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Epidemiologische Untersuchung von Asthma bronchiale im Kindesalter

- protektive Umweltfaktoren und Einschätzung des Schweregrads nach frühkindlichen Symptomen -

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Tabea Brick
aus
Altötting

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Mit Genehmigung der Medizinischen Fakultät
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Tag der mündlichen Prüfung:	19. Januar 2021

1. Eidesstattliche Versicherung

Brick, Tabea

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema „Epidemiologische Untersuchung von Asthma bronchiale im Kindesalter - Protektive Umweltfaktoren und Einschätzung des Schweregrads nach frühkindlichen Symptomen“ selbstständig verfasst, mich außer den angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 26.04.2021	Tabea Brick
Ort, Datum	Unterschrift DoktorandIn

2. Inhaltsverzeichnis

3. Abkürzungsverzeichnis

Et al.	Und andere
z. B.	Zum Beispiel
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analysis
OR	Odds Ratio
LJ	Lebensjahr
CI	Konfidenz-Intervall
PARSIFAL study	Prevention of Allergy Risk factors for Sensitisation in children related to Farming and Anthroposophic Lifestyle study
ALEX study	Protection against the developement of atopy: relevant factors from farming environments study
GABRIEL study	A multidisciplinary study to identify genetic and environmental causes of asthma in the European Community
PASTURE study	Protection against allergy: Study in rural environments
MARTHA study	Milk against respiratory tract infections and asthma study
UHT	Ultra hoch erhitzt
hsCRP Werte	High-sensitive C-Reactive Proteins = Entzündungsmarker im Blut
ISAAC study	International study of asthma and allergies in childhood
FEV1	Forciertes expiratorisches Volumen in 1 Sekunde
CIE	Change-in-Estimate = Änderung des β-Schätzers
LTB5	Leukotriene B5
PGE3	Prostaglandin E3
ESL	Extended-shelf-life
LFQ	Label-free-quantification
SNP	Single Nucleotide Polymorphism
FVC	Forcierte Vitalkapazität
INF-Y	Interferron-Y
HR	Hazard-ratio
LPS	Lipopolysaccharide
miRNA	Micro RNA

4. Publikationsliste

1. **Tabea Brick**, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol. 2016 Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042. Epub 2016 Jan 12.*

2. **Brick T**, Ege M, Boeren S, Böck A, von Mutius E, Vervoort J, Hettinga K. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. *Nutrients. 2017 Aug 31;9(9). pii: E963. doi: 10.3390/nu9090963.*

3. **Brick T**, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTURE study group. Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes. *Pediatr Allergy Immunol. 2019 Aug 23. doi: 10.1111/pai.13118.*

4. **Tabea Brick**, Kasper Hettinga, Benedikt Kirchner, Michael W. Pfaffl, Markus Johannes Ege. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial. *The Journal of Allergy and Clinical Immunology: In Practice. Volume 0, Issue 0. 2019. doi: https://doi.org/10.1016/j.jaip.2019.11.017*

5. Schmidt F, Hose AJ, Mueller-Rompa S, **Brick T**, Hämäläinen AM, Peet A, Tillmann V, Niemelä O, Siljander H, Knip M, Weber J, von Mutius E, Ege MJ; DIABIMMUNE Study Group. Development of atopic sensitization in Finnish and Estonian children: A latent class analysis in a multicenter cohort. *J Allergy Clin Immunol. 2019 May;143(5):1904-1913.e9. doi: 10.1016/j.jaci.2018.12.1014. Epub 2019 Jan 23.*

5. Einleitung

5.1 Asthma bronchiale

Asthma ist eine chronische entzündliche Erkrankung der Atemwege, die sich durch eine bronchiale Hyperreagibilität und variable Atemwegsobstruktion auszeichnet [1, 2]. Akute Exazerbationen zeichnen sich durch Symptome wie pfeifende, keuchende Geräusche beim Atmen, Husten, ein Engegefühl in der Brust, Kurzatmigkeit bis hin zu Atemnot aus, die zu Angst und Panikattacken und im schlimmsten Fall zum Atemstillstand führen können [1]. Zwischen den Anfallsphasen gibt es in der Regel symptomärmere oder symptomfreie Phasen [2].

Asthma bronchiale gehört mit einer Prävalenz von über 5% zu den häufigsten chronischen Erkrankungen weltweit, wobei die Prävalenzen abhängig von Faktoren wie Herkunftsland, sozioökonomischem Status und Altersgruppe sind. Im Kindesalter ist Asthma mit über 10% sogar die häufigste chronische Erkrankung. Bei einem Großteil der asthmatischen Kinder lassen sich die Symptome medikamentös gut behandeln. Bei etwa 5 % der Betroffenen kommt es jedoch trotz Einnahme hoher Dosen oraler oder inhalativer Kortikosteroide zu starken, kaum kontrollierbaren Exazerbationen [3].

Erscheinungsformen von Asthma bronchiale sind sehr heterogen. Definitionen verschiedener Phänotypen zeichnen sich durch den Zeitpunkt des erstmaligen Auftretens, den Krankheitsverlauf, genetische Prädisposition, Schweregrad und Häufigkeit der Exazerbationen, Vorhandensein einer zusätzlichen Sensibilisierung gegen unterschiedlichste Allergene oder krankheitsfördernde Umweltfaktoren aus. Diese Verschiedenartigkeit der Manifestationen von Asthma erschweren die Diagnose und damit die adäquate Behandlung erheblich. Die Pathogenese von Asthma ist trotz intensiver Forschung bis heute nicht vollständig verstanden [4].

Innerhalb der letzten 50 Jahre ließ sich ein Anstieg der Asthma Prävalenz in westlichen Industrienationen beobachten, wobei die Asthmaprävalenz seit einigen Jahren vermutlich ein Plateau erreicht [5, 6] hat. Einen möglichen Erklärungsansatz für diesen Prävalenzanstieg innerhalb der letzten Jahrzehnte bietet die Hygienehypothese, die auf Beobachtungen des britischen Epidemiologen David Strachan zurückgeht [7, 8].

5.2 Die Hygienehypothese

Strachan et al. untersuchten im Rahmen der National Child Developement Study 17.414 britische Kinder, die 1958 geboren wurden von Geburt bis zum Alter von 23 Jahren. Dabei entdeckten sie zwei parallel ablaufende Entwicklungen; die Abnahme von

Infektionskrankheiten im Kindesalter und eine Zunahme von allergischen Erkrankungen wie Heuschnupfen und Ekzemen. Mit Beginn der Industrialisierung änderten sich neben Arbeitsbedingungen, Wohnverhältnissen, Hygienestandards und der Ernährung auch die Familienstrukturen; von der Mehrgenerationen-Großfamilie, die gemeinsam in einem Haus lebte oder einen Bauernhof bewirtschaftete, hin zur kleinen Kernfamilie, die unabhängig von der Großelterngeneration lebt. Zusätzlich nahm die Zahl der Kinder pro Familie deutlich ab. In Deutschland ist die Geburtenziffer pro Frau beispielsweise von 2.5 Kinder in den 1960er Jahren auf heute 1.5 Kinder (Stand 2018) zurückgegangen [9]. Europaweit ist ein vergleichbarer Trend zu beobachten [10].
Durch diese Veränderungen des direkten Lebensumfelds kommen Kinder innerhalb der ersten Lebensjahre weniger in Kontakt mit Bakterien, Viren und Parasiten, was zu einer Abnahme der Häufigkeit von Infektionserkrankungen im Kindesalter führt. Strachan et al. vermuteten genau diese Abnahme von Infektionserkrankungen in der frühen Kindheit als ursächlich für den Anstieg allergischer Erkrankungen in westlichen Industrienationen. Zahlreiche weitere Studien hierzu unterstützen die Hypothese, dass die Konfrontation des Immunsystems mit potentiellen Pathogenen protektiv für eine spätere Entwicklung von Asthma und allergischen Erkrankungen ist [11-13]. Dieses Phänomen ist heute noch beobachtbar beispielsweise bei Kindern, die in weniger industrialisierten Ländern leben, oder Kindern, die in Großfamilien aufwachsen, wie es bei Bauernfamilien üblich ist [14].

5.3 Der Bauernhofeffekt

Weitere Forschung basierend auf den Ideen der Hygienehypothese erbrachte einen Prävalenzunterschied von Asthma und allergischen Erkrankungen zwischen Kindern die auf traditionell bewirtschafteten Bauernhöfen aufwuchsen und Nichtbauern-Kindern aus den gleichen ländlichen Regionen [15-17]. Dieser sogenannten „Bauernhofeffekt“ zeigte, dass Bauernkinder seltener an Asthma und Allergien leiden. Ursprünglich in Zentral-Europa beobachtet, wurden diese Ergebnisse durch Studien weltweit gestützt [18-21]. Zwei Asthma- und Allergie-protektive Faktoren des Aufwachsens auf dem Bauernhof wurden mittlerweile identifiziert: der regelmäßige Kontakt zu Tierstall und/oder Futterscheune (Heustall) und der regelmäßige Konsum von Milch direkt von einem Bauernhof [7, 17, 22, 23] . Kinder, die auf einem Bauernhof leben, kommen bereits in utero mittelbar in Kontakt mit Ställen, Tieren und Futtermitteln, wenn die Mütter beispielsweise während der Schwangerschaft im Stall arbeiten. Auch der Konsum unbehandelter Kuhmilch ist auf Bauernhöfen bereits ab der frühen Kindheit üblich [24]. Es wird daher vermutet, dass bei Nichtbauern-Kindern das Immunsystem durch eine geringere mikrobielle Exposition in den ersten Lebensjahren [25],

durch weniger intensiven Kontakt zu Tieren und unverarbeiteten Lebensmitteln weniger stark beansprucht wird als das Immunsystem von Bauern-Kindern [26]. Diese „Unterforderung“ des Immunsystems wird mit dem Anstieg asthmatischer und allergischer Erkrankungen durch Überreaktionen bei Kontakt mit Allergenen in Zusammenhang gebracht [13].

Obwohl der Konsum unbehandelter Kuhmilch aufgrund teilweise enthaltener, pathogener Mikroorganismen (z.B. Escherichia-coli-Bakterien, Campylobacter-Bakterien) nicht empfohlen werden kann, ist sie als potentielle Maßnahme zur Prävention von Asthma und allergischen Erkrankungen interessant, da ihr protektiver Effekt unabhängig vom direkten Lebensumfeld „Bauernhof“ [27-29] weltweit beobachtbar ist.

6. Publikationen

6.1. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial [30]

Die Publikation *„The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial“* befasst sich mit dem Zusammenhang zwischen Asthma und allergischen Erkrankungen und dem Konsum unbehandelter Milch, beziehungsweise unbehandelter Milchprodukte. Der erste Teil der dreiteiligen Publikation berichtet Ergebnisse einer Metaanalyse, die weltweit beobachtete Assoziationen zwischen unbehandelter Kuhmilch und Asthma oder allergischen Erkrankungen zusammenfasst.

Zuerst wurde eine systematische Literaturrecherche gemäß den PRISMA-Guidelines (Preferred Reporting Items for Systematic Reviews and Meta-analysis) über die Suchmaschinen pubmed und ClinicalTrials.gov durchgeführt. Es wurde weltweit nach Studien mit einer Kombination aus den Suchbegriffen für die Endpunkte *„asthma, wheeze, atopy, hay fever“* und der Exposition *„farm milk“* gesucht. Von den anfänglich 59 gefundenen Publikationen wurden nach Ausschluss von Duplikaten Publikationen, die nicht den vorab definierten Kriterien entsprachen, und Studien, die nicht in englischer oder deutscher Sprache verfasst waren, 8 Publikationen unterschiedlicher Studien in die Metaanalyse einbezogen. Zur Berechnung der Metaschätzer wurden Random-Effekt-Modelle verwendet.

Unabhängig von den Studienregionen, zeigten sich inverse Assoziationen zwischen dem Konsum unbehandelter Kuhmilch (Rohmilch) sowohl im ersten Lebensjahr als auch zum Zeitpunkt der Studiendurchführung (aktuell) mit Asthma (**$OR_{1.LJ}$ =0.58**, 95% CI [0.49-0.69], **$OR_{aktuell}$=0.70**, 95% CI [0.62-0.79]), keuchender/ pfeifender Atmung (engl. Wheeze) (**$OR_{1.LJ}$ =0.66**, 95% CI [0.55-0.78], **$OR_{aktuell}$=0.80**, 95% CI [0.68-0.95]), atopischer Rhinokonjunktivitis (**$OR_{1.LJ}$ =0.68**, 95% CI [0.57-0.82], **$OR_{aktuell}$=0.68**, 95% CI [0.54-0.85]) und Atopie (**$OR_{1.LJ}$ =0.76**, 95% CI [0.62-0.95], **$OR_{aktuell}$=0.65**, 95% CI [0.47-0.90]) in allen Studien (Abbildung 1-2). Die Metaschätzer deuten auf weltweite Generalisierbarkeit der Effekte hin. Variationen der Effektstärken liegen vermutlich in den unterschiedlichen Populationen und Designs der einzelnen Studien begründet. Die Berechnung von Higgings I^2 zeigte jedoch, dass keine signifikante Effekt-Heterogenität zwischen den verschiedenen Studien vorlag. Eine eingehendere Analyse der vier zentral-europäischen Studien (PARSIFAL, ALEX, GABRIEL, PASTURE), mit vergleichbaren Studienpopulationen und

Expositions- sowie Krankheitsdefinitionen lieferte analoge Ergebnisse. Darüber hinaus wurde deutlich, dass die Assoziation zwischen Rohmilchkonsum in der frühen Kindheit und Asthma unabhängig von einem Aufwachsen auf dem Bauernhof bestand (Abbildung 3).
Im zweiten Teil der Publikation wurden die unterschiedlichen, industriell angewendeten Milchverarbeitungsverfahren erklärt und potentielle, aus der Literatur bekannte Kandidaten-Moleküle diskutiert, die für den beobachteten Zusammenhang von Rohmilchkonsum und dem geringeren Auftreten von Asthma und Allergien im Kindesalter verantwortlich sein könnten.

Vor dem Verkauf im Supermarkt durchläuft Kuhmilch mehrere industrielle Verarbeitungsschritte (Tabelle 1). Begonnen wird mit der Zentrifugation, bei der Fremdstoffe und die Milchfettfraktion abgetrennt werden, um ein exaktes Einstellen auf einen gewünschten Fettgehalt (beispielsweise 3.8%, 3.5% oder 1.5%) zu ermöglichen. Danach wird die Milch mindestens pasteurisiert (15 bis 30 Sekunden auf 72 bis 75 Grad Celsius erhitzt), um potentielle Pathogene abzutöten. Erhitzen auf höhere Temperaturen verlängert die Haltbarkeit und wird daher bei einem Großteil der Milchen durchgeführt. Um ein späteres Aufrahmen von Milch zu verhindern, wird Milch in den meisten Fällen zusätzlich homogenisiert. Dabei wird die Milch unter hohem Druck durch feine Düsen auf eine Metallplatte gesprüht, um die Größe der Fettkügelchen zu verringern und ein beständiges homogenes Gemisch zwischen der Fett- und der Molke-Fraktion der Milch zu erzeugen [31]. Hitze- sowie druckempfindliche Milchbestandteile erfahren durch die genannten Verarbeitungsschritte strukturelle Veränderungen, die zu einem Verlust ihrer ursprünglichen biologischen Funktionalität führen können. Einige dieser, durch Verarbeitung veränderten Bestandteile sind möglicherweise für den Asthma- und Allergie-protektiven Effekt von unbehandelter Kuhmilch verantwortlich.

Da Kuhmilch aus über 2000 verschiedenen Bestandteilen [32] besteht, ist es bisher nicht gelungen die genauen Wirkstoffe des Rohmilcheffekts zu ermitteln, allerdings werden verschiedene Kandidaten-Moleküle diskutiert [24, 27, 29, 33-40]. Dazu gehören unter anderem Kohlenhydrate, wie zum Beispiel Laktose oder Oligosaccharide [41-45] denen zum Teil entzündungshemmende sowie präbiotische Eigenschaften zugeschrieben werden, die die Immunantwort beim Kontakt mit Allergenen modulieren könnten [42-45]. Proteine sind weitere Kandidaten-Moleküle. Vor allem Molkeproteine, zu denen α-Laktalbumin, β-Laktoglobulin und die Immunglobuline, aber auch quantitativ weniger vorkommende Moleküle wie Albumin, Laktoferrin, verschiedene Zytokine und Enzyme sind sehr hitzeempfindlich[35]. Durch industrielle Verarbeitung werden Struktur und bioaktive

Eigenschaften dieser Proteine verändert. Einige dieser Molkeproteine könnten die Entwicklung des Darmmikrobioms beeinflussen [46, 47], oder in die Reifung des Immunsystems der Kinder eingreifen [48-50]. Beide Mechanismen führen im Körper möglicherweise zu einer erhöhten Allergentoleranz und dadurch verminderten entzündlichen Reaktionen.

Diskutiert werden auch die Fettsäuren in der Milchfettfraktion. Die im Handel meist erhältliche, in herkömmlicher Weise verarbeitete Milch (Supermarktmilch), enthält im Vergleich zu unbehandelter Kuhmilch meist weniger Fett [24, 32]. Zusätzlich könnte das Homogenisieren die in den Milchfettmolekülen enthaltenen Fettsäurestrukturen und deren Wirkungsweise im Körper, durch die hohe Druckeinwirkung ändern. Kurzkettige Fettsäuren in der Milch zeigten gesundheitsförderliche Eigenschaften bezogen auf Asthma, atopische Sensibilisierung, Nahrungsmittelallergien und allergische Rhinitis [51]. Auch höhere Gehalte an Omega-3-Fettsäuren in der unbehandelten, fettreicheren Kuhmilch zeigten Asthma-protektive Eigenschaften [24]. Ebenso könnten Mineralien, Vitamine und Hormone in der Rohmilch durch industrielle Verarbeitung verändert oder vermindert werden und einen Anteil am Rohmilcheffekt auf Asthma und Allergien haben [52-59]. Bisher liegen hierzu jedoch kaum Forschungsergebnisse vor.

Der letzte Abschnitt der Publikation berichtet von der im Jahr 2018 gestarteten MARTHA-Studie (registriert im Deutschen Register Klinischer Studien (DRKS00014781)). Die MARTHA Studie ist eine Interventionsstudie, in der der gesundheitsförderliche Effekt von minimal verarbeiteter, aber gesundheitlich unbedenklicher Kuhmilch mit UHT-Milch verglichen wird. Die Milchintervention startet sobald die Kleinkinder nicht mehr voll gestillt werden und dauert bis zum dritten Lebensjahr an. Primärer Endpunkt der Studie ist Asthma im Alter von fünf Jahren. Sekundäre Endpunkte sind respiratorische Infektionen, keuchende, pfeifende Atemgeräusche während des Interventionszeitraumes, leichte Infektionen, atopische Sensibilisierung und Ekzeme im Alter von drei und fünf Jahren. Bei Erfolg der Studie wäre mit dieser minimal behandelten Milch eine einfache präventive Maßnahme gegen Asthma im Kindesalter gefunden. Aus wissenschaftlicher Sicht sind zusätzlich weitere Nachforschungen zu den genauen Mechanismen des Rohmilcheffekts wünschenswert.

