plus buffer, which was included in the RNeasy Plus Kit (Qiagen, Hilden, Germany), and subjected to RNA isolation (see below).

NOD2 genotyping

DNA was isolated from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. All patients and controls were genotyped with respect to the three major mutations in the *NOD2* gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844, SNP 12; G908R, NCBI reference SNP ID: rs2066845 and SNP 13; 1007fs, NCBI reference SNP ID: rs2066847). The corresponding regions of the *NOD2* gene were amplified by PCR using a Taq PCR Master Mix Kit (Qiagen) and primers as specified in Table 1. The following PCR conditions were used: 5 min, 94 ℃; 1 min, 94/60/72 ℃ (45 cycles); 7 min, 72 ℃; 4 ℃. After Sanger sequencing (Seqlab, Göttingen, Germany), the data were analyzed using the software Chromas, version 2.6. Individuals with no SNP mutations were considered wild-type (WT) for *NOD2*.

Microarray analysis of RNA expression profiles

RNA was extracted employing an RNeasy Plus Kit according to the manufacturer's protocol. Total RNA samples were quantified with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, United States), and their integrity was confirmed using the Agilent Bioanalyzer 2100 with an RNA Nano chip kit (both from Agilent Technologies, Waldbronn, Germany).

Expression profiling was performed using 200 ng RNA and the Affymetrix Human Clariom S Assay (Affymetrix/Thermo Fisher Scientific), which interrogates over 20000 well-annotated genes. Therefore, the so-called Whole Transcriptome protocol was employed. T7 promoter tags were introduced into all RNA molecules by using N6 3'-ends for DNA strand synthesis, before RNA strand replacement according to Eberwine[29] was conducted. Non-labeled aRNA was produced by *in vitro* transcription. All RNA molecules were amplified in a linear manner, avoiding a 3' bias. Using purified aRNA as a template, a new strand-identical single-strand DNA was produced by adding random primers and dNTPs (including dUTP, which replaced a limited

Table 1 Primer for *NOD2*-genotyping

SNP	Primer
8	Forward: 5′CCTCTTCAATTGTGGCAGGC-3′ Reverse: 5′-CTCCTGCATCTCGTACAGGC-3′
12	Forward: 5′-ATGGAGGCAGGTCCACTTTG-3′ Reverse: 5′-TTACCTGAGCCACCTCAAGC-3′
13	Forward: 5′-GATGGTACTGAGCCTTTGTTGA-3′ Reverse: 5′-CAGACTTCCAGGATGGTGTCAT -3′

amount of dTTP). After digestion with RNase H, endpoint fragmentation was performed with uracil-DNA-glycosylase in combination with apurinic/apyrimidinic endonuclease 1, and biotinylated dNTPs were added to the 3′-ends of the single-stranded DNA fragments with deoxynucleotidyl transferase. Subsequently, hybridization of the microarrays was performed at 45 ℃ in a GeneChip® Hybridization Oven 645 (Affymetrix/Thermo Fisher Scientific). After overnight incubation, the microarrays were scanned using the GeneChip Scanner 3000 (Affymetrix/Thermo Fisher Scientific) at 0.7 μm resolution.

Primary data analysis was performed with the Affymetrix Transcriptome Analysis Console software version 3.1.0.5 including the Robust Multiarray Average module for normalization. Gene expression data were log-transformed. A change was considered significant when the ANOVA *P*-value met the criterion $P < 0.05$ at fold changes >|2|, *i.e.*, expression increments or declines larger than two. Along with the publication of the manuscript, our complete microarray data will be available in the Gene Expression Omnibus database (GEO accession number: GSE110186).

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Unless indicated otherwise, reagents from Thermo Fisher Scientific were used in all subsequent steps. Cellular RNA prepared as described above was treated with a DNA-free kit to remove traces of genomic DNA, and 250 ng of RNA per sample was reverse transcribed into cDNA using TaqMan™ Reverse Transcription Reagents and random priming. Using a ViiA 7 sequence detection system (Thermo Fisher Scientific), target cDNA levels were quantified by real-time PCR. Therefore, qPCR MasterMix (Eurogentec, Seraing, Liège, Belgium) and the following human-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were used: Hs00355476_m1 (*CCL20*), Hs00188627_m1 (*CD101*), Hs00370621_m1 (*CLEC12A*), Hs04398399_m1 (*CLEC5A*), Hs01902549_s1 (*CLEC7A*), Hs01099660_g1 (*CXCL5*), Hs01114274_m1 (*IL24*), Hs00167304_m1 (*ITGAM*), Hs00164957_m1 (*ITGB2*), Hs00426232_m1 (*LYZ*), Hs00234007_m1 (*MSR1*), Hs01065279_m1 (*PECAM1*), Hs00218624_m1 (*TREM1*), and Hs99999905_m1 (*GAPDH*). PCR conditions were as follows: 95 ℃ for 10 min, followed by 40 cycles of 15 s at 95 ℃/ 1 min at 60 ℃. Relative amounts of target mRNA in PBMCs were expressed as $2^{-(\Delta Ct)}$ values.