1. Centrifugation	Separation of dirt particles, somatic cells and cream at 50°C
2. Adjustment of milk fat content	Commercially available milk is offered in four different categories - natural full-cream milk (>3.5% fat) - full-cream milk (3.5% fat) - semi-skimmed milk (1.5-1.8% fat) - skimmed milk (≤0.3% fat)
3. Heat treatment (to kill potential pathogens and prolong shelf life)	
a. Pasteurization	Heating (72-75°C) for 20-30 s
b. High-heat-treatment (extended-shelf life)	Preheating at 95°C for 20 s, direct steam injection at 127°C for 5 s
c. Ultra-high-heat treatment (UHT-milk)	Preheating at 93°C for 23 s, direct steam injection at 142°C for 5 s
d. Sterilization	Milk bottling, heating at 110-120°C for 10-30 min
4. Homogenization	2-stage homogenization* at 250/50 bar

Tabelle 1: Industriell angewendete Milchverarbeitungsschritte

Siehe Tabelle 2 in der Publikation:
Tabea Brick, Kasper Hettinga, Benedikt Kirchner, Michael W. Pfaffl, Markus Johannes Ege. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial. The Journal of Allergy and Clinical Immunology: In Practice. Volume 0, Issue 0. 2019. doi: https://doi.org/10.1016/j.jaip.2019.11.017

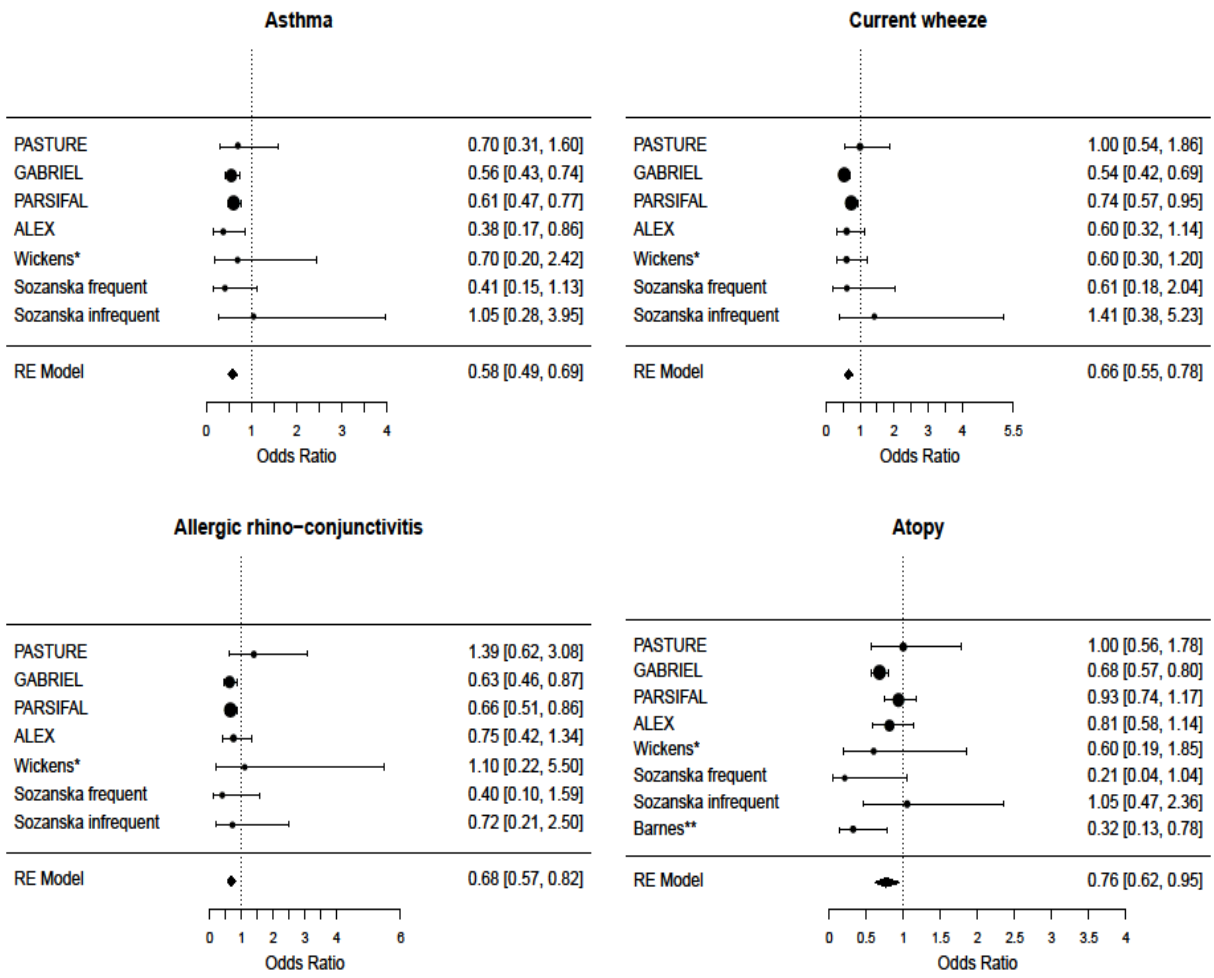

Abbildung 1: Assoziationen zwischen Rohmilchkonsum im ersten Lebensjahr und den Studienendpunkten (OR [95% CI])

Siehe Abbildung 2 in der Publikation:

Tabea Brick, Kasper Hettinga, Benedikt Kirchner, Michael W. Pfaffl, Markus Johannes Ege. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial. The Journal of Allergy and Clinical Immunology: In Practice. Volume 0, Issue 0. 2019. doi: https://doi.org/10.1016/j.jaip.2019.11.017

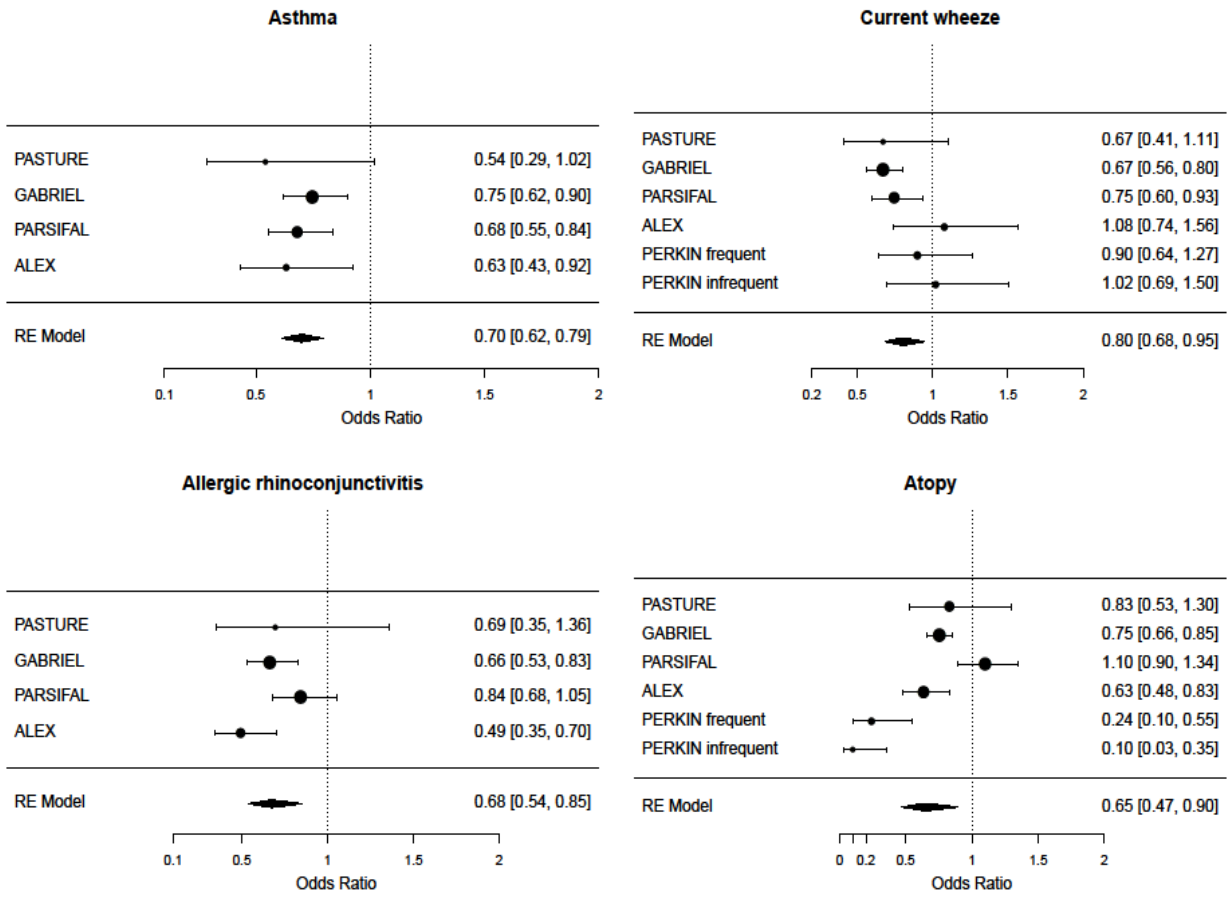

Abbildung 2: Assoziation zwischen Rohmilchkonsum aktuell und den Studienendpunkten (OR [95% CI])

Siehe Abbildung 3 in der Publikation:

Tabea Brick, Kasper Hettinga, Benedikt Kirchner, Michael W. Pfaffl, Markus Johannes Ege. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial. The Journal of Allergy and Clinical Immunology: In Practice. Volume 0, Issue 0. 2019. doi: https://doi.org/10.1016/j.jaip.2019.11.017

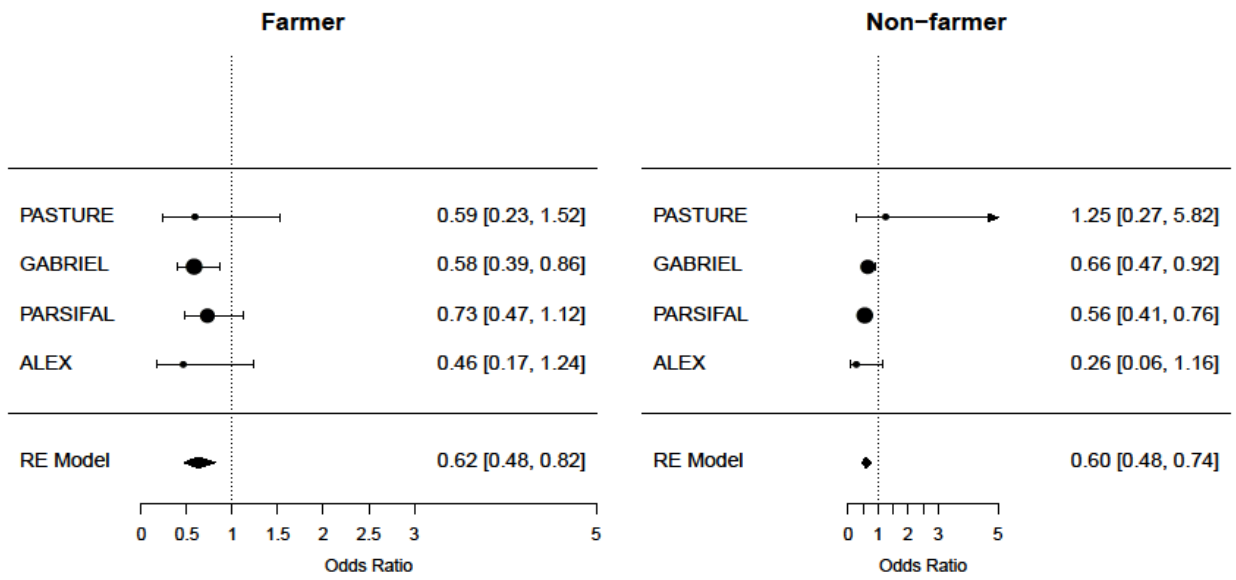

Abbildung 3: Rohmilchkonsum im ersten Lebensjahr und Asthma in der Kindheit (OR [95% CI])
Siehe Abbildung 4 in der Publikation:
Tabea Brick, Kasper Hettinga, Benedikt Kirchner, Michael W. Pfaffl, Markus Johannes Ege. The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial. The Journal of Allergy and Clinical Immunology: In Practice. Volume 0, Issue 0. 2019. doi: https://doi.org/10.1016/j.jaip.2019.11.017

Bei der Suche nach den gesundheitsfördernden Inhaltsstoffen in der unbehandelten Kuhmilch beobachteten Wjiga et al. und Waser et al., dass Kinder, die Produkte mit einem höheren Milchfettanteil konsumierten, weniger häufig unter Allergien litten [60-62]. Basierend auf dieser Beobachtung entstand die Idee, Unterschiede in den Fettfraktionen unbehandelter und industriell verarbeiteter Milch zu untersuchen. Milchfettsäuren sind zwar nicht in gleichem Maße temperatursensitiv wie beispielsweise Proteine [40], bei der industriellen Milchaufbereitung werden jedoch zusätzliche Zentrifugations- und Homogenisierungsschritte durchgeführt, die nachgewiesenermaßen den Gehalt der Milchfettsäuren, ihre Verhältnisse zueinander und ihre Bioverfügbarkeit verändern [60].

6.2. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk [24]

Für die Publikation **„***Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk"* wurde im Rahmen der PASTURE Studie der Asthma-protektive Effekt des Konsums unbehandelter Kuhmilch auf longitudinaler Ebene betrachtet und zusätzlich untersucht, ob unterschiedliche Gehalte ungesättigter, langkettiger Fettsäuren in unbehandelter, bzw. verarbeiteter Kuhmilch einen Anteil an diesem Effekt haben könnten.

Bei der Milchverarbeitung werden nicht nur hitzelabile Milchkomponenten wie Proteine oder Vitamine verändert, sondern durch Zentrifugation, Homogenisierung und häufig Reduzierung des Fettgehalts auch die Fettfraktion in der Kuhmilch. Quantität und biologische Verfügbarkeit der enthaltenen Fettsäuren könnten sich dabei ändern, was möglicherweise eine Abnahme des gesundheitsförderlichen Effekts bei industriell verarbeiteter Milch auf Asthma und Allergien zur Folge hat.

751 Kinder, die bis zum Alter von sechs Jahren an der PASTURE Studie teilgenommen hatten, wurden in die nachfolgenden Analysen eingeschlossen. Davon waren 351 Kinder, die auf einem Bauernhof aufwuchsen und 400 Nichtbauern-Kinder, die in denselben ländlichen Regionen lebten. Angaben zu sozioökonomischem Status und Lebensstil, Bauernhofexpositionen und respiratorischen und anderen Gesundheitsfaktoren der Kinder sowie deren Familien wurden mithilfe regelmäßiger, mindestens jährlicher Fragebögen von der Geburt bis zum Alter von sechs Jahren erhoben. Zusätzlich wurden im Alter von vier Jahren Proben der üblicherweise getrunkenen Milch gesammelt und Blutproben zur Analyse der hsCRP Werte (hochsensitives C-reaktives Protein = Entzündungsmarker im Blut [63]) entnommen. Mit sechs Jahren wurde nach einer ärztlichen Asthmadiagnose, beziehungsweise nach mindestens zweimalig vorliegender ärztlicher Diagnose einer obstruktiven Bronchitis gefragt. Kinder, die diese Kriterien erfüllten, wurden als Asthmatiker definiert (entsprechend der Definition in der ISAAC Studie [64]). Zusätzlich wurden Lungenfunktionsmessungen durchgeführt und eine positive Bronchodilatatoren-Antwort als relative Abnahme des FEV1-Wertes (forciertes Ausatemvolumen innerhalb 1 Sekunde) um mindestens 12% definiert. Milchen wurden in verschiedene Kategorien eingeteilt: Bauernmilch, die wiederum unterschieden wurde in rohe und gekochte Bauernmilch, Ladenmilch und Milch mit hohem (≥3.5%) beziehungsweise niedrigem (<3.5%) Fettgehalt. In einer Subpopulation, die im Fall-Kontroll-Design angelegt war (n= 49 Asthmatiker, n=35 gesunde Kinder) wurde die Quantität 46 vorab definierter Fettsäuren in den Milchproben analysiert.

Die Beständigkeit des Milchkonsumverhaltens der Kinder über den Verlauf des Studienzeitraums wurde mit Pearsons Korrelationskoeffizient untersucht. In beiden Populationen wurde der Zusammenhang zwischen Art der konsumierten Milch und Asthma mit jährlichen logistischen Regressionsmodellen berechnet. Der Anteil der verschiedenen Fettsäuregruppen (konjugierte Linolsäuren, gesättigte Fettsäuren, einfach ungesättigte Fettsäuren, trans-Fettsäuren, mehrfach ungesättigte Fettsäuren, Omega-3-Fettsäuren, Omega-6-Fettsäuren) an der beobachteten Assoziation zwischen Rohmilchkonsum und Asthma wurde mithilfe der Änderung des β-Schätzers (Change-in-Estimate (CIE)) geschätzt. Die Korrelation der einzelnen Fettsäuren mit ihrer Gruppenvariable wurde mit Spearmans rho berechnet, um herauszufinden, welche Fettsäure innerhalb einer Gruppe den größten Anteil an der beobachteten Änderung des β-Schätzers hatte.

Es zeigte sich ein Anstieg des Milchkonsums bei den Kindern bis zum Alter von drei Jahren und eine leichte Abnahme ab fünf Jahren. Entgegen Empfehlungen der Ärzte tranken Kinder ab dem Alter von zwei Jahren häufig unbehandelte Kuhmilch (Abbildung 4). In der hier untersuchten Studienpopulation zeigte sich ein beständiger Asthma-protektiver Effekt des Konsums unbehandelter Kuhmilch verglichen mit abgekochter Milch vom Bauernhof und Milch aus dem Laden über die Zeit der Studiendauer (**OR=0.51**, 95% CI [0.15- 1.73] im Alter von 1 Jahr, **OR=0.29**, 95% CI [0.11-0.76] im Alter von sechs Jahren) (Tabelle 2). Beobachtet wurde außerdem eine geringere Asthmaprävalenz bei Kindern, die Milch mit einem höheren Fettanteil (≥3.5 % Fettanteil versus <3.5% Fettanteil) tranken (**OR=0.37**, 95% CI [0.19-0.75] im Alter von sechs Jahren) (Tabelle 2). Alle Effekte wurden mit kürzer zurückliegendem Rohmilchkonsum stärker.
Bauernmilch hatte in dieser Studie einen durchschnittlichen Fettgehalt von 4%, wohingegen etwa 60% der Milchen aus Supermärkten fettreduziert (<3.5%) gekauft wurden. Der Zusammenhang zwischen einem höheren Milchfettanteil und Asthma konnte nur zu etwa 20% durch den hohen Anteil unbehandelter Kuhmilch in der Gruppe der fettreichen Milchen erklärt werden. Dieser eigenständige „Fetteffekt“ wiederum wurde zum Großteil auf den höheren Gehalt an Omega-3-Fettsäuren zurückgeführt (CIE=111%) (Tabelle 3), beziehungsweise auf ein günstigeres Verhältnis zwischen Omega-6- und Omega-3-Fettsäuren in der unbehandelten Kuhmilch, verglichen mit industriell verarbeiteter Milch. Anders als der Gehalt an Omega-3-Fettsäuren war der Gehalt an Omega-6-Fettsäuren in fettreduzierter Milch kaum vermindert.

Omega-3-Fettsäuren sind Vorstufen anti-inflammatorisch wirkender Stoffwechselprodukte wie LTB5 Leukotriene oder PGE3 Prostaglandine, wohingegen Omega-6-Fettsäuren häufiger zu inflammatorischen Derivaten verstoffwechselt werden (Abbildung 5). Für die Stoffwechselprozesse beider Fettsäuregruppen werden die gleichen Enzyme benötigt. Diesen Enzymen wird eine höhere Affinität zu Omega-3-Fettsäuren zugeschrieben; daher führt eine geringere Omega-6/Omega-3-Ratio zu mehrheitlich entzündungshemmenden Stoffwechselprodukten [65, 66]. Zusätzlich wird diese Annahme durch die Beobachtung eines positiven Zusammenhangs zwischen einer geringeren Omega-6/Omega-3-Ratio in den konsumierten Milchen, und geringeren hsCRP-Entzündungsmarkern im Serum der Kinder in der untersuchten Studienpopulation gestützt.

Zusammenfassend war der regelmäßige Konsum unbehandelter Kuhmilch vom Bauernhof invers mit einer Asthmadiagnose im Alter von 6 Jahre assoziiert. Dieser Effekt ließ sich zu einem erheblichen Teil durch einen höheren Omega-3-Fettgehalt, beziehungsweise ein günstigeres Mengenverhältnis zwischen Omega-3- und Omega-6-Fettsäuren erklären. Obwohl der Konsum unbehandelter Bauernmilch aufgrund der potentiell enthaltenen Pathogene nicht empfohlen werden kann, wird eine permanente, entzündungshemmende Wirkung von Rohmilch vermutet die zu einem geringeren Auftreten asthmatischer Symptome führen könnte.

Age of children (mo)	Farm milk vs. shop milk, OR (95 % CI)	Unprocessed farm milk vs. shop milk, OR (95 % CI)	Unprocessed vs. boiled farm milk, OR (95 % CI)	High fat milk (≥3.5%) vs. low fat milk (<3.5%) , OR (95 % CI)
12	0.54 (0.20-1.45)	0.51 (0.15-1.73)	0.95 (0.31-2.90)	---
18	0.51 (0.23-1.14)	0.38 (0.13-1.12)	0.66 (0.23-1.89)	---
24	0.71 (0.34-1.51)	0.61 (0.24-1.59)	0.83 (0.33-2.06)	0.72 (0.39-1.33)
36	0.37 (0.18-0.78)	0.23 (0.09-0.59)	0.51 (0.19-1.38)	0.63 (0.33-1.20)
48	0.34 (0.16-0.71)	0.26 (0.10-0.67)	0.77 (0.26-2.32)	0.38 (0.18-0.78)
60	0.40 (0.19-0.85)	0.21 (0.08-0.54)	0.28 (0.11-0.75)	0.61 (0.32-1.15)
72	0.48 (0.22-1.03)	0.29 (0.11-0.76)	0.35 (0.12-1.02)	0.37 (0.19-0.75)

*Adjusted for center and farming.

Tabelle 2: Oddsratios und Konfidenzintervalle der Effekte des Konsums unterschiedlicher Milcharten über die Zeit auf Asthma (im Alter von sechs Jahren)

Siehe Tabelle E4 in der Publikation:
Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016 Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042. Epub 2016 Jan 12.

Adjustment	OR [CI 95%]	β estimate	CIE	CIE [%]
----	0.43 [0.17-1.11]	-0.83	-----	----
∑-FA	0.53 [0.19-1.49]	-0.63	(-0.83-(-0.63))/ -0.83= 0.24	24
SFA	0.45 [0.17-1.16]	-0.81	(-0.83-(-0.81))/ -0.83= 0.02	2
MUFA	0.42 [0.15-1.13]	-0.87	(-0.83-(-0.87))/ -0.83= -0.05	-5
PUFA	0.56 [0.21-1.52]	-0.58	(-0.83-(-0.58))/ -0.83= 0.30	30
ω-3 PUFA	1.09 [0.33-3.65]	0.09	(-0.83-(0.09))/ -0.83= 1.11	111
ω-6 PUFA	0.42 [0.16-1.10]	-0.87	(-0.83-(-0.87))/ -0.83= -0.05	-5
Trans-FA	0.40 [0.13-1.26]	-0.92	(-0.83-(-0.92))/ -0.83= -0.11	-11
CLA	0.49 [0.15-1.59]	-0.71	(-0.83-(-0.71))/ -0.83= 0.14	14

CIE, Change in estimate; *CLA*, conjugated linoleic acid; *MUFA*, monounsaturated fatty acid; *SFA*, saturated fatty acid.
*Because of skewed distributions of some FA variables, all FA variables were used after rank transformation;

Tabelle 3: Oddsratios des Bauernmilcheffekt auf Asthma (im Alter von sechs Jahren): Roher Schätzer und nach dem Adjustieren auf die rangtransformierten Fettsäuregruppen

Siehe Tabelle E7 in der Publikation:
Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016 Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042. Epub 2016 Jan 12.