Statistical analysis

Real-time PCR data were analyzed with repeated-measures ANOVA. Mean group differences were compared for "disease" (patients with CD *vs* controls) and "*NOD2*-status" (WT, heterozygote, homozygote), as well as for (within-subject factors) "vitamin D application" (yes *vs* no) and "stimulation" (LPS, PGN, LPS + PGN, controls), employing a *general linear model* for repeated measurements. Age was considered a covariate in the disease model because disease groups were not balanced by age, and *NOD2* groups were tested post hoc by LSD. Normal distribution of measurements was assessed using the Kolmogorov-Smirnov test. $P < 0.05$ was considered statistically significant. All data were processed using IBM® SPSS® Advanced Statistics 22.0.

RESULTS

PBMCs provide an easily accessible tool to investigate disease-associated peculiarities of the antipathogenic immune response of patients with CD. To study transcripts in an unbiased manner, we initially chose a microarray approach. Therefore, PBMCs from healthy individuals and patients with CD in remission (n = 3 each; all of them *NOD2*-WT) were pretreated with vitamin D3 for 20 h before they were challenged simultaneously with LPS and PGN for 6 h. Subsequently, global gene expression was analyzed employing Clariom S assays, and data were compared with those of untreated controls. Table 2 gives an overview of the significant differences between patients with CD and controls under identical conditions of PBMC treatment.

Under basal conditions and any treatment regimen, genes upregulated in patients with CD exceeded downregulated genes both in number and maximum change. Complete lists of the 267 genes are presented as Supplementary Table 1.

Many of the differentially expressed genes are well-known modulators of immune cell functions in the context of innate and adaptive immunity, and unsurprisingly, some of them have previously been implicated in the pathogenesis of CD, including several immune cell receptors, cytokines/chemokines and their cognate receptors and the antimicrobial peptide lysozyme[30-36]. The latter transcript was found to be upregulated in PBMCs of patients with CD in response to LPS/PGN treatment, independent of the presence or absence of vitamin D3. Intriguingly, expression of various genes was synchronously up- or downregulated under different conditions, suggesting a robustness of the expression profile against external perturbations.

For in-depth analysis, we selected a panel of 11 genes from the list of candidates shown in Table 2 that fulfilled the following criteria: (1) differential expression in PBMCs from patients with CD and controls under basal conditions and/or under at least two

Table 2 Numbers and maximum changes of up- and downregulated genes in peripheral blood mononuclear cells from Crohn's disease patient *vs* identically treated controls

Treatment of PBMCs	Upregulated genes	Downregulated genes
Untreated	85 (59-fold)	39 (39-fold)
Vitamin D3	25 (21-fold)	12 (9-fold)
LPS/PGN	54 (6-fold)	15 (5-fold)
Vitamin D3 + LPS/PGN	29 (15-fold)	8 (5-fold)

$P < 0.05$; Threshold: ⩾ 2-fold change. PBMCs: Peripheral blood mononuclear cells; LPS: Lipopolysaccharide; PGN: Peptidoglycan.

Table 3 Genes selected for real-time PCR studies

Transcript	Fold changes: patients *vs* controls				Details on function/ reasons to study
	Basal	+D, -L/P	-D, +L/P	+D, +L/P	
MSR1	9.56	13.19		14.72	macrophage scavenger receptor[49]; differentially expressed in 3 of 4 groups
CD101	2.66				expressed on various immune cells; inhibits expansion of colitogenic T cells[30]
CLEC5A		2.82		3.11	C-type lectin member 5A, pattern recognition receptor; involved in antibacterial/ antiviral defense[43]
CLEC7A	4.53			3.94	C-type lectin member 7A, pattern recognition receptor; control of fungal infections[50]
CLEC12A	6.04	4.52			C-type lectin member 12A, pattern recognition receptor, inhibits cell death-induced inflammation[51]
ITGAM	2.18				CD11b; integrin αM; expressed by many immune cells; polymorphisms linked to autoimmunity[52]
LYZ			3.51	2.03	antimicrobial enzyme; essential role in innate immunity; increased production linked to CD[31]
PECAM1	3.15				CD31; implicated in transendothelial leukocyte migration in experimental colitis[32]
CCL20	-2.33		-5.08		chemokine expressed by neutrophils, enterocytes, B-cells and dendritic cell; IBD predilection gene[33]
CXCL5	-38.89		-5.32		regulates neutrophil homeostasis and chemotaxis; increased serum levels in IBD patients reported[34]
IL-24			-3.49	-4.82	Involved in host defence against bacteria and fungi; increased expression in patients with active IBD[35]
TREM1					amplifier of antimicrobial immune responses and inflammation in experimental colitis and IBD[36]