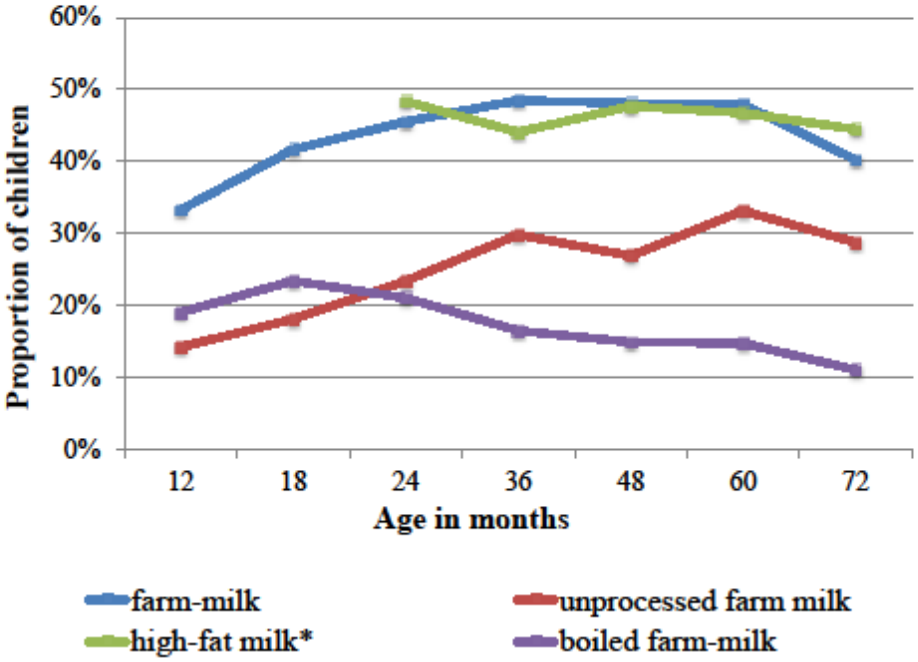

Abbildung 4: Häufigkeit des Konsums unterschiedlicher Milcharten im Verlauf des Studienzeitraums

Siehe Abbildung 2A in der Publikation:

Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016 Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042. Epub 2016 Jan 12.

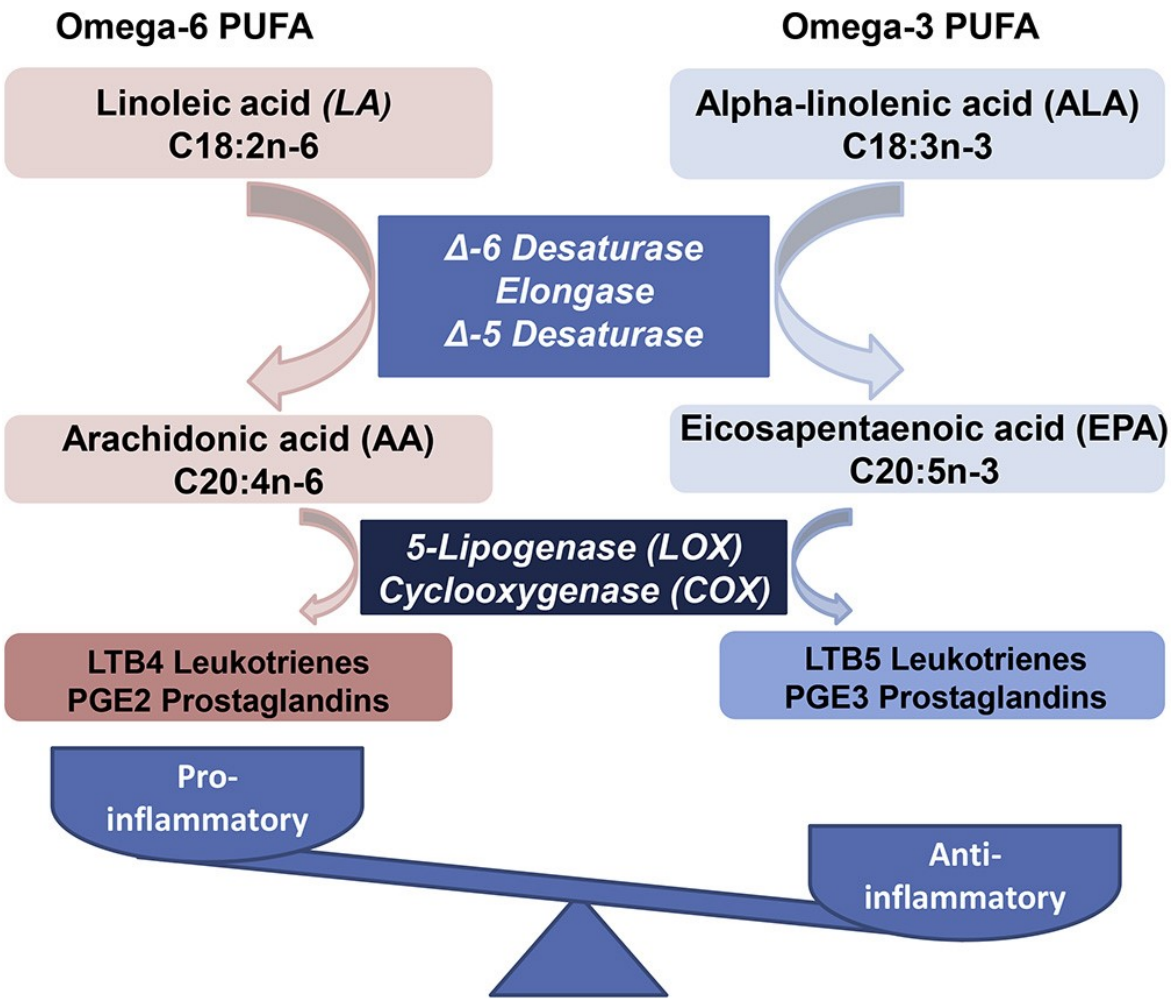

Abbildung 5: Metabolismus von Linolsäure und Alpha-Linolensäure

Siehe Abbildung 6 in der Publikation:

Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016 Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042. Epub 2016 Jan 12.

6.3. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins [35]

Ein weiterer Teil dieser Arbeit beschäftigt sich mit dem Thema Proteine, beziehungsweise deren unterschiedlichen Quantität in verschieden verarbeiteten Milchen. Proteinstrukturen und -funktionalitäten können sich beispielsweise durch Erhitzung und Druckeinwirkung verändern und sind daher potentielle Moleküle, die zum Asthma-und Allergie-protektiven Effekt unbehandelter Kuhmilch im Vergleich zu industriell verarbeiteter Milch beitragen könnten. Die Publikation *„Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins“* beschäftigt sich mit Proteinen in der unbehandelten Kuhmilch, die zu deren asthma-und allergie-protektivem Effekt beitragen könnten. Ziel der Analysen war der Vergleich des nativen Serumproteoms in unbehandelten Kuhmilchproben mit dem Serumproteom aus unterschiedlich industriell verarbeiteten Kuhmilchproben (Tabelle 4).
In vielen westlichen Ländern wird Kuhmilch mindestens pasteurisiert, um eine Gesundheitsgefährdung durch Pathogene in der Milch zu verhindern. Wegen der längeren Haltbarkeit der Milch ist heutzutage auch der Konsum hocherhitzter Milchen wie ESL oder UHT-Milch üblich. Proteine im Milchserum sind hitzelabil [67, 68], vor allem solche, die immunaktive Eigenschaften besitzen [69]. Die angewendeten industriellen Milchverarbeitungsverfahren können bei Proteinen zu Denaturierungs- und Aggregationsprozessen führen, die den Verlust der biologischen Funktionalität zur Folge haben. Auch chemische Veränderungen der Proteinstrukturen durch Erhitzung (Maillard-Reaktion) sind denkbar [70].

Für die Analysen wurden 24 Milchproben von drei verschiedenen Bauernhöfen in Süddeutschland gesammelt. Jede Bauernhofcharge umfasste acht Einzelproben, von denen jeweils eine als unbehandelte Milchprobe, die anderen sieben nach unterschiedlichen, industriell üblichen Verarbeitungsschritten (Tabelle 4) auf Anzahl und Quantität der enthaltenen, messbaren Proteine analysiert wurden. Die Proteinmenge wurden mit Flüssigchromatographie/ Tandemmassenspektrometrie bestimmt und als LFQ-Werte (label-free quantification) angegeben. Der Anteil der messbaren Proteine in den verschieden verarbeiteten Milchproben wurde prozentual berechnet, wobei die Rohmilchproben die Referenz darstellten. Proteine, bei denen bei über 33% der Milchproben keine Werte messbar waren, wurden von den nachfolgenden Analysen ausgeschlossen. Von allen anderen Proteinen wurde zuerst die proteinspezifische Verteilung mithilfe von linearen Tobit-Modellen geschätzt, und danach wurden fehlende Werte entsprechend dieser Verteilung imputiert. Mithilfe hierarchischer Clustering-Methoden (basierend auf Pearson's

Korrelationskoeffizienten) wurden Proteinprofile der unterschiedlich verarbeiteten Milchen auf Ähnlichkeit überprüft. Nach Einteilung der Milchproben in hoch- und niedrig-erhitzte Milchen (Erhitzung >80°C versus Erhitzung <80°C, [71]) wurde ein logistisches Regressionsmodell gerechnet, um Unterschiede der Gesamtproteingehalte zwischen den beiden Milchgruppen zu finden. Zur Schätzung des Quantitätsunterschiedes und damit der proteinspezifischen Hitzelabilität, wurden die Log-2-fold-Changes der einzelnen Proteine zwischen den beiden Milchgruppen berechnet.
Insgesamt konnten 364 unterschiedliche Proteine in mindestens einer Milchprobe detektiert werden. Die statistischen Analysen basierten auf 169 Proteinen mit ausreichend Messwerten (Proteine, bei denen bei über 66% der Milchproben Werte messbar waren). Je höher der Grad der Erhitzung, desto höher der Anteil der Proteine unterhalb der Detektionsgrenze. Trotz geringer Erhitzung während des Homogenisierungsprozesses, zeigten auch diese Milchproben einen deutlich geringeren Anteil messbarer Proteine verglichen mit Rohmilchproben. Ähnlich stark erhitzte Milchproben wiesen vergleichbare Proteinprofile auf (Abbildung 6). Rohmilch, pasteurisierte und entrahmte Milch bildeten zusammen ein Cluster, genauso wie UHT-Milch, ESL-Milch und gekochte Milch. Bei der einzelnen Betrachtung der Proteine zeigten 23 signifikant geringere Quantitäten in der Gruppe der hoch-erhitzten Milchen im Vergleich zur Gruppe der niedrig-erhitzten Milchen. Zehn dieser Proteine (beispielsweise Xanthindehydrogenase/-oxidase, Lactoferrin, Lactoperoxidase) werden mit Immunfunktionen im menschlichen Körper in Verbindung gebracht und könnten Einfluss auf die Ausbildung des Immunsystems oder auf die Immunantwort nach Allergenkontakt nehmen (Tabelle 5). Zusammenfassend lässt sich sagen, dass die Erhitzung der Milchproben zu einer Verringerung sowohl der Anzahl der messbaren Proteine als auch der Proteinmenge führt. Ein Großteil dieser Proteine ist mit Immunfunktionen im menschlichen Körper assoziiert; diese Proteine könnten daher Kandidaten sein, die zum asthma-und allergieprotektiven Effekt von Rohmilch beitragen.

Code	Milk fraction	Processing conditions	Day of processing*	Grouping of milk types**
RAW	Native raw milk	-	3	No-low heat
PAS	Pasteurized	72°C for 20 sec	3	No-low heat
SKI	Skim milk	Separation at 50°C	2	No-low heat
FAT	Fat fraction/cream	Separation at 50°C	2	-
HOM	Homogenized milk	Preheating to 55°C, 2-stage homogenization at 250/50 bar	2	-
ESL	Extended shelf life milk	Preheating at 95°C for 20 sec, direct steam injection at 127°C for 5 sec	1	High heat
UHT	Ultra-high heat treated	Preheating at 93°C for 23 sec, direct steam injection at 142°C for 5 sec	1	High heat
BOI	Boiled milk	Preheating at >80°C for > 300sec, boiling at 100°C for 30 sec	2	High heat

* Milk samples were stored at -20°C until analysis. ** for further analysis of heat treatment on milk proteins, grouping of milk types according to the heat treatment was conducted; homogenized milk was excluded due to additional treatment with pressure; cream was excluded because it contains only the milk fat fraction

Tabelle 4: Details zur Verarbeitung der Milchproben

Siehe Tabelle 2 in der Publikation:

Brick T, Ege M, Boeren S, Böck A, von Mutius E, Vervoort J, Hettinga K. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. Nutrients. 2017 Aug 31;9(9). pii: E963. doi: 10.3390/nu9090963.

Protein code	p-value*	Log2 fold change	Protein name	*Protein function*
P80457	**0.001**	**-0.44**	**Xanthine dehydrogenase/ oxidase**	*immunity*
P24627	**0.004**	**-0.37**	**Lactoferrin**	*immunity*
G3X6N3	**0.006**	**-0.35**	**Serotransferrin**	*transport*
F1MR22	**0.004**	**-0.34**	**Polymeric immunoglobulin receptor**	*immunity*
P80025	**0.001**	**-0.33**	**Lactoperoxidase**	*immunity*
G3N1R1	**0.002**	**-0.32**	**Uncharacterized protein**	*unknown*
F1MGU7	**0.04**	**-0.30**	**Fibrinogen gamma-B chain**	*Blood coagulation*
G3X7A5	**0.002**	**-0.29**	**Complement C3**	*immunity*
F1MZ96	**0.002**	**-0.27**	**Uncharacterized protein**	*unknown*
F1MX50	**0.01**	**-0.27**	**Uncharacterized protein**	*cell*
F1MM32	**0.026**	**-0.26**	**Sulfhydryl oxidase**	*enzyme*
P81265	**0.006**	**-0.24**	**Polymeric immunoglobulin receptor**	*immunity*
F1N076	**0.001**	**-0.23**	**Ceruloplasmin**	*cell*
F1MXX6	**0.02**	**-0.22**	**Lactadherin**	*cell*
Q08DQ0	**0.017**	**-0.21**	**Plakophilin-3**	*cell*
P07589	**0.004**	**-0.20**	**Fibronectin**	*immunity*
A6QNL0	**0.01**	**-0.20**	**Monocyte differentiation antigen CD 14**	*immunity*
P10152	**0.048**	**-0.20**	**Angiogenin-1 (ribonuclease 5)**	*cell*
F1MMD7	**0.031**	**-0.198**	**Inter-alpha-trypsin inhibitor heavy chain H4**	*Protease inhibitor*
Q3MHN2	**0.043**	**-0.196**	**Complement component C9**	*immunity*
P00735	**0.028**	**-0.18**	**Prothrombin**	*immunity*
F1MCF8	**0.001**	**-0.17**	**Uncharacterized protein**	*immunity*
P17690	**0.005**	**-0.16**	**Beta-2-glycoprotein 1**	*Blood coagulation*

*p-values are adjusted for multiple testing.

Tabelle 5: Proteine mit signifikanten Quantitätsunterschieden (≥ 10%) zwischen hoch und nicht/ niedrig erhitzten Milcharten

Siehe Tabelle 3 in der Publikation:

Brick T, Ege M, Boeren S, Böck A, von Mutius E, Vervoort J, Hettinga K. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. Nutrients. 2017 Aug 31;9(9). pii: E963. doi: 10.3390/nu9090963.

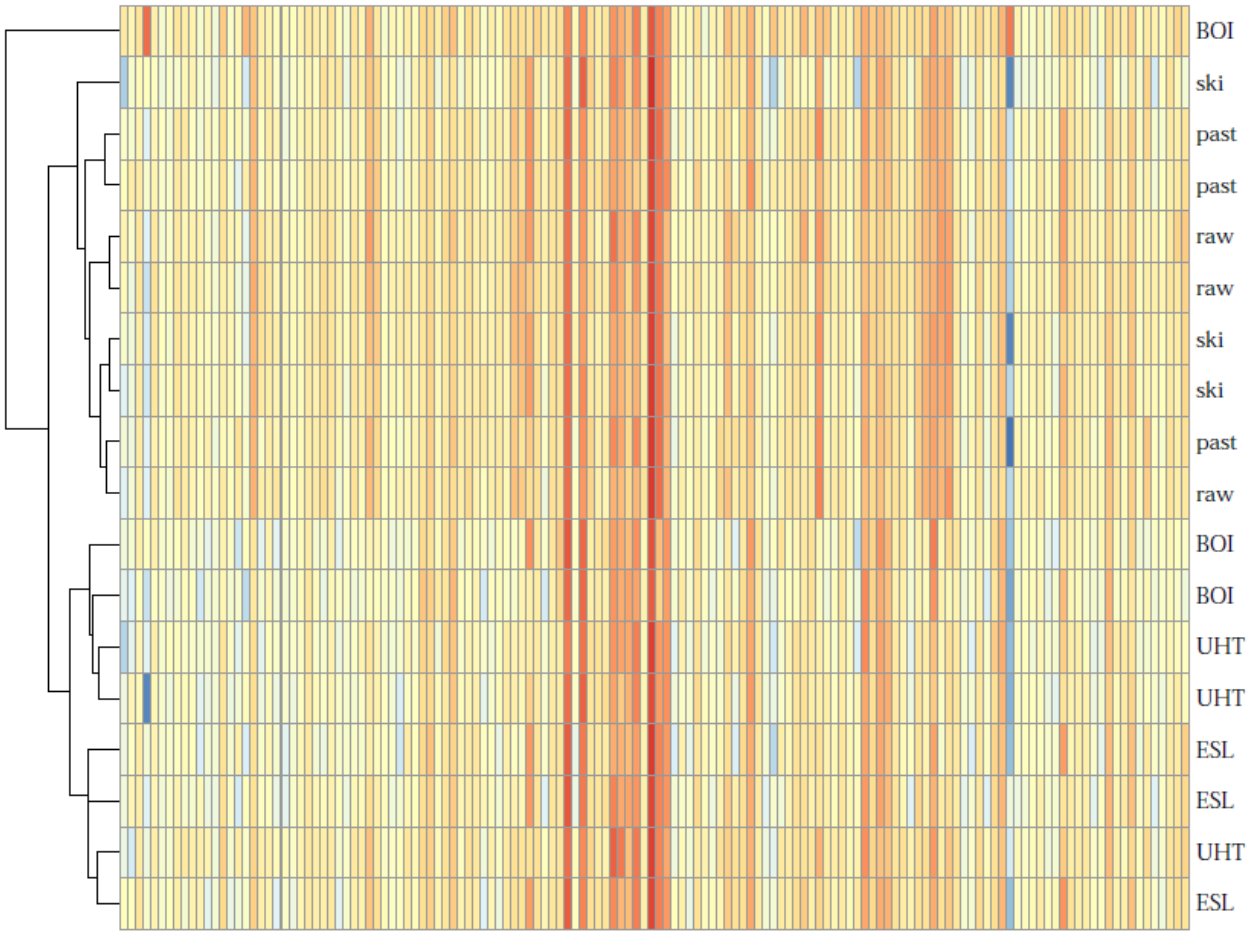

Abbildung 6: Heatmap der Korrelationen zwischen Proteinquantitäten und Milcharten
Siehe Abbildung 3 in der Publikation:
Brick T, Ege M, Boeren S, Böck A, von Mutius E, Vervoort J, Hettinga K. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. Nutrients. 2017 Aug 31;9(9). pii: E963. doi: 10.3390/nu9090963.

Neben der Erforschung ursächlicher oder schützender Faktoren asthmatischer und allergischer Erkrankungen, ist auch die Suche nach Indikatoren eines zukünftigen Erkrankungsrisikos bei Kindern mit möglichst gering-invasiven und kostengünstigen Mitteln von zentraler Bedeutung. Kinder, denen ein erhöhtes Erkrankungsrisiko attestiert wird, könnten dann engmaschiger beobachtet und auftretende Symptome zeitnah und adäquat medizinisch behandelt werden, um die bestmögliche Lebensqualität zu erhalten und Langzeitfolgeschäden der Lunge durch unzureichend behandelte Exazerbationen zu verhindern [72, 73].
Die Heterogenität der Asthmasymptome [74] in Kombination mit wenig passenden Lungenfunktionsmessgeräten und fehlenden Referenzwerten stellen gerade bei Kleinkindern ein großes Problem bei der Beurteilung der klinischen Relevanz der beobachteten Symptome dar. Die meisten Lungenfunktionstests liefern erst ab einem Alter von circa drei Jahren valide Ergebnisse. Bei jüngeren Kindern ist die Qualität der Messungen durch fehlende Kooperation

oft vermindert und der Aufwand zur Qualitätssicherung deutlich erhöht, weil wiederholte Messungen und Trainingsdurchläufe nötig sind. Daher basieren ärztliche Prognose- und Diagnosestellungen bei Kleinkindern auf Berichten der Eltern zu Asthmasymptomen ihrer Kinder, auf Familienanamnese und basalen körperlichen Untersuchungen. Kinder unter drei Jahren werden dadurch häufiger nicht, beziehungsweise falsch diagnostiziert [75].

6.4. Parents know it best: prediction of asthma and lung-function by parental perception of early wheezing episodes [76]

Die Publikation *„Parents know it best: prediction of asthma and lung-function by parental perception of early wheezing episodes“* beschäftigt sich mit der Frage, inwieweit sich Angaben der Eltern zu Asthmasymptomen ihrer Kinder im ersten Lebensjahr zur Einschätzung einer späteren Asthmadiagnose eignen. Zur Vereinfachung wird im Folgenden der englische Begriff „wheeze“ für diese Asthma-typischen Symptome wie keuchende, pfeifende Atmung verwendet. Ziel der Untersuchung war herauszufinden, wie sich Eltern an Wheeze-Episoden im ersten Lebensjahr ihrer Kinder erinnern, und ob sich aus dem Erinnerungsvermögen Implikationen für die spätere Lungenfunktion der Kinder, beziehungsweise eine spätere Asthmadiagnose ableiten lassen.

Dazu wurden 880 Kinder der PASTURE Studie, die von der Geburt bis zum Alter von sechs Jahren regelmäßig beobachtet und deren Eltern zu Asthma-Symptomen befragt wurden, in die Analysen eingeschlossen.
Im ersten Lebensjahr wurden die Eltern wöchentlich via Tagebuchfragebögen nach Wheeze-Episoden ihres Kindes in der vorangegangenen Woche gefragt. Im Alter von einem Jahr wurden die Eltern retrospektiv gefragt, ob sie sich an Wheeze-Episoden erinnern. Die Tagebuchdaten zu Wheeze-Episoden wurden mit den Antworten aus dem 1-Jahresfragebogen kombiniert und vier Kategorien des richtigen beziehungsweise falschen Erinnerns der Eltern abgeleitet (Abbildung 7). Die beiden Kategorien „true positive recall“ (richtiges Erinnern an Wheeze-Episoden im ersten Lebensjahr) und „false negative recall“ (kein Erinnern der Wheeze-Episoden im ersten Lebensjahr) wurden in dieser Arbeit genauer untersucht. Eltern in beiden Kategorien gaben im Laufe des ersten Lebensjahres an, dass ihr Kind Wheeze-Episoden hatte, nur etwa die Hälfte erinnerte sich jedoch nach einem Jahr an diese Episoden.

Als ersten Endpunkt der Analysen wurde Asthma (entsprechend der Definition in der ISAAC-Studie [64]) wie folgt definiert: Im Alter von sechs Jahren wurde nach dem Vorliegen einer ärztlichen Asthmadiagnose, beziehungsweise wiederholter ärztlicher Diagnose einer obstruktiven Bronchitis gefragt. Darüber hinaus wurden Lungenfunktionsmessungen im Alter von sechs Jahren durchgeführt. Die Ratio FEV1/FVC (FEV1 = forciertes expiratorisches Volumen in einer Sekunde/ FVC = forcierte Vitalkapazität) wurde als Maßzahl für die Güte der Lungenfunktion verwendet. Zusätzlich wurden die Kinder genotypisiert und die Interferron-y (IFNy)-Produktion im Alter von zwölf Monaten gemessen [28]. Variablen, die Einfluss auf das Erinnern der Eltern, beziehungsweise auf eine spätere Asthmadiagnose oder

Lungenfunktion der Kinder hatten, wurden mit Hilfe einer Rückwärtsselektion bestimmt (level of entry $p \leq 0.2$, level of stay $p < 0.05$) und in multivariate logistische Regressionsmodelle eingeschlossen. Der Zusammenhang zwischen IFN-y-Werten und dem elterlichen Erinnerungsvermögen wurde via Kaplan-Meier-Schätzung und Cox-proportionalem Hazard-Modellen berechnet. Alle Schätzer werden als Odds-Ratios (OR), Hazard-Ratios (HR) oder ß-Schätzer mit zugehörigem 95%-Konfidenzintervall (95%-CI) berichtet.

Von den 307 Kindern die laut Tagebuchangaben im ersten Lebensjahr Wheeze-Episoden zeigten, erinnerten sich nur 54.4% der Eltern (n=167) korrekt daran zurück. Determinanten die das „richtige Erinnern" positiv beeinflussten waren eigenes Asthma der Eltern, Gesamtanzahl der Wheeze-Episoden im ersten Lebensjahr und ein kurzer zeitlicher Abstand zwischen der letzten Wheeze-Episode und dem Ausfüllen des 1-Jahresfragebogens (Tabelle 6). Asthmamedikation wurde nur gelegentlich berichtet und zeigte keine signifikante Assoziation mit dem Erinnerungsvermögen der Eltern. Kinder deren Eltern sich richtig an die Wheeze-Episoden im ersten Lebensjahr erinnerten hatten ein deutlich erhöhtes Risiko für eine spätere Asthmadiagnose und schlechtere Lungenfunktionswerte im Alter von sechs Jahren (**OR=3.45**, 95% CI [1.59-7.51]) als Kinder, deren Eltern die Wheeze-Episoden vergaßen. Neben dem richtigen Erinnern der Wheeze-Episoden waren Heuschnupfen der Eltern und das Asthma-Risiko-Allel auf Chromosom 17q21 weitere prädizierende Faktoren für eine spätere Asthmadiagnose in der untersuchten Studienpopulation (Tabelle 7). Bei genauerer Betrachtung zeigte sich, dass der Zusammenhang zwischen richtigem Erinnern und einer späteren Asthmadiagnose nur bei Kindern existierte, die Träger des Asthma-Risiko-Allels waren (Abbildung 8). Dies deutet darauf hin, dass Träger des Risiko-Allels schon in früher Kindheit einprägsamere Symptome zeigen. Zusätzlich war „true positive recall" signifikant mit einer schlechteren Lungenfunktion im Alter von sechs Jahren assoziiert. Kinder, deren Eltern sich an Wheeze-Episoden erinnerten, hatten zusätzlich etwa 50% höhere IFN-y Werte bei in-vitro Stimulation mit LPS verglichen mit Kindern, deren Eltern sich nicht erinnerten.

Zusammenfassend zeigte sich in unseren Analysen, dass elterliche Beobachtungen anfällig für den sogenannten „Recall-Bias" (Erinnerungs-Bias) sind, dass jedoch in unserem Setting genau dieser Recall-Bias informativ war. Hauptergebnis unserer Analysen war der deutliche Zusammenhang zwischen richtigem Erinnern an Wheeze-Episoden und einer späteren Asthmadiagnose, der nicht durch andere Faktoren erklärt werden konnte. Bei Asthma im Kindesalter wird eine frühe Diagnosestellung und unverzügliche Behandlung von Asthmasymptomen empfohlen, um ein ungehindertes Lungenwachstum zu erreichen, spätere

Einschränkungen der Lungenfunktion zu verhindern und einer Verschlimmerung hin zu Asthma vorzubeugen. Adäquate Einschätzung der Lungenfunktion bei Kleinkindern ist durch fehlende medizinische Ausstattung und Schwierigkeiten der Kleinkinder die Anweisungen des medizinischen Personals zu verstehen oft limitiert, weswegen sich Ärzte auf elterliche Angaben zu Asthma-Symptomen verlassen müssen. Unsere Ergebnisse deuten darauf hin, dass richtiges elterliches Erinnern frühkindlicher Wheeze-Episoden eine klinische Relevanz für eine spätere Asthmadiagnose und eingeschränkte Lungenfunktion haben und damit einen wichtigen Beitrag zur Prognose- und frühen Diagnosestellung von Asthma leisten können.

Determinants	Crude odds ratio	Adjusted odds ratio
Concomitant rhinitis	0.91 [0.50-1.64]	-
Timing of last wheeze episode (in weeks of age)	1.05 [1.03-1.07]	1.04 [1.01-1.06]
Number of wheeze episodes	1.49 [1.26-1.76]	1.38 [1.17-1.65]
Average duration of wheeze episodes (in days)	0.91 [0.74-1.12]	-
Number of cough episodes	1.09 [1.03-1.16]	-
Number of rhinitis episodes	1.03 [0.98-1.09]	-
Sex	1.56 [0.99-2.47]	-
Farming status	0.75 [0.48-1.18]	-
Mode of birth	1.61 [0.8-3.23]	-
Breastfeeding (>6 months)	1.12 [0.72-1.76]	-
Smoking in pregnancy	1.01 [0.53-1.91]	-
Older siblings	1.18 [0.73-1.91]	-
Parental hay fever	1.09 [0.69-1.71]	-
Parental asthma	2.3 [1.24-4.27]	2.46 [1.27-4.95]
Parental atopic eczema	1.1 [0.5-2.4]	-
Parental atopy	1.32 [0.83-2.08]	-
Parental education	1.06 [0.67-1.67]	-
Having pets in the 4th year	0.71 [0.43-1.19]	-
Day care attendance in the first 3 years	1.44 [0.46-4.55]	-

Odds ratios are given with 95%-confidence intervals. For crude models, only determinants with p-values <0.2 are listed. Adjusted models were established by backward elimination.

Tabelle 6: Determinanten des richtigen Erinnerns an Wheeze-Episoden bei Kindern deren Eltern Wheeze im ersten Lebensjahr (Tagebuchfragebögen) berichteten (n=307)
Siehe Tabelle 1 in der Publikation:
Brick T, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTURE study group. Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes. Pediatr Allergy Immunol. 2019 Aug 23. doi: 10.1111/pai.13118.

Determinants	Crude odds ratio	Adjusted odds ratio
True positive recall	3.45 [1.59-7.51]	4.54 [1.75-14.16]
Sex	2.21 [1.31-3.74]	-
Farmer	0.56 [0.34-0.93]	-
Parental asthma	3.94 [2.3-6.73]	-
Parental hay fever	3.11 [1.83-5.3]	2.86 [1.23-7.09]
Parental atopic eczema	4.08 [1.5-11.13]	-
Parental atopy	2.83 [1.61-4.97]	-
Chr 17q21 (rs7216389)	1.68 [1.12-2.52]	1.92 [1.05-3.61]

Odds ratios are given with 95%-confidence intervals. For crude models, only determinants with p-values <0.05 are listed. Adjusted models were established by backward elimination.

Tabelle 7: Assoziationen zwischen richtigem Erinnern von Wheeze-Episoden mit einer späteren Asthmadiagnose (n=880)

Siehe Tabelle 2 in der Publikation:

Brick T, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTURE study group. Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes. Pediatr Allergy Immunol. 2019 Aug 23. doi: 10.1111/pai.13118.

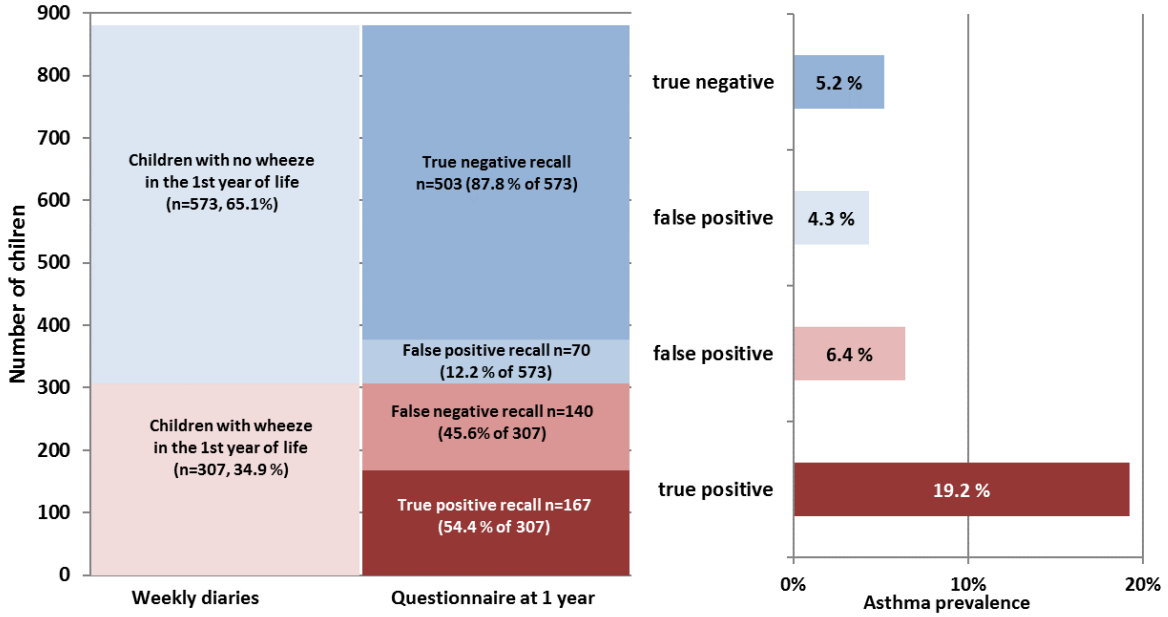

Abbildung 7: Richtiges Erinnern an Wheeze-Episoden und späteres Asthma

Siehe Abbildung 2 in der Publikation:

Brick T, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTURE study group. Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes. Pediatr Allergy Immunol. 2019 Aug 23. doi: 10.1111/pai.13118.

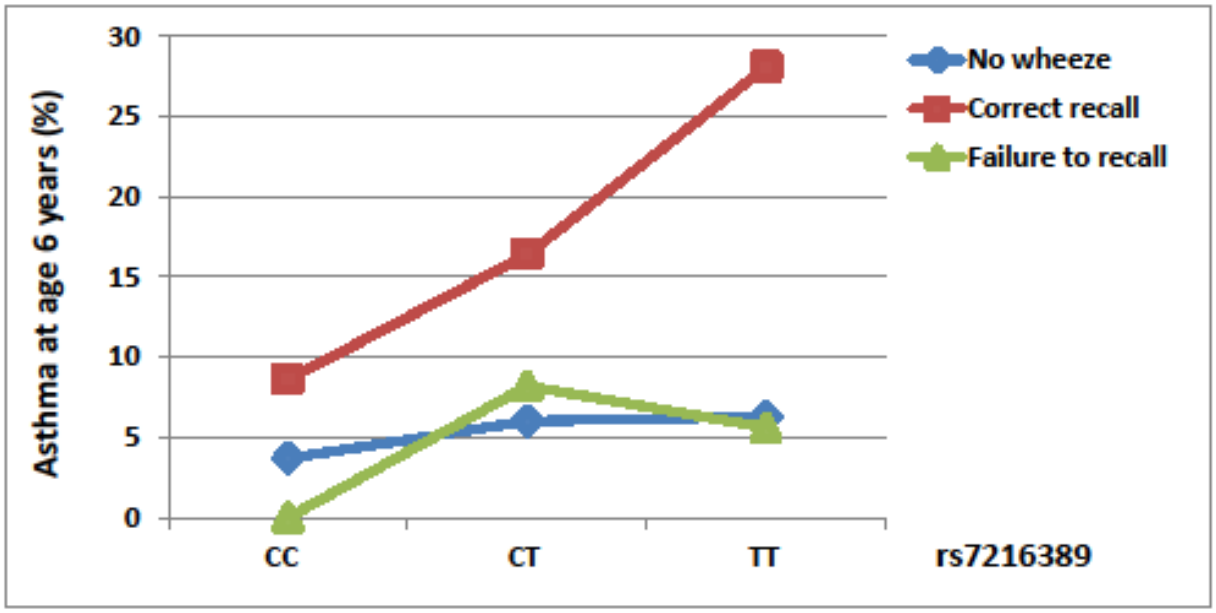

Abbildung 8: Das Asthmarisiko stratifiziert für richtiges Erinnern und die Genotypen des Asthma-Risiko-Allels (rs7126389) auf Chromosom 17q21.

Siehe Abbildung 3 in der Publikation:

Brick T, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTURE study group. Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes. Pediatr Allergy Immunol. 2019 Aug 23. doi: 10.1111/pai.13118.

6.5 Abschließende Zusammenfassung

Im Rahmen einer Metaanalyse wurde der vornehmlich in europäischen Ländern beobachtete asthma- und allergieprotektive Effekt des Konsums unbehandelter Kuhmilch weltweit untersucht und bestätigt. Zusätzlich wurden quantitative Unterschiede von Proteinen und Fettsäuren in unbehandelten und unterschiedlich verarbeiteten Milchen untersucht. Industriell verarbeitete Milchen zeigten insgesamt deutlich geringere Mengen an nativem Protein und an Fettsäuren als Rohmilchen. Bei den Proteinen wurde vor allem bei immunaktiven Proteinen ein deutlicher Rückgang nach industrieller Verarbeitung festgestellt. Der Konsum von Rohmilch mit höheren Omega-3-Fettsäuregehalten war mit einer geringeren Asthmaprävalenz der Kinder assoziiert. Darüber hinaus wurde gezeigt, dass die elterliche Wahrnehmung früher Asthmasymptome einen prädiktiven Wert für eine spätere Asthmaerkrankung ihrer Kinder hat und somit im klinischen Alltag bei der frühen Einschätzung und Behandlung von Asthmasymptomen eine Rolle spielen sollte.

7. Eigenanteil an den Manuskripten

Publikation 1 (Originalarbeit): Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk.

- Bereinigung der Datensätze
- Statistische Analysen
- Interpretation und kritische Diskussion der Ergebnisse
- Verfassen der ersten Manuskriptversion
- Überarbeitung des Manuskripts zusammen mit den Koautoren

Publikation 2 (Originalarbeit): Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins.

- Bereinigung der Datensätze
- Vergleich verschiedener Ansätze zur Ersetzung von fehlenden Messwerten
- Statistische Analyse
- Interpretation und kritische Diskussion der Ergebnisse
- Verfassen der ersten Manuskriptversion
- Überarbeitung des Manuskripts zusammen mit den Koautoren

Publikation 3 (Originalarbeit): Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes.

- Bereinigung der Datensätze
- Statistischen Analysen
- Interpretation und kritische Diskussion der Ergebnisse
- Verfassen der ersten Manuskriptversion
- Überarbeitung des Manuskripts zusammen mit den Koautoren

Publikation 4 (Metaanalyse): The beneficial effect of farm milk consumption on asthma, allergies, and infections: from meta-analysis of evidence to clinical trial.

- Systematische Literaturrecherche
- Statistischen Analysen
- Interpretation und kritische Diskussion der Ergebnisse
- Verfassen der ersten Manuskriptversion (Abschnitt 1-2)
- Überarbeitung des Manuskripts zusammen mit den Koautoren

8. Zusammenfassung auf Deutsch

Asthma gehört in der westlichen Welt mit einer Prävalenz von etwa 10 Prozent zu den häufigsten chronischen Erkrankungen im Kindesalter. Die Erforschung der genauen Erkrankungsmechanismen gestaltet sich wegen der hohen Heterogenität der Asthmaphänotypen schwierig. Auch gibt es bisher keine wirksamen Präventionsmaßnahmen. Forschungsergebnisse der letzten Jahrzehnte haben jedoch aufgezeigt, dass es protektive Umweltfaktoren gibt, die vor Asthma und anderen atopischen Erkrankungen wie Heuschnupfen oder Dermatitis schützen. Einer dieser Faktoren ist das Aufwachsen auf, beziehungsweise der regelmäßige Kontakt mit einem traditionell bewirtschafteten Bauernhof in der Kindheit. Zahlreiche Studien zeigten, dass sich dieser sogenannte *„Bauernhof-Effekt“* auf zwei Faktoren zurückführen lässt, nämlich auf den Kontakt zu Ställen, Kühen und Futtermitteln und auf den Konsum unbehandelter Kuhmilch. Der Konsum unbehandelter Kuhmilch ist für die medizinische Forschung von besonderem Interesse, weil sein asthma- und allergie-protektives Potential unabhängig von einem Leben auf dem Bauernhof besteht.

Im ersten Abschnitt dieser Doktorarbeit wird im Rahmen einer weltweiten Metaanalyse der Zusammenhang zwischen dem Auftreten von Asthma und atopischen Erkrankungen im Kindesalter und dem Konsum unbehandelter Kuhmilch untersucht. Es zeigte sich studienübergreifend ein klarer asthma-protektiver Effekt für den Konsum unbehandelter Kuhmilch, der nicht auf einzelne Regionen beschränkt war. Bezüglich der anderen untersuchten Endpunkte (keuchende/pfeifende Atmung, atopische Rhinokonjunktivitis, Atopie) variierten die Ergebnisse zwischen den Studien stärker. Diese Heterogenität wurde auf Variationen des Studiendesigns, der Expositions- und Endpunktdefinition, sowie des Alters der eingeschlossenen Studienteilnehmer zurückgeführt. Eine eingehende Analyse der vier zentral-europäischen Studien bestätigte zudem, dass der Rohmilch-Effekt nicht an ein Aufwachsen der Kinder auf einem Bauernhof geknüpft war.
Darüber hinaus wurden Milchkomponenten diskutiert, die basierend auf dem aktuellen Stand der Wissenschaft mitverantwortlich für den Effekt unbehandelter Kuhmilch auf Asthma und Allergien sein könnten. Dazu gehören unter anderem Kohlenhydrate, Proteine, Fettsäuren, Mineralien, Vitamine, Hormone, miRNAs und zelluläre Strukturen wie Exosomen, somatische Zellen oder das Milchmikrobiom. Neben den diskutierten Milchkomponenten zeigten sich außerdem Unterschiede in Quantität und Häufigkeit der getrunkenen Milch zwischen den Konsumenten von Roh- und industriell verarbeiteter Milch. Kinder, die vornehmlich rohe oder gering behandelte Kuhmilch direkt von einem Bauernhof tranken, konsumierten insgesamt größere Mengen Milch als Kinder, deren Eltern die Milch im

Supermarkt kauften. Im letzten Abschnitt wurde die MARTHA-Studie vorgestellt, eine 2019 gestartete Interventionsstudie, die die gesundheitlichen Auswirkungen eines regelmäßigen Konsums minimal verarbeiteter Kuhmilch versus UHT-Milch in der frühen Kindheit untersucht. Primärer Endpunkt der MARTHA-Studie ist eine Asthmadiagnose im Alter von fünf Jahren. Zusätzlich werden respiratorische Infektionen, keuchende und pfeifende Atemgeräusche, sowie atopische Sensibilisierung und Ekzeme beobachtet.