$P < 0.05$; Threshold: ⩾ 2-fold change. Positive values refer to genes upregulated and negative values to genes downregulated in CD patients. LPS: Lipopolysaccharide; PGN: Peptidoglycan; PBMCs: Peripheral blood mononuclear cells. CD: Crohn's disease; IBD: Inflammatory bowel diseases.

treatment regimens (vitamin D3, LPS+PGN and their combination, respectively) and (2) an established or potential role in inflammation and/or regulation of the immune response. Table 3 shows details regarding all selected genes as well as a twelfth gene, *TREM1*, that was included as a control as an established vitamin D-responsive gene with immunomodulatory function[37]. Interestingly, three pro-inflammatory mediators, *CCL20*, *CXCL5* and *IL-24*, displayed lower expression levels in patients with CD, which might be a consequence of their disease-specific medication (see below).

The expression profiles of the selected genes were subsequently studied by real-time PCR. In addition to WT-*NOD2* patients and healthy controls (n = 6 each, including the samples previously analyzed by microarray technology), we also included patients with heterozygous and homozygous mutations of *NOD2* (n = 5 each). Furthermore, we refined the protocol of PBMC treatment using LPS and PGN both in combination and as individual factors (Supplementary Table 2).

The clinical characteristics, laboratory findings and the medication of all 16 patients are shown in Table 4. Except for one person with a slightly increased CDAI of 166, all patients presented with a CDAI of < 150, indicating disease remission[27]. The CRP-values of 13 patients were in the normal range (below 5 mg/L). In the remaining three patients, modestly elevated CRP-values (all below 14 mg/L) were detected. Disease activity in all patients could still be considered low. All but two patients presented with vitamin D levels below 75 nmol/L, suggesting an insufficiency or even deficiency (levels below 50 nmol/L). This finding was not unexpected, as all of the samples were collected during the European winter season. As a consequence, a vitamin D substitution therapy was initiated, if appropriate. Three of the patients were on steroids (> 10 mg prednisolone/d) at the time of the study, 7 received azathioprine, and 12 were treated with anti-TFN-α antibodies. Healthy controls consisted of 3 males and 3 females with an age range from 25 to 53 years.

For statistical data analysis, a general linear model repeated measure was chosen to assess mean differ-

Table 4 Characteristics of the patients -nucleotide-binding oligomerization domain 2 status, classification and activity of the disease, C-reactive protein and vitamin D levels, and medication at the time of the study

No.	Sex	Age	NOD2	Montreal classification	CDAI	CRP (mg/L)	Vitamin D (nmol/L)	Prednisolon (> 10 mg/d)	Azathio-prine	Anti-TNF-α
1	M	24	WT[1]	A2 L3 L4 B1	121	2.58	104.0	Yes	No	Yes
2	M	28	WT[1]	A2 L3 B3p	46	< 1.0	62.9	No	No	Yes
3	M	64	WT[1]	A3 L3 B2p	111	4.58	27.0	Yes	No	Yes
4	F	60	WT	A2 L3 B2	100	2.27	72.5	No	No	Yes
5	F	62	WT	A3 L3 B3p	82	< 1.0	58.2	Yes	Yes	Yes
6	M	46	WT	A2 L3 L4 B2p	110	4.97	32.9	No	No	Yes
7	M	38	HO	A2 L3 B3p	92	13.30	40.2	No	Yes	No
8	M	64	HO	A3 L3 B1	34	< 1.0	53.0	No	No	Yes
9	F	48	HO[2]	A2 L3 B2	54	< 1.0	67.6	No	No	Yes
10	M	54	HO	A2 L3 B2p	103	1.63	51.9	No	Yes	No
11	M	26	HO	A1 L3 B2p	120	7.86	31.0	No	Yes	Yes
12	M	27	HT	A2 L3 B1	106	1.41	47.2	No	No	Yes
13	M	37	HT	A2 L3 B2p	166	4.56	37.9	No	Yes	No
14	F	48	HT	A2 L1 B3p	115	1.01	55.2	No	Yes	Yes
15	M	57	HT	A1 L3 B2p	132	12.30	52.3	No	No	Yes
16	M	47	HT	A2 L3 B3	60	2.62	92.0	No	Yes	No

[1]Patients who were also included into microarray analysis; [2]SNP8 mutation. All other HO/HT mutations refer to SNP13. NOD2: Nucleotide-binding oligomerization domain 2; CDAI: Crohn's disease activity index; CRP: C-reactive protein; TNF : Tumor necrosis factor; WT: Wild-type; HO: Homozygous mutation; HT: Heterozygous mutation.

Table 5 differentially expressed transcripts in vitamin D-pretreated peripheral blood mononuclear cells from Crohn's disease patients and controls (with and without additional stimulation with lipopolysaccharide and peptidoglycan, respectively, age adjusted)

Gene	*P* value	Upregulated in
CLEC5A	0.030	CD patients
LYZ	0.047	CD patients
TREM1	0.023	CD patients

CD: Crohn's disease.

ences between groups. Comparing controls to CD patients, no significant differences were detected when samples with and without vitamin D incubation were considered conjointly, due to the strong overlaying effect of vitamin D. However, focusing on the subgroup of vitamin D treated samples, disease-related differences were observed (no. 5-8 in Supplementary Table 2). Here, with age adjustment, three genes displayed a disease-dependent expression pattern (Table 5): consistent with the microarray data, significantly higher *CLEC5A* and *LYZ* transcript levels were observed in PBMCs of patients with CD. The same finding was observed for *TREM1*, which was included due to its vitamin D-dependent expression pattern but not because of the microarray results. Together, these findings suggest that CD-associated changes in gene expression could be attributed to treatment protocols that include vitamin D, as analyses in the remaining subgroup (w/o vitamin D) did not show any differences (all $P > 0.20$).

We also analyzed the influence of *NOD2* mutations on the expression of the gene panel described above. Considering all treated and untreated samples (Supplementary Table 2), a significant effect of the *NOD2* status was observed for 5 of these genes, including *CLEC5A* and *LYZ*, and one additional gene from Table 2, integrin subunit beta 2 (*ITGB2*) (Table 6). With a *P*-value of 0.053, *TREM1* just missed statistical significance.

Unexpectedly, heterozygous CD patients displayed the highest expression levels for all of the genes, whereas no statistically significant differences between persons with WT-*NOD2* (patients and controls) and homozygous *NOD2* mutations were detected. The phenomenon is apparently unrelated to medication, which was very similar in the groups of patients with heterozygous and homozygous *NOD2* mutations (Table 4). This conclusion is also supported by statistical evaluations, which did not show any significant association between treatment with prednisolone, azathioprine or anti-TNF-α and expression of *CLEC5A*, *LYZ* and *TREM1* in PBMCs of patients with CD.

DISCUSSION

Many studies have shown that numerous risk genes of CD code for molecules involved in host defense against pathogens, such as nucleotide-binding oligomerization domain 2 (*NOD2*), *ATG16L1*, and those implicated in the T helper type 17 (Th17) pathway[38-43]. Here, we tested the hypothesis that PBMCs of patients with CD, even at the stage of clinical remission, exhibit an

Table 6 Transcripts with a *NOD2*-dependent expression pattern

Gene	*P* value	Highest levels
CD101	0.002	Heterozygotes
CLEC5A	0.020	Heterozygotes
CXCL5	0.009	Heterozygotes
IL-24	0.044	Heterozygotes
ITGB2	0.041	Heterozygotes
LYZ	0.042	Heterozygotes

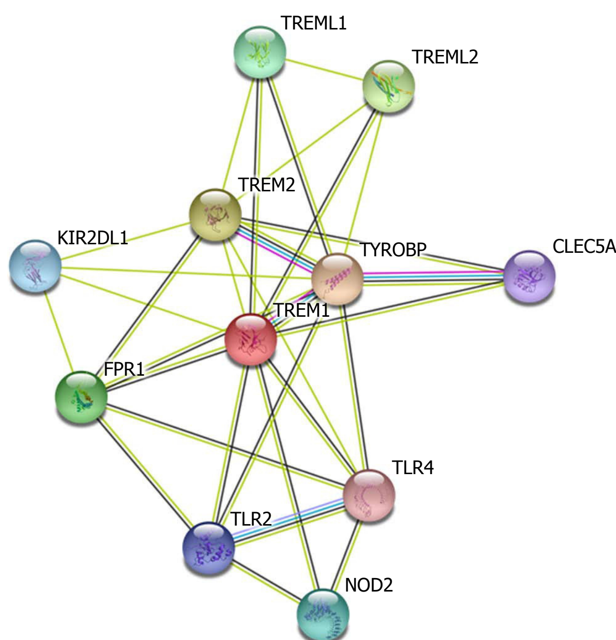

Figure 2 Network analysis using the STRING database[48]. The network was derived employing human TREM1 as the search term (https://string-db.org/cgi/network.pl?taskId=PmXpOD7RMwaM).