Da Kuhmilch aus einer großen Anzahl an Komponenten besteht, ist die Erforschung der genauen Träger des Rohmilcheffekts schwierig. Der zweite Abschnitt der Doktorarbeit behandelt zwei, bisher wenig untersuchte potenzielle Einflussfaktoren des genannten Milcheffekts: Fettsäuren und Proteine. In der PASTURE-Studie konnte der gesundheitsfördernde Effekt von unbehandelter Kuhmilch im Vergleich zu Kuhmilch aus dem Laden zum Teil durch den höheren Fettgehalt in der Rohmilch erklärt werden. Untersuchungen der Fettfraktion und der enthaltenen Fettsäuren zeigten, dass dieser „Fetteffekt“ wiederum auf einen höheren Anteil von Omega-3-Fettsäuren in der Rohmilch zurückzuführen ist. Im menschlichen Organismus werden aus Omega-3-Fettsäuren hauptsächlich anti-inflammatorische Derivate gebildet. Bei regelmäßigem Rohmilchkonsum wird daher eine permanente entzündungshemmende Wirkung vermutet, die zu einem geringeren Auftreten von Asthmasymptomen führen könnte.

Bei der Proteom-Analyse in Proben von unterschiedlich verarbeiteter Milch stellte sich heraus, dass die Anzahl der messbaren Proteine und deren Quantität mit steigenden Erhitzungstemperaturen abnahmen. 23 Proteine zeigten signifikante Quantitätsunterschiede zwischen hoch (>72°C) und niedrig (<72°C) erhitzten Milchen. Vornehmlich Proteine, die bereits mit Immunfunktionen in Verbindung gebracht worden sind, waren in hoch erhitzter Milch signifikant vermindert zu finden. Die Abnahme dieser Proteine könnte Einfluss auf die Maturation des Immunsystems haben oder die Immunantwort des Körpers auf Allergene verändern und daher mitverantwortlich für den Verlust der Asthma-protektiven Wirkung der Milch nach industrieller Verarbeitung sein.

Abschließend wurde im Rahmen der Doktorarbeit untersucht, inwieweit die elterliche Wahrnehmung beziehungsweise die Erinnerung an Asthmasymptome im ersten Lebensjahr der Kinder Aufschluss über die Wahrscheinlichkeit einer späteren Asthmaerkrankung geben kann. Tatsächlich zeigte sich, dass Kinder, deren Eltern sich an Asthmasymptome (keuchende, pfeifende Atmung) erinnerten, ein 3,5-fach erhöhtes Risiko für eine spätere Asthmadiagnose hatten und schlechtere Lungenfunktionswerte im Alter von 6 Jahren

aufwiesen als Kinder, deren Eltern sich nicht an die Asthmasymptome erinnerten. Eltern scheinen intuitiv die Relevanz der beobachteten Symptome zu erkennen und könnten damit bei der frühen Einschätzung des Asthmarisikos ihrer Kinder helfen.

Die Ergebnisse dieser Doktorarbeit weisen darauf hin, dass es einen weltweiten asthma-und atopie-protektiven Effekt des Konsums unbehandelter Kuhmilch gibt. Unterschiedliche Mengen einzelner Milchkomponenten wie bestimmte Fettsäuren und Proteine, könnten mitverantwortlich für diesen Rohmilcheffekt sein. Diese Ergebnisse, wie auch die Erkenntnis, dass die Erinnerung der Eltern an frühere Asthmasymptome ihrer Kinder einen prädiktiven Wert für eine spätere Asthmaerkrankung hat, bilden die Grundlage für weitere Untersuchungen zu präventiven Maßnahmen von Asthma und Atopie im Kindesalter und können dazu beitragen, das Risiko einer späteren Asthmamanifestation frühzeitig zu erkennen.

9. Abstract (in English)

With a prevalence exceeding 10%, bronchial asthma is the most common chronic disease in childhood in the Western world. Due to a high heterogeneity of asthma phenotypes, it is difficult to identify the mechanisms underlying the development of asthma. So far, no preventive measures exist. Over the last decades, however, environmental factors have been identified which have a protective effect on the development of asthma and other atopic diseases such as hay fever or atopic dermatitis. One of these factors is growing up on, or regular contact with a traditionally husbanded farm. Several studies revealed that this so-called „farm effect“can be traced back to two farm-specific exposures: 1. contact to cows, stables and sheds and 2. consumption of unprocessed cow's milk. The asthma and allergy protective potential of unprocessed cow’s milk is of special interest, as it exists irrespective of a farm surrounding.

The first section of this dissertation describes the conducted meta-analysis of the association between asthma and atopic diseases in childhood and the consumption of unprocessed cow’s milk worldwide. The asthma-protective effect of unprocessed cow’s milk persisted irrespectively of the study region. The slight heterogeneity regarding the other outcomes (wheeze, atopic rhinoconjunctivitis, and atopy) probably resulted from variations in study performance, definition of exposures, outcomes and age of study participants. In depth analyses of the four central-European studies further revealed that the milk effect was independent of growing up on a farm. Moreover, milk components that could partly be responsible for the raw milk effect were mentioned and discussed afterwards. These include among others carbohydrates, proteins, fatty acids, minerals, vitamins, hormones, miRNAs and cellular components such as exosomes, somatic cells and the milk microbiome. Besides differences of the milk components, also frequency and quantity of consumed milk differed between the children drinking unprocessed cow‘s milk and children drinking milk bought in a supermarket. Unprocessed cow’s milk directly derived from a farm was consumed at higher quantity. Lastly the MARTHA-study was presented, an intervention study, which started in 2019 and investigates health outcomes comparing children regularly consuming minimally processed cow’s milk and children consuming UHT-milk in early childhood. Primary outcome is an asthma diagnose at the age of five years. Additionally respiratory infections, wheeze, atopic sensitization and eczema were observed.

Cow’s milk comprises a large variety of different components, thus investigating the active ingredient of raw cow’s milk effect is difficult. In the second part of this dissertation, two

specific groups of milk components that could have an impact on mentioned milk effect were discussed: fatty acids and proteins. The PASTURE study revealed that parts of the beneficial effect of unprocessed cow's milk compared to shop milk can be explained by a higher fat content in unprocessed milk. Assessment of fatty acids in the milk fat fraction suggested higher quantities of omega-3 fatty acids in raw cow's milk as an explanation for the observed „fat effect". The human organism metabolizes omega-3 fatty acids mainly to anti-inflammatory derivatives. Regular consumption of unprocessed cow's milk is thus hypothesized to have an ongoing anti-inflammatory effect and might therefor reduce asthma symptoms.

Investigations in protein quantities of differently processed milk samples showed reduced numbers of measurable proteins with increasing heating temperatures. Quantities of 23 proteins significantly differed between high (>72°C) and low (<72°) heat treated milks. Especially proteins associated with immune functions were considerably diminished after high heat treatment. A reduction of these proteins might influence the maturation of the children's immune systems or alter immune responses after contact with allergens and could thus be responsible for the loss of the asthma protective effect of industrially processed milks.

Finally, we investigated parental perception and recall of asthma symptoms within the first year of the child's life and its potential informational value regarding a later asthma diagnosis. In fact, children whose parents correctly remembered early asthma symptoms (wheeze) had a 3.5-fold increased risk for a later asthma diagnosis and had worse lung-function parameters at age 6 years compared to children whose parents did not remember early wheeze episodes. Parents seemed to recognize intuitively the severity of observed symptoms, thus their reports could help improving the early estimation of asthma risks in children.

The results of this dissertation point towards a worldwide validity of the asthma and atopy protective effect of unprocessed cow's milk. Different quantities of milk components such as fatty acids or proteins could be responsible for this milk effect. These findings, as well as the asthma-predictive potential of parental recall of early asthma symptoms provide a basis for future investigations into preventive measures of asthma and atopy in childhood and could improve early risk assessment of a later asthma diagnosis.

10. Publikation 1 (Originalarbeit)

ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk

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Background: Living on a farm has repeatedly been shown to protect children from asthma and allergies. A major factor involved in this effect is consumption of unprocessed cow's milk obtained directly from a farm. However, this phenomenon has never been shown in a longitudinal design, and the responsible milk components are still unknown.

Objectives: We sought to assess the asthma-protective effect of unprocessed cow's milk consumption in a birth cohort and to determine whether the differences in the fatty acid (FA) composition of unprocessed farm milk and industrially processed milk contributed to this effect.

Methods: The Protection Against Allergy—Study in Rural Environments (PASTURE) study followed 1133 children living in rural areas in 5 European countries from birth to age 6 years. In 934 children milk consumption was assessed by using yearly questionnaires, and samples of the "usually" consumed milk and serum samples of the children were collected at age 4 years. Doctor-diagnosed asthma was parent reported at age 6 years. In a nested case-control study of 35 asthmatic and 49 nonasthmatic children, 42 FAs were quantified in milk samples.

Results: The risk of asthma at 6 years of age was reduced by previous consumption of unprocessed farm milk compared with shop milk (adjusted odds ratio for consumption at 4 years, 0.26; 95% CI, 0.10-0.67). Part of the effect was explained by the higher fat content of farm milk, particularly the higher levels of ω-3 polyunsaturated FAs (adjusted odds ratio, 0.29; 95% CI, 0.11-0.81).

Conclusion: Continuous farm milk consumption in childhood protects against asthma at school age partially by means of higher intake of ω-3 polyunsaturated FAs, which are precursors of anti-inflammatory mediators. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Allergy protection, farm milk effect, ω-3 fatty acid, asthma*

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Abbreviations used
aOR: Adjusted odds ratio
BDR: Bronchodilator response
FA: Fatty acid
hsCRP: High-sensitivity C-reactive protein
OR: Odds ratio
PASTURE: Protection Against Allergy—Study in Rural Environments
PUFA: Polyunsaturated fatty acids

Currently, there are no effective preventive measures for asthma and allergies, but there is natural prevention. An example can be found in children growing up on farms, who are at a significantly lower risk for asthma, allergic rhinoconjunctivitis, and atopic sensitization than children living in the same rural area but not directly living on farms. This protective "farm effect" has been shown in many populations and is sustained into adult life.[1,2] The farm exposures contributing to the reduced risk of asthma and allergies have been identified as contact with livestock and animal feed and consumption of unprocessed cow's milk.[3-5] The latter effect is of particular interest because it has been found to work similarly in children from nonfarming families[6-8]; this suggests that a general population might equally benefit from the consumption of unprocessed cow's milk or its native ingredients as well.

Commercially available cow's milk is usually heat treated for inactivating potentially hazardous microorganisms. Thereby other thermolabile milk ingredients, such as proteins, are altered chemically, which might in part explain the loss of the beneficial farm milk effect after pasteurization.[9] Dairy processes also affect the milk lipid fraction because centrifugation and homogenization modify content, balance, and bioavailability of milk fatty acids (FAs).[10] This is important because consumption of milk fat–containing products, such as full-cream milk and butter, has been implied in the protective effect on asthma.[6,11] Likewise, we have previously observed in the Protection Against Allergy—Study in Rural Environments (PASTURE) study that maternal consumption during pregnancy of unskimmed cow's milk and homemade butter affected the fetal immune system in that cord blood mononuclear cells of the exposed neonates produced more of the allergy-protective cytokine IFN-γ on stimulation with mitogens.[12]

Previously, only cross-sectional studies examined the effect of milk consumption on asthma. PASTURE offered the opportunity to step beyond the cross-sectional design and analyze the effect from a longitudinal point of view. The aims of the present analysis were (1) to evaluate the protective effect of farm milk consumption on asthma, (2) to disentangle the effects of heat treatment and alteration of fat composition, and (3) to evaluate the possible role of specific components of milk fat. For the latter, we assessed FA composition in milk samples usually consumed by asthmatic children and healthy control subjects in a nested case-control design.

METHODS

Study design and population

PASTURE is a prospective birth cohort study conducted in rural areas of 5 European countries: Germany, Austria, Switzerland, Finland, and France.[13]

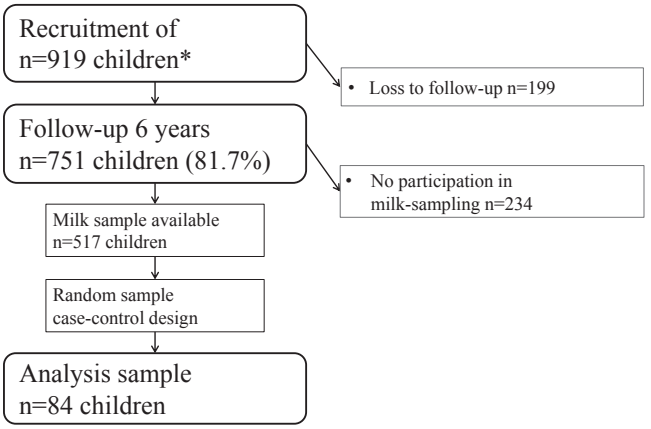

FIG 1. Selection of study population. *Exclusion of Finnish children because the milk module was not implemented in Finland.

The study was approved by local research ethics committees in each country, and written informed consent was obtained from the children's parents. Women were recruited during the last trimester of pregnancy (Fig 1). Women living on an animal husbandry farm were assigned to the farming group (n = 351), and women living in the same area but not on a farm were assigned to the reference group (n = 400). Because in Finland no milk samples were taken, Finish children and those having not completed the follow-up period of 6 years (n = 199) were excluded for later analysis. For measuring milk FA content, a case-control population (1:1.5) consisting of 84 children was selected from all children participating in the milk sampling (n = 517) at age 4 years. This case-control population contained all asthmatic patients with available milk samples (n = 35) and a random sample of healthy children (n = 49) exceeding the cases by about 50%. Cases were defined as children having a lifetime diagnosis of asthma once or obstructive bronchitis at least twice, as reported by the parents at the age of 6 years (n = 35), and control subjects were defined as children without such a diagnosis (n = 49).

Measurements

The questionnaires were based on items of the International Study of Allergy and Asthma in Childhood, the Allergy and Endotoxin study, the Prevention of Allergy—Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle study, and the American Thoracic Society questionnaire. Socioeconomic and lifestyle factors, agricultural exposures, and respiratory and other health factors of these women, their husbands, and their children were assessed through questionnaires during pregnancy and regularly up to age 6 years. At the age of 4 years, samples of the children's "usually" consumed milk were taken in the nested case-control sample. A short questionnaire accompanying the milk sample collected data on type of milk. Milk types were defined as follows: farm milk (cow's milk directly derived from a traditionally husbanded farm), shop milk (any cow's milk bought from a shop or supermarket), unprocessed farm milk (farm milk consumed exclusively without any prior boiling), and boiled farm milk (farm milk normally boiled before consumption). Furthermore, the milk fat content was dichotomized at 3.5%, which is consistent with the usual definitions of whole milk in Europe and the United States.[14,15] Thus high-fat milk was defined as shop milk with a fat content of at least 3.5% or farm milk without skimming.[13] At the same time, serum samples were taken from children and quantified for high-sensitivity C-reactive protein (hsCRP) values as a marker of inflammation.[16] At age 6 years, FEV_1 and bronchodilator response (BDR; relative change of at least 12% in FEV_1 after versus before administration of a short-acting β-agonist) were measured, as previously described.[17] Combination variables reflecting functional airway obstruction and reversibility were defined as follows: asthma with FEV_1 greater than or less than the median (1.22 L) and asthma responding or not responding to a bronchodilator with 12% improvement in FEV_1.

FA assessment in cow's milk samples

Characteristics of milk samples are given in Table E1 in this article's Online Repository at www.jacionline.org. After collection, the milk samples were stored at −80°C and thawed shortly before measurement. All samples were measured in duplicates according to the method of Bocking et al.[16] FAs were extracted from 100 μL of cow's milk with 2 mL of MeOH containing internal standard (C18iso, 250 mg/L) and 1 mL of chloroform. After 5 minutes of mixing, again, 1 mL of chloroform and 1 mL of NaCl solution (0.9%) were added to facilitate separation of the phases, followed by 10 minutes of centrifugation at 2100*g*. The chloroform phase was transferred to a fresh vial and evaporated with nitrogen at 37°C until dry. Afterward, the extract was dissolved in 2 mL of hexane. Derivatization of the FAs was performed by adding 0.3 mL of a 2N KOH/MeOH solution and mixing for 5 minutes. The reaction was stopped with the addition of 0.5 g of $NaHSO_4$, followed by another centrifugation step for 5 minutes at 1100*g*. The upper phase containing the FA methyl esters was transferred to a fresh vial and again evaporated under nitrogen. The residue was dissolved in 250 μL of hexane. A panel of 42 FAs was determined by using gas chromatography coupled to a mass spectrometer (for detailed quality controls, see the Methods section in this article's Online Repository at www.jacionline.org). Quantities of FAs were given as arbitrary units approximately corresponding to milligrams per liter. After validation in preliminary analyses, arbitrary unit values were entered in the models without further adjustment for the total amount of FA. FA arbitrary units were summed up within FA groups defined by their chemical properties: saturated FAs, monounsaturated FAs, polyunsaturated fatty acids (PUFAs), ω-3 PUFAs, ω-6 omega-6 PUFAs, trans-FAs, and conjugated linoleic acids.

Statistical analysis

For all statistical analysis, R 3.1.0 software (R Core Team, 2014) was used. To discover disparities in the populations, the children in the recruitment population, the follow-up population, and the analysis sample were compared with respect to socioeconomic and nutritional characteristics by using the Fisher exact test. Milk consumption patterns were compared between 2 subsequent years by using the Pearson correlation coefficient. Logistic regression models were applied to estimate odds ratios (ORs) with 95% CIs for doctor-diagnosed asthma and consumption of different milk types in the follow-up population and analysis sample. Because of the skewed distributions of some FA variables, all FA variables were used after rank transformation for mutual comparisons. The variables for ω-3 and ω-6 PUFAs followed a log-normal distribution and were used log-transformed. The proportion of the respective milk effects on asthma explained by distinct FA groups was quantified by using the change-in-estimate method. Correlation between distinct ω-3 PUFA species and their group variable were assessed by using Spearman rho. *P* values of less than .05 were considered statistically significant. hsCRP values were classified into 3 categories: nondetectable values (<0.20 mg/L), less than the median of detectable values (0.81 mg/L), and greater than the median of detectable values.

RESULTS

The selection process of the population is illustrated in Fig 1. In the follow-up population breast-feeding for at least 6 months was more common and smoking during pregnancy was less common compared with the recruitment population (see Table E2 in this article's Online Repository at www.jacionline.org). As expected, children with and without asthma differed with respect to sex, family history of asthma, and milk consumption (see Table E3 in this article's Online Repository at www.jacionline.org). In the analysis sample the proportion of children with a doctor's diagnosis of asthma was increased by design, and consequently, children consuming shop milk were enriched (see Table E2); asthma cases were more exposed to smoking during pregnancy (see Table E3).

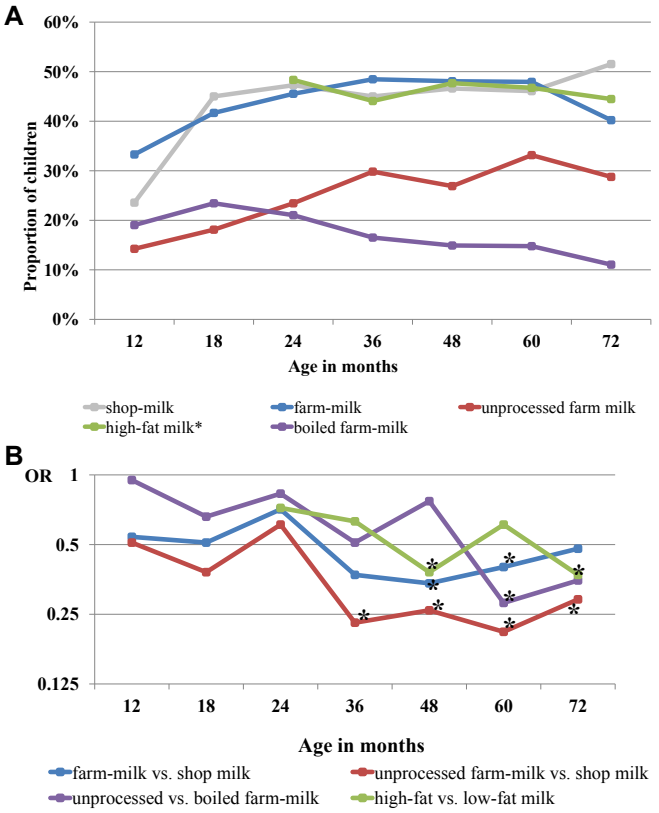

FIG 2. Frequency of milk types consumed over time **(A)** and effects on asthma (in the follow-up population, n = 751; **B**). Fig 2, *A*, Frequency of consumption of milk types from age 1 year until age 6 years. *No data on fat content of consumed milk was collected until age 2 years. Fig 2, *B*, Effects of consuming different milk types at different time points on asthma as defined by age 6 years. ORs were adjusted for center and farming because of the study design. *Significant values, $P < .05$.

In the follow-up population overall consumption of farm milk increased from 1 to 3 years and decreased slightly after 5 years (Fig 2, *A*). At the age of 2 years, consumption of unprocessed farm milk became increasingly more common and replaced successively boiled farm milk; consumption of high-fat milk did not change with age. The high correlations with the preceding years (see Fig E1 in this article's Online Repository at www.jacionline.org) indicate a rather constant consumer behavior with respect to milk type. The effects on asthma of drinking unprocessed farm versus shop milk, unprocessed versus boiled farm milk, or high-fat versus low-fat milk tended to increase with age (Fig 2, *B*); the adjusted odds ratio (aOR) of unprocessed milk versus shop milk consumption decreased, for example, from 0.51 (95% CI, 0.15-1.73) at 1 year to 0.29 (95% CI, 0.11-0.76) at 6 years of age (see Table E4 in this article's Online Repository at www.jacionline.org). The mutually adjusted effects on asthma of unprocessed farm milk versus shop milk and high-fat versus low-fat milk consumption over time were 0.50 (95% CI, 0.25-0.98) and 0.60 (95% CI, 0.36-1.01), respectively. As shown in Table E5 in this article's Online Repository at www.jacionline.org, mutual adjustment of both effects led to a reduction of the respective estimates by about 20%, which means that 20% of both effects overlap. The protective effect of high-fat milk tended to be stronger for asthmatic patients with FEV_1 greater than the

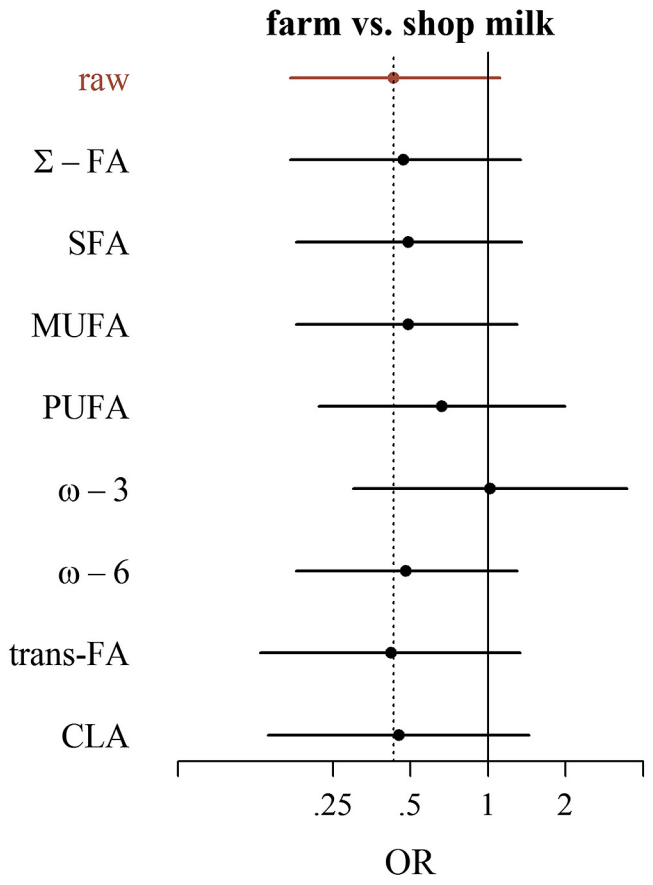

FIG 3. Change in estimate of the farm milk effect on asthma by distinct FA groups. Because of skewed distributions of some FA variables, all FA variables were used after rank transformation. *CLA*, Conjugated linoleic acid; *MUFA*, monounsaturated fatty acid; *SFA*, saturated fatty acid.

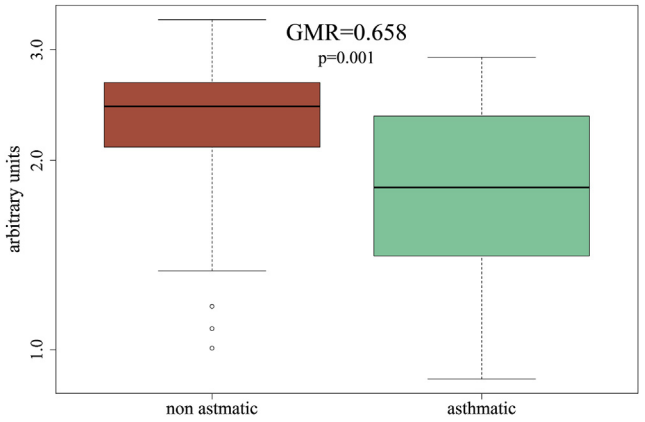

FIG 4. ω-3 PUFA levels (log-transformed) in milk samples consumed by asthmatic and nonasthmatic children and geometric mean ratio *(GMR)*. The GMR was calculated because of log-normal distribution of ω-3 levels.

median or a positive BDR, respectively, although the sample size did not allow for formal confirmation of heterogeneity of effects (see Table E6 in this article's Online Repository at www.jacionline.org). In contrast, the effect of heating was not related to disease severity, as reflected by FEV_1 and BDR (data not shown).

The case-control sample for the analysis of FA contents showed associations similar to those for the follow-up population, thereby showing its representativeness (see Fig E2 in this article's Online Repository at www.jacionline.org). When adjusting the association of farm milk and asthma for the FA groups (Fig 3), the ω-3 group had the strongest change in estimate (111%, see Table E7 in this article's Online Repository at www.jacionline.org). The ω-3 PUFA contents differed significantly between cases and control subjects, with asthmatic children consuming milk with a 35% poorer ω-3 PUFA content (geometric mean ratio, 0.658; $P = .001$; Fig 4). Moreover, the inverse association of ω-3 PUFAs with asthma (aOR, 0.29; 95% CI, 0.11-0.81) was not explained by any potential confounder (see Table E8 in this article's Online Repository at www.jacionline.org).

The overall fat content of unprocessed farm milk was 4.0%, on average, with only a few families (15.6%) skimming the milk before consumption. Conversely, consumption of skimmed or semiskimmed shop milk (<3.5%) was rather common (61.2%). The ω-3 content of unprocessed farm milk was substantially higher compared with that of full-fat shop milk ($P = .03$), which again exceeded the ω-3 content of low-fat shop milk considerably ($P = .0001$; Fig 5, *A*). In contrast, the ω-6 content was only marginally related to the overall fat content (data not shown), resulting in a profoundly skewed ω-6/ω-3 ratio of 3.38:1 in low-fat milk compared with 1.75:1 in high-fat milk (see Table E9 in this article's Online Repository at www.jacionline.org). Yet among high-fat milk samples, the ω-6/ω-3 ratio was affected by thermal treatment and industrial processing, with a significant trend from unprocessed over boiled farm milk to shop milk ($P = .015$; Fig 5, *B*). The ω-6/ω-3 ratio of milk samples collected at age 4 years was positively associated with hsCRP values in serum measured at the same age ($P = .038$, see Fig E3).

DISCUSSION

Regular unprocessed milk consumption was inversely related to asthma onset by age 6 years. The association was stronger with recent exposure compared with exposure in early childhood. This protective effect of native milk was explained partly by absent heating and partly by a higher fat content. The effect of fat content was largely attributable to higher ω-3 PUFA levels and a lower ω-6/ω-3 ratio in unprocessed milk compared with industrially processed milk. The inverse effect of ω-3 PUFA contents on asthma itself was strong and not explained by any potential confounder.

The asthma-protective effect of farm milk consumption in childhood is well in line with findings from several other countries in and beyond Europe.[1,2,5-7,9,18-20] This milk effect is independent from other farm-related exposures, such as animal shed visits, and it is not confounded by family size, family history of atopy, or any other known confounder.[5]

Cow's milk is consumed predominantly by young children, for whom it serves as a breast milk replacement after weaning. Therefore the protective effect of farm milk has previously been attributed to consumption in infancy.[20] Moreover, an anti-infectious effect of unprocessed milk during the first year of life has been found in the PASTURE cohort.[21] However, our present analyses suggest that at least the beneficial effect on asthma increases over time. Recent consumption of farm milk seems to be more relevant than consumption in the first years of life, which extends the concept of early prevention to sustained prevention

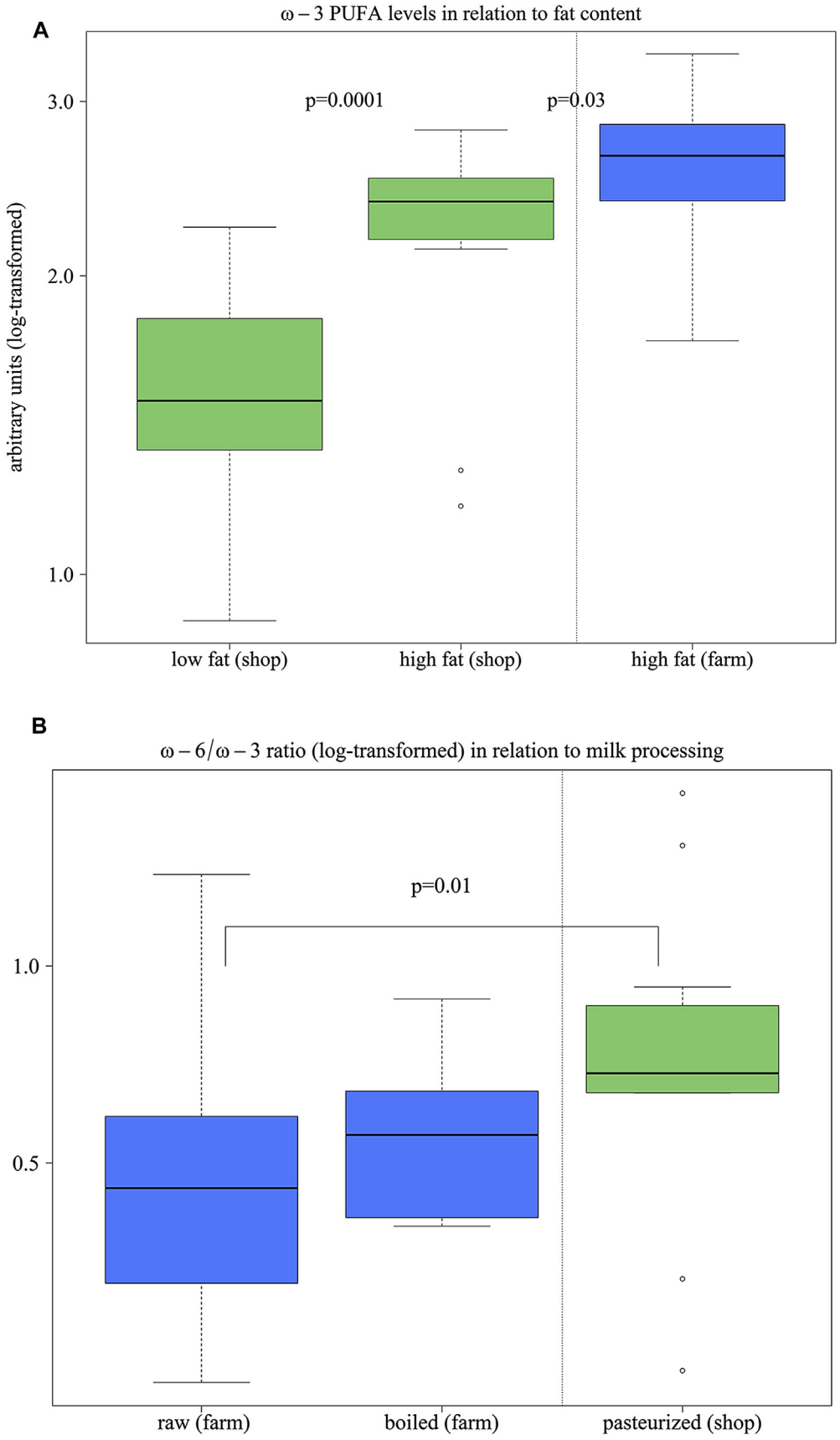

FIG 5. ω-3 PUFA levels in milk samples consumed by PASTURE children **(A)** and ω-6/ω-3 ratio in relation to milk processing **(B)**. Fig 5, *A*, Different ω-3 PUFA levels (log-transformed) in milk samples "usually" consumed by PASTURE children. Fig 5, *B*, Different ω-6/ω-3 ratios (log-transformed) in milk samples "usually" consumed by PASTURE children in relation to milk processing. In Fig 5, *A*, the *t* test (2-sided) was used to calculate differences between milk variables. Fig 5, *B*, shows differences among high-fat milk samples. The *P* value refers to a trend test.

until school age and beyond. An alternative explanation for the increasing effect size might be found in the growing prevalence of farm milk consumption with age. Nevertheless, the proportion of full-fat milk consumption remained stable over time, although its effect strengthened. Thus this phenomenon invalidates the explanation by growing prevalence.

The discrepancy between the effects of heating and fat content brings us to the following question: What is the critical difference between unprocessed farm milk and industrially processed milk that drives the effect? Essentially, industrial processing involves centrifugation, homogenization, and heat treatment. Obviously, the latter process affects thermolabile milk components, such as microorganisms, whey proteins,[9] or microRNA. Alteration of microRNA content and composition is demonstrated in a separate article.[22] Because farm milk is not homogenized but shop milk generally is, it is difficult to disentangle the effects of homogenization and heat treatment. Centrifugation removes particles, microorganisms, and somatic cells; however, its main goal is to regulate the fat content of the final product. The fat content of native cow's milk varies with breed, feeding, and regional origin[23] and reaches values of 6% or greater, whereas the content of commercially available milk is usually adjusted to 3.5%, 2%, or 1.5%.

Thus a further aim of our analysis was to allocate the farm milk effect to the respective procedures involved in industrial milk processing. The answer to this question was somewhat ambiguous because both fat content and heating exerted strong independent effects on asthma. Consumption of unprocessed versus boiled farm milk clearly mattered, thereby supporting an important role of thermolabile ingredients (Fig 2, *B*). Moreover, adjustment for fat content weakened the effect of unprocessed farm versus shop milk on asthma substantially (see Table E5). This corresponds well to previous findings of inverse associations between asthma and the consumption of butter or full-fat farm milk.[6,11,12]

In contrast to heating, the fat content was associated with disease severity: high-fat milk exerted a somewhat stronger effect on asthma with an FEV_1 of greater than the median or a positive BDR, respectively, compared with asthma with an FEV_1 of less than the median or a negative BDR, respectively (see Table E6). The limited sample size precludes formal evidence of effect heterogeneity; nevertheless, this tendency was consistent over time, thus implying a systematic difference. The more pronounced effect of milk fat on milder forms of asthma suggests a more susceptible phenotype that might be alleviated by a natural form of symptomatic treatment in contrast to more severe asthma phenotypes.[17]

Therefore we were particularly interested in the composition of the fat compartment of cow's milk. Milk fat mainly consists of triglycerides, which comprise esters of the trivalent alcohol glycerol with FAs. The latter vary predominantly with the number of carbon atoms and the proportion of unsaturated bonds between them. Because of limited power, we assessed the FAs in groups of similar chemical properties, such as saturated FAs or PUFAs, or by the distance of the last double bond to the last carbon atom (ie, ω-3 vs ω-6 PUFAs). First, we confirmed that the 35 cases and their control subjects, exceeding them by one and a half times, were only selected for asthma status and related variables, such as family history of asthma (see Table E3). Second, we verified that the associations between milk types and asthma in this analysis population matched those of the entire follow-up cohort (see Fig E2).

TABLE I. Spearman correlation* of single ω-3 PUFAs with the sum of ω-3 PUFAs

	FAs	Correlation
C18.3n3	α-Linolenic acid	0.994
C20.3n3	Eicosatrienoic acid	0.909
C20.5n3	Eicosapentaenoic acid	0.937
C22.4n3	Docosatetratetraenoic acid	0.073
C22.5n3	Docosapentaenoic acid	0.751
C22.6n3	Docosahexaenoic acid	0.624

*Spearman correlation was used because of skewed distributions of some ω-3 PUFA variables.

To figure out which of the FA groups best explained the effect of farm versus shop milk and whether FAs contributed to the effect of fat content and milk processing, we adjusted the respective logistic regression models for the FA groups (Fig 3). In contrast to all other FA groups, only the PUFA group and particularly the ω-3 PUFAs changed the estimate of the effect of farm milk consumption on asthma. We interpret these findings that the ω-3 PUFA group is specifically involved in the protective effect of farm milk consumption on asthma. Hence we assessed the ω-3 PUFA levels in more detail and found a much stronger gradient of ω-3 PUFA levels between high- and low-fat milk compared with ω-6 PUFA levels (see Table E9). This phenomenon might be attributed to 3 factors.

First, the average fat content of the farm milk samples of 4% exceeds the standard value of full cream shop milk by 14%.

Second, most of the farm milk samples in our study were derived from grass-fed or grazing cows, which generally have a lower ω-6/ω-3 PUFA ratio compared with cows fed a mixed diet based on hay, silage, and concentrate.[24] Conversely, shop milk is a blend of various milk batches from all over the European common market with a rather low proportion of milk from pasturing animals.

Third, enzymes metabolizing ω-6 and ω-3 PUFAs differently might be released by mechanical damage to microvesicles or inactivated by industrial processing or heating.[25,26] The latter assumption is suggested by the different ω-6/ω-3 ratios between raw and boiled farm milk (Fig 5, *B*).

The relevant ω-3 PUFA species driving the effect were identified by the highest individual correlations with their group (Table I) as α-linolenic acid (C18.3n3) and its 20-carbon chain derivatives, eicosatrienoic acid (C20.3n3) and eicosapentaenoic acid (C20.5n3). α-Linolenic acid and its ω-6 counterpart, linoleic acid (C18:2n-6), are essential PUFAs metabolized by the same set of enzymes (ie, elongases and Δ5 and Δ6 desaturases; Fig 6). However, ω-3 PUFAs are precursors of anti-inflammatory mediators, whereas ω-6 PUFAs are precursors of proinflammatory mediators. In general, the mentioned enzymes metabolize ω-3 PUFAs with higher affinity than ω-6 PUFAs. In our unprocessed milk samples the overall ω-6/ω-3 ratio of 1.59:1 (see Table E9) was rather favorable with respect to recommended values of 1:1 to 4:1 in foods.[27,28] A lower ω-6/ω-3 ratio limits the metabolization of the proinflammatory prostaglandin D_2 and cysteinyl leukotrienes,[29] although the metabolic pathways might be more complex.[30,31] Ultimately, ω-3 PUFAs interfere with the synthesis of proinflammatory leukotrienes, thereby acting against asthma in a similar way as the widely used leukotriene receptor antagonists.[29] Indeed, supplementation with ω-3 PUFAs has been suggested for prevention and amelioration of asthma,

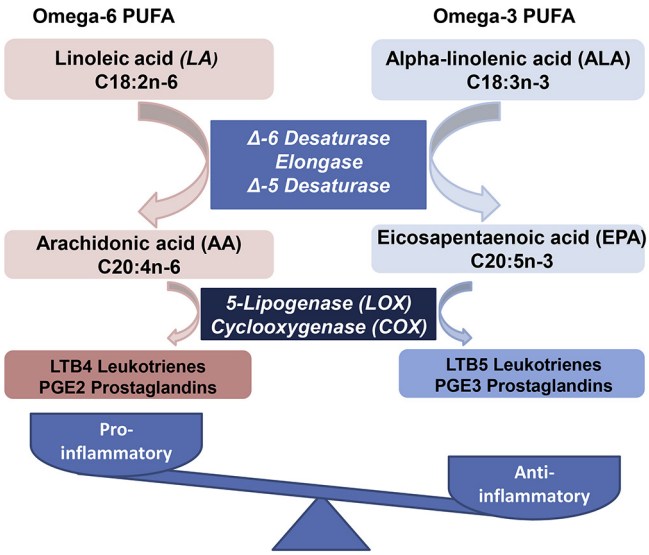

FIG 6. Metabolism of linoleic and α-linolenic acid. In mammalians the PUFA profile is derived from essential FA precursors of both ω-3 and ω-6 PUFAs (α-linolenic acid [18:3 ω-3] and linoleic acid [18:2 ω-6], respectively). Long-chain PUFAs are synthesized endogenously through reactions of both insertion of additional double bonds (desaturases) and elongation of the acyl chain (elongase). ω-3 and ω-6 PUFAs compete for the same set of enzymes in this pathway, with a preferential affinity of ω-3 over ω-6 PUFAs. *LTB*, Leukotriene B; *PGE*, prostaglandin E.

allergies, and inflammatory diseases.[27,29,32] Moreover, in our population we found a positive association of the ω-6/ω-3 ratio in milk with levels of serum hsCRP, a marker of low-grade inflammation[16]; this again supports the suggested anti-inflammatory effect of cow's milk.

Admittedly, several trials supplementing mothers during pregnancy or infants during the first years with ω-3 PUFAs failed with respect to prevention of atopic disease.[33-37] However, most of these studies are hampered by a limited duration of the intervention or an insufficient follow-up time. Only one study compared diets enriched for ω-3 versus ω-6 PUFAs starting from 6 months until assessment of asthma at age 5 years. The authors explained the failure of the intervention by insufficient adherence to the protocol. In contrast, in our study the exposure to farm milk was part of the children's usual diet and did not require profound changes in nutritional habits. Nevertheless, we acknowledge that our observational data are not immune to residual confounding, although the specificity of the ω-3 PUFA effect was remarkable.