altered gene expression profile upon challenge with pathogen-associated molecular patterns (PAMPs) and/or the immunomodulatory hormone vitamin D, which has previously been shown to exert differential effects on the expression of NOD2- and TLR-induced cytokines in the context of CD[26].

Initial microarray experiments identified more than 200 genes with different expression patterns among patients with CD and controls. Based on predefined expression criteria, genes with roles in inflammation and immunity were selected for in-depth analysis by real-time PCR. A disease-associated expression pattern was identified for *CLEC5A*, *lysozyme* and *TREM1*. Six genes, including *CLEC5A* and *lysozyme*, displayed a *NOD2*-dependent expression pattern. With respect to lysozyme and TREM1, our findings are consistent with previous reports, which found that increased levels of both proteins in serum were implicated in the pathophysiology of IBD[44,45]. To the best of our knowledge, however, this is the first report of an association between *CLEC5A* expression and CD. CLEC5A has most recently been identified as an important receptor in innate immunity by neutrophil trap formation and secretion of different proinflammatory cytokines after stimulation with *Listeria monocytogenes*[43]. This finding is especially interesting, as defective bacterial clearance was shown to play a crucial role in the pathogenesis of CD[46,47]. Of note, both CLEC5A and TREM1 proteins can be linked to the product of the best-established CD risk gene, *NOD2*, by the STRING database[48] (Figure 2).

In conclusion, we found that PBMCs of patients with CD display alterations in their response to vitamin D and PAMPs. Disease-associated and *NOD2*-dependent gene expression profiles are preserved even at the stage of clinical remission. Our data identify *CLEC5A*, *LYZ* and *TREM1* as genes of particular interest for follow-up studies. We hypothesize that these genes may act in a common network relevant to CD pathogenesis. Establishment of biomarkers to better predict the clinical course of the disease remains a long-term goal of our studies.

ARTICLE HIGHLIGHTS

Research background

In Crohn's disease (CD), the interplay of genetic and environmental factors converges at the level of an altered antipathogenic immune response, which is incompletely understood. Peripheral blood mononuclear cells (PBMCs) provide a useful tool to study elements of the immunopathogenesis of the disease *in vitro*.

Research motivation

Currently, there is a lack of biomarkers to predict the clinical course of CD. Furthermore, the development of specific therapies would benefit from an improved mechanistic understanding of the pathogenesis of the disease.

Research objectives

The aim of this study was to identify disease-specific gene expression profiles of PBMCs from patients with CD in clinical remission. Specifically, we were interested in alterations of the gene expression profile after challenging PBMCs with pathogen-associated molecular patterns (PAMPs) and the immunomodulatory hormone vitamin D.

Research methods

PBMCs from patients with CD and healthy donors were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS), before RNA was isolated and subjected to microarray analysis and quantitative real-time PCR. Disease-specific gene expression profiles were evaluated by *general linear model repeated measure* analysis, paying particular attention to the well-established CD risk gene *NOD2*.

Research results

Microarray experiments yielded a total of 267 genes that were significantly up- or downregulated in PBMCs of patients with CD, compared to healthy donors, after challenge with vitamin D and/or a combination of LPS and PGN. For further analysis by real-time PCR, genes with roles in inflammation and immunity were selected. For three of these genes, *CLEC5A*, *lysozyme* and *TREM1*, a disease-associated expression pattern was validated. Six genes, including *CLEC5A* and *lysozyme*, were found to be expressed in a *NOD2*-dependent manner.

Research conclusions

PBMCs of patients with CD display alterations of their response to vitamin D and PAMPs that are preserved even at the stage of clinical remission. *CLEC5A*, *TREM1* and *NOD2* may act in a common network relevant to CD pathogenesis.

Research perspectives

Follow-up studies on alterations of the antipathogenic immune response may provide novel insights into the pathogenesis of CD and may also help to establish biomarkers to better predict the clinical course of the disease.

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