The major strength of this analysis is the longitudinal study design, with several points of exposure assessment before determining the outcome based on a physician's diagnosis. The restriction of the study population to rural regions of Europe might be considered a potential shortcoming. However, previous studies have shown that the effects of farm milk consumption on asthma and atopy can be found in both suburban and urban settings.[6] In contrast to other farm-related exposures, such as stable visits, the effect of farm milk consumption can be considered as a paradigm for preventive strategies in a general population. Parent-administered questionnaires might be examined with respect to potential bias by social desirability because consumption of unprocessed milk is clearly discouraged. However, parental answers on milk types and mode of milk consumption have previously been validated by objective measures of heat treatment and fat content in a similar population.[9]

In summary, our data demonstrate that continuous consumption of unprocessed farm milk contributes to protection from childhood-onset asthma. To a substantial extent, this effect is attributable to the higher ω-3 PUFA content in unprocessed cow's milk compared with processed shop milk. The advantageous ω-6/ω-3 ratio in cow's milk might shift the metabolic balance of eicosanoid synthesis from proinflammatory to anti-inflammatory mediators, thereby suggesting that the farm milk effect partially consists of an anti-inflammatory treatment of subclinical asthma. Future interventional studies will determine whether fortification of industrially processed milk with ω-3 PUFAs might be a promising approach to primary or secondary prevention of childhood asthma.

We thank Lydia Lerch and Alexandra Fischer for excellent technical assistance. Samples were stored in the Marburg Biobank CBBMR.

Clinical implications: Higher ω-3 PUFA levels, as contained in unprocessed cow's milk, might contribute to natural asthma prevention.

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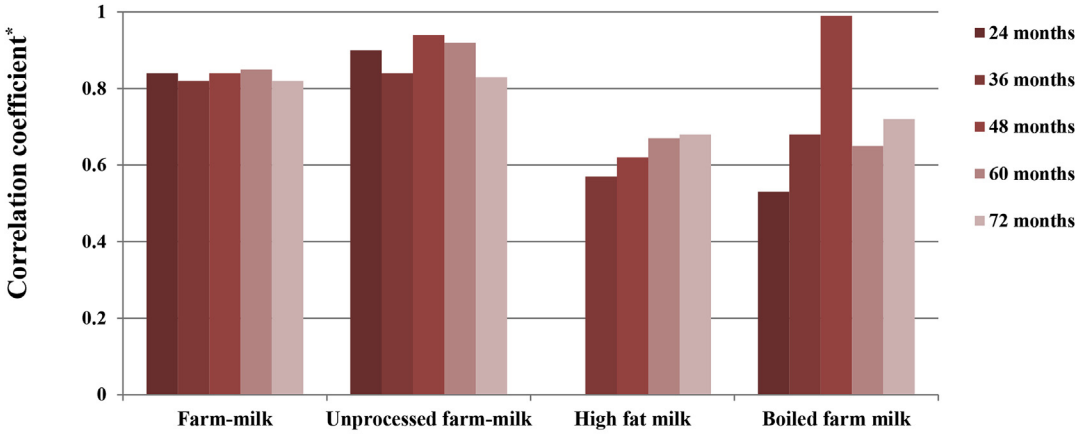
METHODS

Quality control of FA determination

Quality control was conducted as follows. Thawing effects were controlled by establishing comparative measurements in fresh milk samples or samples thawed once or twice, showing that FA pattern and content were not significantly affected by freezing and thawing. For internal quality control, each sample was spiked with C18-iso as an internal artificial standard not occurring in natural sources. For external control, a standard panel containing 42 FAs was measured 2 times per day or at least after the run of 12 samples.



*Correlation coefficient respectively relating to previous year

FIG E1. Correlation of milk consumption over time.

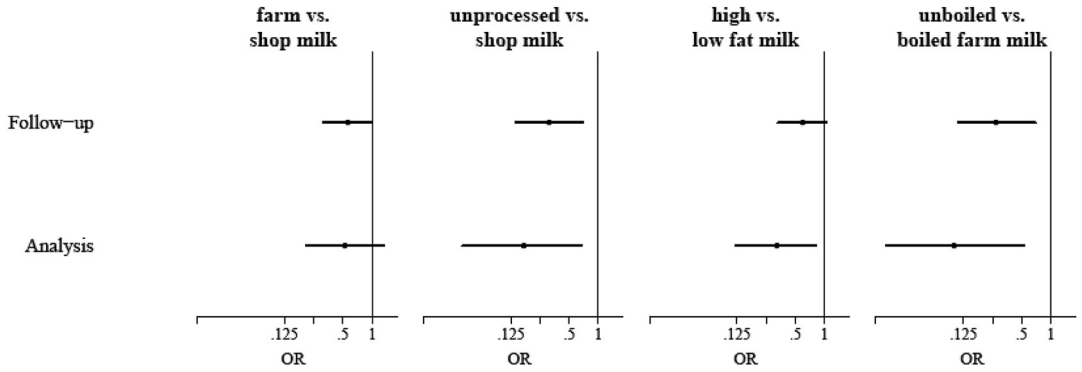
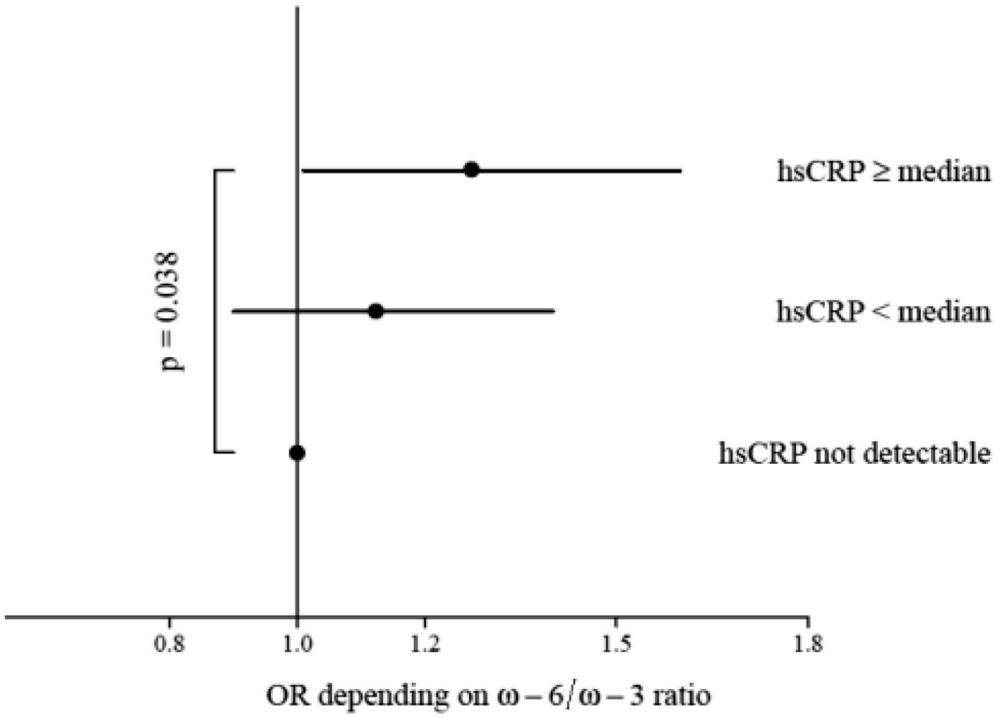


FIG E2. Milk effect in the follow-up population compared with that in the analysis population (5 years).



hsCRP not detectable (detection limit 0.20 mg/L): reference group.

FIG E3. ORs for higher hsCRP values depending on ω-6/ω-3 ratios in consumed milk.

TABLE E1. Characteristics of milk samples assessed in the case-control sample (n = 84)*

	Shop milk (n = 46)			Farm milk (n = 37)	
	Pasteurized (n = 13)	UHT (n = 30)	Other (n = 3)	Raw (n = 25)	Boiled (n = 12)
High fat (≥3.5% fat)	9	4	2	25	12
Low fat (<3.5% fat or skimmed)	2	22	1	0	0
No information about fat content	2	4	0	0	0

*One child provided no information about type of collected milk sample at age 4 years.

TABLE E2. Characteristics of the recruitment, follow-up, and analysis populations

	Recruitment population		Follow-up population			Analysis population		
	n = 919 children	Percent	n = 751 children	Percent	*P* value*	n = 84 children	Percent	*P* value†
Male sex	456	49.6	392	52.3	.63	46	54.8	.65
Farmer	418	45.5	351	46.7	.12	42	50.0	.56
Older siblings	553	60.2	459	62.5	.22	53	63.1	.72
Parental asthma	134	14.6	115	15.3	.79	19	22.6	.05‡
Parental atopy	450	49.0	382	50.9	.71	47	56.0	.66
Breast-feeding ≥6 mo	423	46.0	382	50.9	.01‡	36	42.9	.19
Smoking during pregnancy	125	13.6	86	11.5	.00‡	8	9.5	.72
Cesarean section	213	23.2	155	20.6	.14	16	19.0	.77
Domestic animals (cat/dog)	197	21.4	179	23.8	.66	18	21.4	.59
High parental education§	414	45.0	353	47.0	.36	46	54.8	.16
At age 3 y								
Day care attendance	18	2.0	16	2.1	.69	1	1.2	1.0
At age 5 y								
Milk consumption, never	NA		36	4.8	NA	4	4.8	1.0
Mainly unprocessed farm milk			249	33.2		20	23.8	.06
Mainly boiled farm milk			111	14.8		14	16.7	.18
Mainly shop milk			346	46.1		45	53.6	.06
Mainly milk with a fat content ≥3.5%			351	46.7		37	44.0	.63
Mainly milk with a fat content <3.5%			351	46.7		42	50.0	.63
Stable visits ≥5 times per week	NA		151	20.1	NA	15	17.9	.49
At age 6 y								
Doctor's diagnosis of asthma	NA		57	7.6	NA	35	41.7	.00‡

NA, Not applicable.

**P* values are derived from comparisons of participants (n = 751) and nonparticipants (n = 168) in the follow-up.

†*P* values are derived from comparisons of participants (n = 84) and nonparticipants (n = 667) in the follow-up.

‡Significant difference, $P \leq .05$.

§At least 1 parent with education of greater than 10 years.

TABLE E3. Characteristics between children with and without asthma and cases and control subjects, respectively, in the follow-up and analysis populations

	Study population (n = 751 children)			Analysis population (n = 84 children)		
	Children without asthma (n = 689)	Children with asthma* (n = 57)	*P* value (Fisher exact test)	Control subjects (n = 49)	Cases (n = 35)	*P* value
Male/female sex	350/338	38/19	.03	20/29	26/9	<.01
Farmer/nonfarmer	324/365	25/32	.68	26/23	16/19	.66
Older siblings, yes/no	423/266	34/23	.78	30/19	23/12	.82
Parental asthma, yes/no	94/591	21/33	<.01	5/44	14/19	.01
Parental atopy, yes/no	337/348	39/17	<.01	23/26	24/11	.07
Breast-feeding ≥6 mo/<6 mo	352/312	28/23	.88	21/28	15/15	.64
Smoking during pregnancy, yes/no	976/611	9/46	.27	1/48	7/27	<.01
Cesarean section, yes/no	136/545	10/47	.73	7/41	8/27	.39
Domestic animals (cat/dog), yes/no	168/517	10/45	.33	13/36	5/30	.28
High parental education,† yes/no	320/360	29/26	.48	26/23	20/14	.66
At age 3 y						
Day care attendance, yes/no	14/489	2/35	.30	0/31	1/19	.39
At age 5 y						
Milk consumption, yes/no	648/33	53/3	.75	44/4	35/0	.13
Mainly unprocessed farm milk/shop milk	240/310	8/33	<.01	17/22	3/23	<.01
Mainly farm milk, boiled/unboiled	98/240	12/8	<.01	5/17	9/3	<.01
Mainly milk with a fat content ≥ 3.5%/<3.5%	327/317	20/33	.09	26/18	11/24	.02
Stable per week visits ≥5 times/<5 times	141/173	9/11	1	9/16	6/8	.74

*Five children without information about asthma status.
†At least 1 parent with education of greater than 10 years.

TABLE E4. ORs* and CIs for the effects of consuming different milk types over time (from age 1 to age 6 years) on asthma (age 6 years)

Age of children (mo)	Farm milk vs shop milk, OR (95% CI)	Unprocessed farm milk vs shop milk, OR (95% CI)	Unprocessed vs boiled farm milk, OR (95% CI)	High-fat milk (≥3.5%) vs low-fat milk (<3.5%), OR (95% CI)
12	0.54 (0.20-1.45)	0.51 (0.15-1.73)	0.95 (0.31-2.90)	—
18	0.51 (0.23-1.14)	0.38 (0.13-1.12)	0.66 (0.23-1.89)	—
24	0.71 (0.34-1.51)	0.61 (0.24-1.59)	0.83 (0.33-2.06)	0.72 (0.39-1.33)
36	0.37 (0.18-0.78)	0.23 (0.09-0.59)	0.51 (0.19-1.38)	0.63 (0.33-1.20)
48	0.34 (0.16-0.71)	0.26 (0.10-0.67)	0.77 (0.26-2.32)	0.38 (0.18-0.78)
60	0.40 (0.19-0.85)	0.21 (0.08-0.54)	0.28 (0.11-0.75)	0.61 (0.32-1.15)
72	0.48 (0.22-1.03)	0.29 (0.11-0.76)	0.35 (0.12-1.02)	0.37 (0.19-0.75)

*Adjusted for center and farming.

TABLE E5. Separate and mutually adjusted effects of industrial processing and fat content on asthma

	Separate model		Mutually adjusted model		
	OR (95% CI)	*P* value	aOR (95% CI)	*P* value	CIE*
Unprocessed farm vs shop milk	0.40 (0.20-0.80)	.01	0.50 (0.25-0.98)	.04	24%
High- vs low-fat milk	0.53 (0.34-0.83)	.01	0.60 (0.36-1.01)	.05	20%

CIE, Change in estimate.
*Estimates are averaged over the first 6 years.

TABLE E6. Effect of high-fat milk on asthma (follow-up population) over time

	Effect of high-fat milk on asthma (at age 6 y)			
Age of children (mo)	+ FEV_1 > median (1.22 L), OR (95% CI)	+ FEV_1 < median (1.22 L), OR (95% CI)	+ responding to a bronchodilator by 12% improvement in FEV_1, OR (95% CI)	+ not responding to a bronchodilator by 12% improvement in FEV_1, OR (95% CI)
36	0.21 (0.06-0.74)	0.95 (0.38-2.36)	0.42 (0.13-1.36)	0.57 (0.22-1.44)
48	0.29 (0.09-0.91)	0.53 (0.21-1.31)	0.13 (0.03-0.59)	0.79 (0.31-2.02)
54	0.27 (0.09-0.84)	0.48 (0.18-1.28)	0.25 (0.07-0.86)	0.43 (0.16-1.17)
60	0.35 (0.12-0.97)	1.08 (0.43-2.69)	0.43 (0.13-1.42)	0.67 (0.28-1.59)
72	0.20 (0.06-0.69)	0.58 (0.22-1.53)	0.08 (0.01-0.65)	0.49 (0.19-1.26)

TABLE E7. Farm milk effect on asthma (at age 6 years): raw estimate and after adjustment for FA groups (rank transformed*)

Adjustment	OR (95% CI)	β estimate	CIE	CIE (%)
—	0.43 (0.17 to 1.11)	−0.83	—	—
∑-FA	0.53 (0.19 to 1.49)	−0.63	(−0.83 − [−0.63])/−0.83 = 0.24	24
SFA	0.45 (0.17 to 1.16)	−0.81	(−0.83 − [−0.81])/−0.83 = 0.02	2
MUFA	0.42 (0.15 to 1.13)	−0.87	(−0.83 − [−0.87])/−0.83 = −0.05	−5
PUFA	0.56 (0.21 to 1.52)	−0.58	(−0.83 − [−0.58])/−0.83 = 0.30	30
ω-3 PUFA	1.09 (0.33 to 3.65)	0.09	(−0.83 − [0.09])/−0.83 = 1.11	111
ω-6 PUFA	0.42 (0.16 to 1.10)	−0.87	(−0.83 − [−0.87])/−0.83 = −0.05	−5
Trans-FA	0.40 (0.13 to 1.26)	−0.92	(−0.83 − [−0.92])/−0.83 = −0.11	−11
CLA	0.49 (0.15 to 1.59)	−0.71	(−0.83 − [−0.71])/−0.83 = 0.14	14

CIE, Change in estimate; *CLA*, conjugated linoleic acid; *MUFA*, monounsaturated fatty acid; *SFA*, saturated fatty acid.
*Because of skewed distributions of some FA variables, all FA variables were used after rank transformation.

TABLE E8. Adjustment of the effect of ω-3 PUFA levels on asthma

Exposure	Outcome	Adjustment	OR (95% CI)*
ω-3 PUFA levels (log-transformed)†	Asthma	—	0.29 (0.11-0.81)
		Farm vs shop milk	0.34 (0.10-1.15)
		Unprocessed farm vs shop milk	0.20 (0.04-0.95)
		High-fat vs low-fat milk	0.41 (0.11-1.50)
		Sex	0.30 (0.10-0.89)
		Stable visits at 5 y	0.23 (0.04-1.16)
		Smoking during pregnancy	0.31 (0.10-0.99)
		Breast-feeding ≥6 mo	0.30 (0.10-0.90)

*ORs are shown for separate models assessing 1 confounder at a time.
†Adjusted for center and farming.

TABLE E9. Contents of ω-3 and ω-6 FAs in high- and low-fat milk

	ω-3 PUFAs	ω-6 PUFAs	ω-6/ω-3 ratio
High-fat milk (≥3.5%)	1.18 (1.09-1.28)	2.07 (2.03-2.12)	1.75 (1.66-1.84)
Low-fat milk (<3.5%)	0.66 (0.53-0.79)	2.24 (2.18-2.31)	3.38 (3.25-3.52)
Unprocessed cow's milk	1.34 (1.21-1.47)	2.14 (2.06-2.21)	1.59 (1.47-1.72)
Shop milk	0.76 (0.64-0.89)	2.18 (2.13-2.22)	2.85 (2.72-2.99)

Values represent geometric means of ω-3 and ω-6 FA variables, with 95% CIs in parentheses.

11. Publikation 2 (Originalarbeit)

nutrients

MDPI

Article

Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins

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Abstract: Consumption of raw cow's milk instead of industrially processed milk has been reported to protect children from developing asthma, allergies, and respiratory infections. Several heat-sensitive milk serum proteins have been implied in this effect though unbiased assessment of milk proteins in general is missing. The aim of this study was to compare the native milk serum proteome between raw cow's milk and various industrially applied processing methods, i.e., homogenization, fat separation, pasteurization, ultra-heat treatment (UHT), treatment for extended shelf-life (ESL), and conventional boiling. Each processing method was applied to the same three pools of raw milk. Levels of detectable proteins were quantified by liquid chromatography/tandem mass spectrometry following filter aided sample preparation. In total, 364 milk serum proteins were identified. The 140 proteins detectable in 66% of all samples were entered in a hierarchical cluster analysis. The resulting proteomics pattern separated mainly as high (boiling, UHT, ESL) versus no/low heat treatment (raw, skimmed, pasteurized). Comparing these two groups revealed 23 individual proteins significantly reduced by heating, e.g., lactoferrin (log2-fold change = −0.37, p = 0.004), lactoperoxidase (log2-fold change = −0.33, p = 0.001), and lactadherin (log2-fold change = −0.22, p = 0.020). The abundance of these heat sensitive proteins found in higher quantity in native cow's milk compared to heat treated milk, renders them potential candidates for protection from asthma, allergies, and respiratory infections.

Keywords: proteomics; heat stability; milk serum proteins; immune-active proteins

1. Introduction

Consuming raw milk has been associated with a reduction in risk of childhood asthma and atopy [1,2] as well as respiratory infections [3]. However, consumption of raw milk poses significant risks, due to potential presence of pathogens in raw milk [3]. As an alternative to raw milk, specific milk ingredients for supplementing heat treated milk have become the focus of recent research, and a wide range of components have been hypothesized to be related to the allergy and asthma protective potential of raw milk versus commercially available milk [4].

After industrial processing, cow's milk considerably differs from raw milk in several aspects, with fat content and heat-treatment being the most obvious. Although the effects of fat content and heat treatment on reduction of asthma partially overlap, both factors exert strong independent effects [2]. The effect of fat content was mainly attributed to the levels of ω-3 polyunsaturated fatty acids [2]. Similarly, heat treatment reduced the levels of milk serum proteins such as β-lactoglobulin and α-lactalbumin, which in turn were found to be inversely related to asthma risk in children of the GABRIELA study with statistical significance [1].

Although it remains open whether these proteins actually reduce the asthma risk themselves, these findings suggest an allergy preventive potential by heat-sensitive proteins in general. Generally whey proteins are susceptible to heat treatment [5,6], particularly immunoactive proteins such as lactoferrin or lactadherin [7]. Heating of heat-labile proteins results in denaturation and aggregation processes [8] and thereby leads to a loss of biological functionality (e.g., lactoferrin [9]). Denatured and aggregated proteins can be extracted from the milk with a combination of pH reduction and ultracentrifugation [10], after which remaining levels of non-aggregated milk serum proteins can be determined [7]. Besides denaturation and aggregation, heating may also lead to chemical modifications, especially the Maillard reaction [11]. During industrial milk processing, relatively short heating times are applied, thus we expect relatively low levels of such chemical modifications, although especially for UHT processing a certain level of chemical modifications has previously been observed [12]. The aim of this study was to assess the native protein profile of bovine milk serum after different industrially applied processing steps with varying heating intensity for the identification of potential asthma- and allergy-protective candidate proteins.

2. Materials and Methods

2.1. Milk Samples

The milk samples used for this analysis were derived from three different farms located in Southern Germany. The origins and characteristics of these three milk batches are shown in Table 1. Each milk batch was processed on three consecutive days in a pilot plant. From milk collection to the last processing step, milk samples were stored at 1 °C. After processing, the milk samples were stored at −20 °C until proteomics analysis. The milk types resulting from the various processing procedures are listed in Table 2. Industrial milk processing was not done with technical replicates because these procedures are laborious, expensive, and time-consuming and there were biological replicates represented by the three milk batches from the respective farms. In total, eight milk samples from each of the three milk batches were assessed for proteomics. The same 24 milk samples were previously used to assess the effect of different processing methods on microRNA (miRNA) levels [13].

The subsequent proteomics analyses of the 24 samples including sample preparation and mass spectrometry were performed without technical replicates since technical reproducibility proved to be high in previous experiments [14] and most of the variation was expected to come from the three separate batches of milk.

Table 1. Sources of raw milk.

Sample Origin	Farms (in Bavaria)		
	Traunstein	**Freising**	**Starnberg**
No. of cows	13	60	30
Time point of milking for pooled samples	Morning and evening	Morning and evening	Morning and evening
No. of detectable milk serum proteins in raw milk samples	143	153	158

Table 2. Processing details of the milk samples.

Code	Milk Fraction	Processing Conditions	Day of Processing *	Grouping of Milk Types **
RAW	Native raw milk	-	Wednesday	No-low heat
PAS	Pasteurized	72 °C for 20 s Total processing time *** 60 s	Wednesday	No-low heat
SKI	Skim milk	Separation at 50 °C	Tuesday	No-low heat
FAT	Fat fraction/cream	Separation at 50 °C	Tuesday	-
HOM	Homogenized milk	Preheating to 55 °C, 2-stage homogenization at 250/50 bar	Tuesday	-
ESL	Extended shelf life milk	Preheating at 95 °C for 20 s, direct steam injection at 127 °C for 5 s Total processing time *** 60 s	Monday	High heat
UHT	Ultra-high heat treated	Preheating at 93 °C for 23 s, direct steam injection at 142 °C for 5 s Total processing time *** 85 s	Monday	High heat
BOI	Boiled milk	Preheating at >80 °C for >300 s, boiling at 100 °C for 30 s Total processing time *** 2000 s	Tuesday	High heat

* Milk samples were collected on a Monday and stored at 1 °C until they were processed. Processing occurred on the same day or the two subsequent days. After processing samples were frozen to −20 °C and stored until analysis. ** For further analysis of heat treatment on milk proteins, grouping of milk types according to the heat treatment was conducted; homogenized milk was excluded due to additional treatment with pressure; cream was excluded because it contains only the milk fat fraction. *** Total processing time includes heating and cooling stage.

2.2. Removal of Fat and Denatured Protein

All samples were centrifuged at 1500× *g* for 10 min at 10 °C (with a rotor 25.15, Avanti Centrifuge J-26 XP, Beckman Coulter, Miami, FL, USA). After centrifugation, all skimmed milk samples were acidified by drop-wise addition of 1 M HCl under stirring, until a pH of 4.6 was reached. The samples were then kept at 4 °C for 30 min to equilibrate. When needed, pH was adjusted before the final pH reading. This pH adjustment was done to separate the denatured serum proteins from the native serum proteins during ultracentrifugation, as previously described [7,10]. The acidified skim milk was transferred to ultracentrifuge tubes followed by ultracentrifugation at 100,000× *g* for 90 min at 30 °C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three phases. The top layer was remaining milk fat, the middle layer was milk serum, and the bottom layer (pellet) was casein with denatured proteins. Milk serum was used for filter aided sample preparation (FASP) as described below.

2.3. Filter Aided Sample Preparation (FASP)

FASP method was carried out according to Wisniewski et al., 2009 [15], with adaptations according to Zhang et al., 2016 [7]. Milk serum samples (20 µL) were diluted in SDT-lysis buffer (4% SDS with 0.1 M dithiotreitol and 100 mM Tris/HCl pH 8.0) to get a 1 µg/µL protein solution. Samples were then incubated for 10 min at 95 °C. They were centrifuged at 21,540× *g* for 10 min after being cooled down to room temperature. Of each sample 20 µL were directly added to the middle of 180 µL 0.05 M iodoacetamide (IAA) in 8 M urea with 100 mM Tris/HCl pH 8.0 (called UT) in a low binding Eppendorf tube and incubated for 10 min while mildly shaking at room temperature. The entire volume of the sample (200 µL) was transferred to a Pall 3K omega filter (10–20 kDa cutoff, OD003C34; Pall, Washington, NY, USA) and centrifuged at 20,000× *g* for 30 min. Another three centrifugations at 20,000× *g* for 30 min were carried out after adding three times 100 µL UT. Afterwards 110 µL 0.05 M NH_4HCO_3 (ABC) in water was added to the filter unit and centrifuged at 20,000× *g* for 30 min. Then, the filter was transferred to a new low-binding Eppendorf tube. On the filter, 100 µL ABC containing 0.5 µg trypsin was added and centrifuged at 20,000× *g* for 30 min after incubation overnight. Finally, the filter was removed and 5 µL 10% trifluoroacetic acid (TFA) was added to adjust the pH of

the sample to around 2. These samples were ready for analysis by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.4. LC-MS/MS Analysis

A volume of 18 µL of the trypsin digested milk fractions was injected in a 0.10 × 30 mm Magic C18AQ 200A 5 µm beads (Bruker Nederland B.V., Leiderdorp, The Netherlands) pre-concentration column (prepared in house) at a maximum pressure of 270 bar. Peptides were eluted from the pre-concentration column onto a 0.10 × 200 mm Magic C18AQ 200A 3 µm beads analytical column with an acetonitrile gradient at a flow of 0.5 µL/min, using gradient elution from 8 to 33% acetonitrile in water with 0.5 v/v % acetic acid in 50 min. The column was washed using an increase in the percentage of acetonitrile to 80% (with 20% water and 0.5 v/v % acetic acid in the acetonitrile and the water) in 3 min. Between the pre-concentration and analytical columns, an electrospray potential of 3.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line of a P777 Upchurch microcross. Full scan positive mode FTMS spectra were measured between m/z 380 and 1400 on a LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA) in the Orbitrap at high resolution (60,000). IT and FT AGC targets were set to 10,000 and 500,000, respectively, or maximum ion times of 100 µs (IT) and 500 ms (FT) were used. Collision-induced dissociation (CID) fragmented MS/MS scans (isolation width 2 m/z, 30% normalized collision energy, activation Q 0.25 and activation time 15 ms) of the four most abundant 2+ and 3+ charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold = 5.000, 45 s exclusion duration for the selected m/z ±25 ppm).

2.5. Data Analysis

Each run with all MS/MS spectra obtained was analysed with Maxquant 1.3.0.5 with Andromeda search engine [16]. Carbamidomethylation of cysteines was set as a fixed modification (enzyme = trypsin, maximally 2 missed cleavages, peptide tolerance for the first search 20 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The bovine reference database for peptides and protein searches was downloaded as fasta file from Uniprot with reverse sequences generated by Maxquant (fasta file downloaded from Uniprot 2013 [17]). A set of 31 protein sequences of common contaminants was used as well, which included Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and a mass deviation of 0.5 Da was set as limit for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. The false discovery rate (FDR) was set to 1% on both peptide and protein levels. The length of peptides was set to at least seven amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique and at least one unmodified. Match between runs was used with a time window of 10 min. Both unmodified and modified peptides were used for quantification. Only unique or razor peptides were used for quantification. Minimum ratio count for label-free quantification (LFQ) was set as 2.

The quantification of the full proteome is based on the extracted ion current and is taking the whole three-dimensional isotope pattern into account, using peak volumes of all measured isotopes for quantification [16]. At least two quantitation events were required for a quantifiable protein. MaxQuant was used with the Intensity based absolute quantification (IBAQ) algorithms for quantification [18]. The IBAQ algorithm estimates the absolute amount of a protein as the sum of the intensities of all peptides (based on peak volumes), divided by the number of tryptic peptides that can theoretically be generated. Proteins had to have at least three valid IBAQ intensities in the individual samples for counting of the number of identified proteins.

The function of the identified proteins was checked in the UniprotKB database released February 2014 [17].

2.6. Statistical Analysis

Statistical analysis was performed with R 3.3.2 software [19]. The average number of measurable proteins in raw milk was calculated and related to the respective numbers of milks after different processing methods.

Proteins with ≤33% non-detects were included in further analysis. Non-detects of these proteins were either simply replaced by zero or imputed by simple imputation. For the imputation firstly the mean and standard deviation of each protein was estimated including the non-detected values as censored observations by a linear Tobit model to determine protein specific distributions. Subsequently, non-detects were replaced by random samples from the lower tail of the respective distribution, i.e., below the protein specific detection limit as defined by the minimum of the measured protein levels. The quality of imputation was examined via Wilcoxon tests, comparing median protein levels of the imputed data against the raw data. For subsequent analyses, the imputed data were used.

Hierarchical clustering of milk samples was based on Pearson's correlation of the specific protein profiles following imputation.

For assessment of the effect of heating, milks were categorized in two groups by temperatures above and below 80 °C [20]; high heated milk samples, defined as UHT, ESL and boiled milk and no-/low heat treated milks, represented by pasteurized, skimmed and raw milk samples (Table 2).

A logistic regression model adjusted for the milk origin (Traunstein, Freising, Starnberg) was used to calculate the differences in high vs. low heat treated milks. The log2 fold-changes of the protein levels in low versus high heat treated milks were calculated to rank the proteins according to their heat sensitivity, and plotted against the corresponding negative decadic logarithm of the *p*-values in a volcano plot. Resulting *p*-values were adjusted for the false discovery rate according to Benjamini–Hochberg, and a corrected *p*-value < 0.05 was considered statistically significant.

3. Results

A total of 364 milk serum proteins were identified and quantified in at least one of the 24 milk samples, of which 44 could be quantified in all 24 samples. Subsequent analyses were based on the 169 proteins found in at least three different milk samples; 130 of those proteins were detected in all three raw milk samples and further 28 proteins in two raw milk samples. The average LFQ levels of proteins in the raw milk samples did not differ significantly between the three farms ($p = 0.49$), thereby ruling out major differences in original milk batches.

Figure 1 shows a substantial loss of detectable proteins after the various processing procedures.

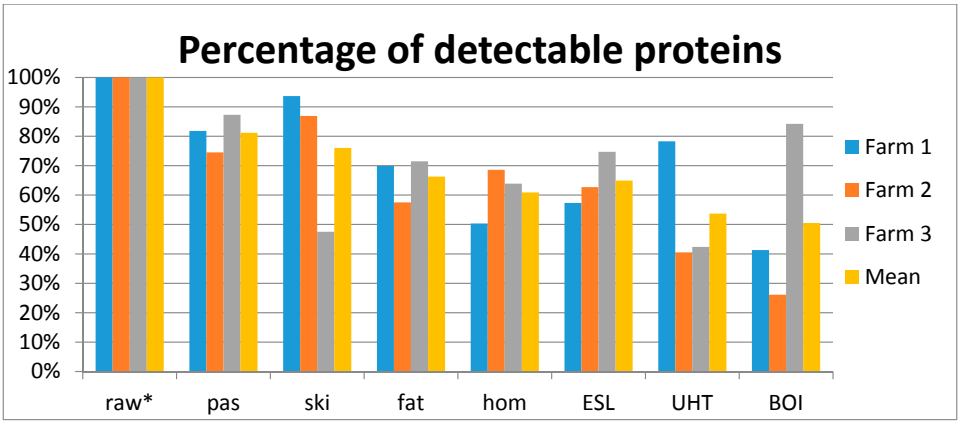

Figure 1. Proportion of number of detectable proteins in milk samples (each sample per farm individually and averaged over the three different samples) after different processing compared to raw cow's milk. * No. of detectable native proteins in raw milk is the reference, i.e., 151 distinct proteins were detected in the three raw milk samples on average.

For further statistical analysis, proteins with >33% non-detects were excluded. Non-detects in the remaining proteins (n = 140) were either replaced by imputed values below detection limit or simply by zeros. Figure 2 shows the superiority of the imputation method in contrast to the simple replacement of missing values by zero. The median of the individual protein LFQ levels averaged over all 24 samples is solely slightly reduced after imputation compared to the raw data set (median value was calculated after exclusion of missing values). In contrast, replacement of non-detects by zero resulted in a clear distortion of the distribution and was not considered for further analysis.

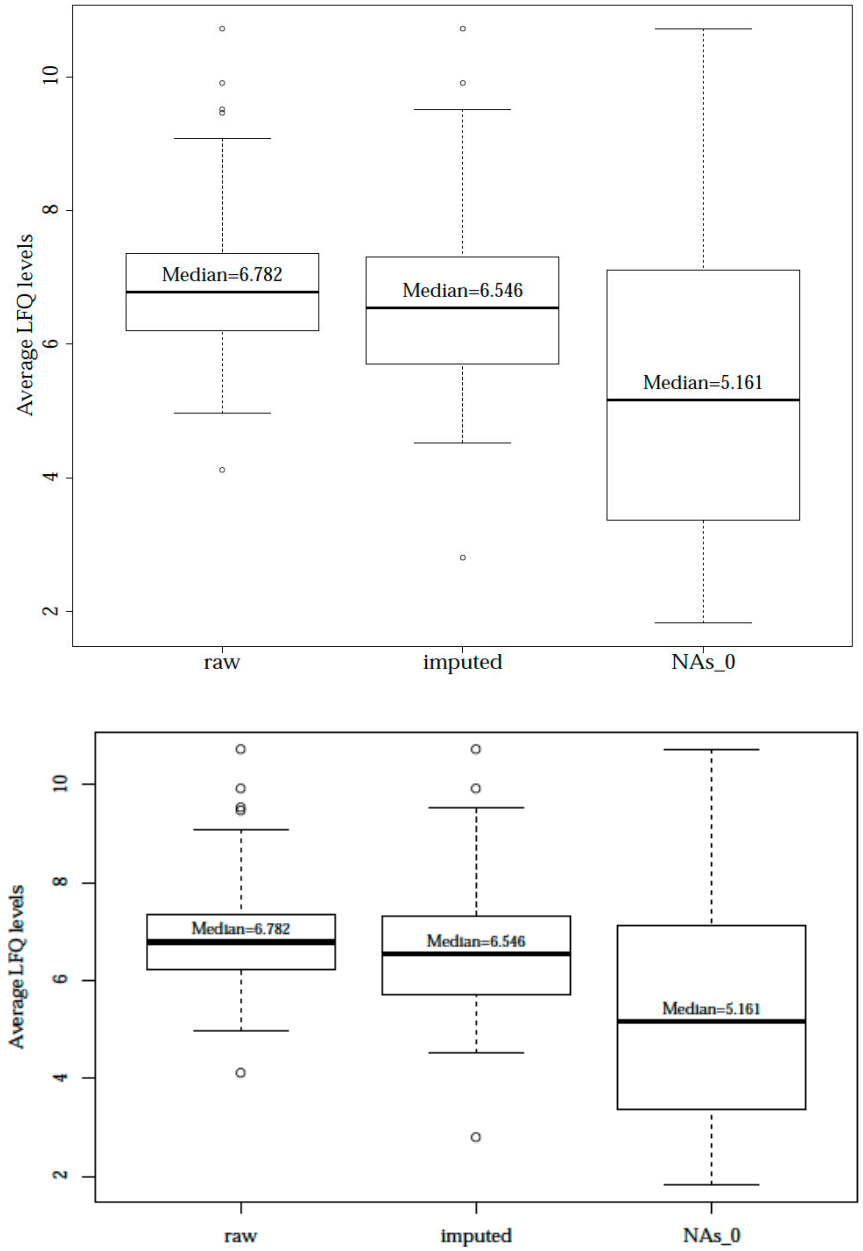

Figure 2. Boxplots of average protein LFQ levels after different NA replacement. Comparison of mean LFQ protein values in different data (raw, imputed, and NAs replaced by 0). Replacement by 0 differed significantly from the raw data ($p < 0.0001$).

Similar protein patterns resulted from similar heating temperatures of the milk samples as demonstrated by hierarchical clustering of the specific protein profiles (Figure 3): raw, skimmed and pasteurized milk samples formed one cluster, whereas UHT, ESL, and boiled milk samples formed

another cluster with the exception of one boiled milk sample, which differed substantially from both main clusters. Under the assumption that this milk was partially overcooked, it was excluded from subsequent analyses.

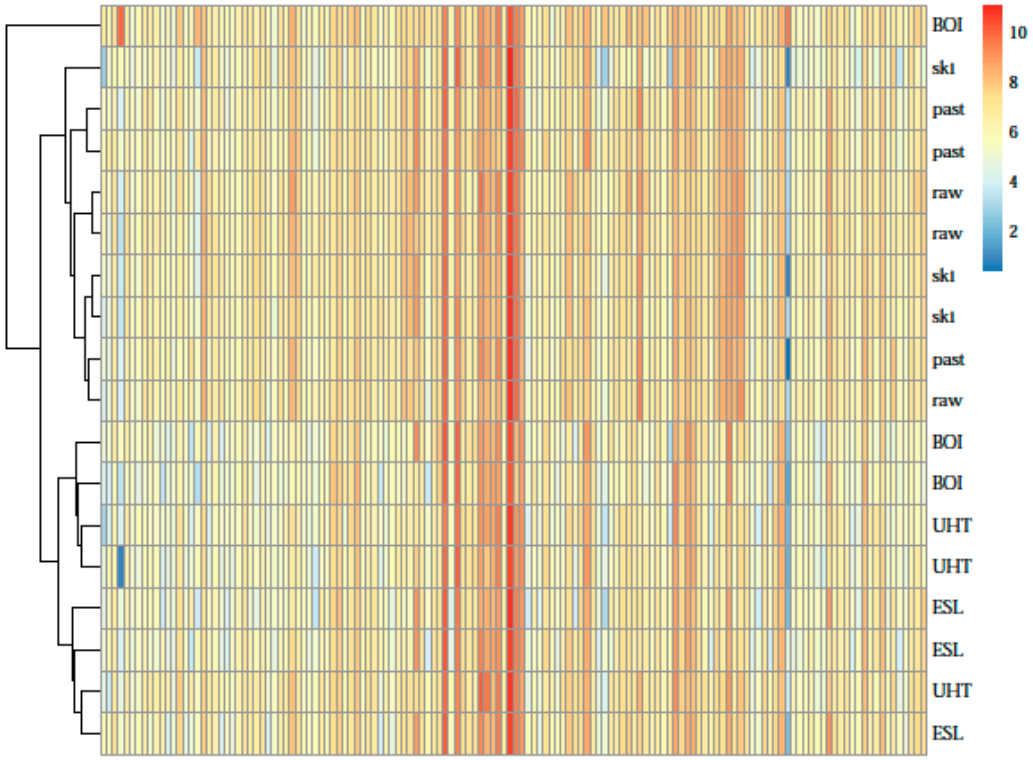

Figure 3. Heat map for protein levels and milk types. Rows reflect individual samples, whereas individual proteins are given in columns. Their LFQ values are represented by different colors according to the color code from low (blue) to high (red) expression.

Comparison of milks in the high heat versus the low heat treated group revealed a significant reduction of the total protein LFQ levels in high heated milks compared to no/low heat treated milks, as shown in Figure 4. Boiled milk showed the lowest protein levels; other heat treated milks contained total protein LFQ levels ranging between boiled and raw milk and were inversely related to heating temperatures (Figure 4).

When focusing on individual proteins, a significant reduction in quantity of at least 10% was found in 23 proteins after high heat treatment compared to low heat treatment (Figure 5).

Ten of these proteins were related to immune functions (Table 3).

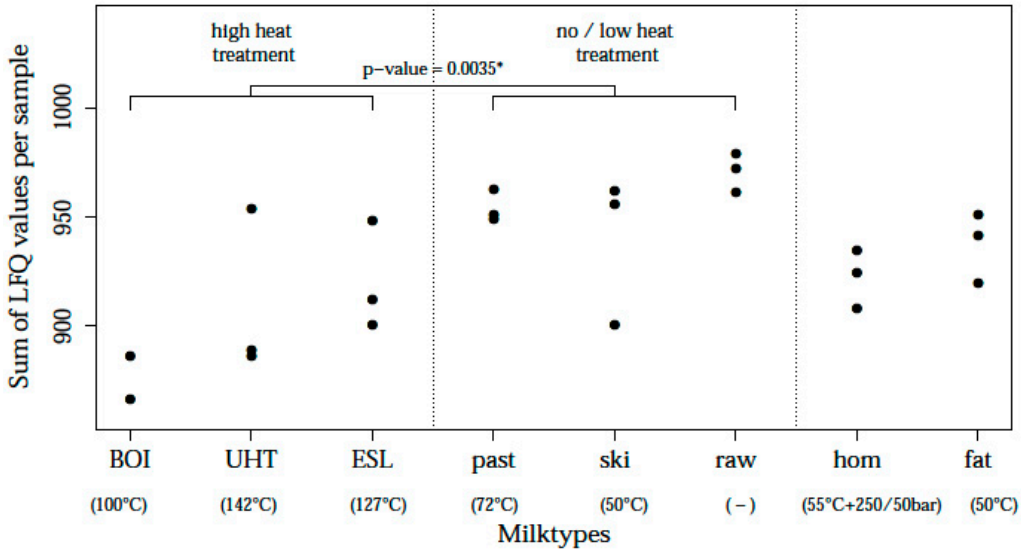

Figure 4. Total protein contents (sum of LFQ values per sample) in differently processed milks. * *p*-value derived from a logistic regression with adjustment for milk batch.

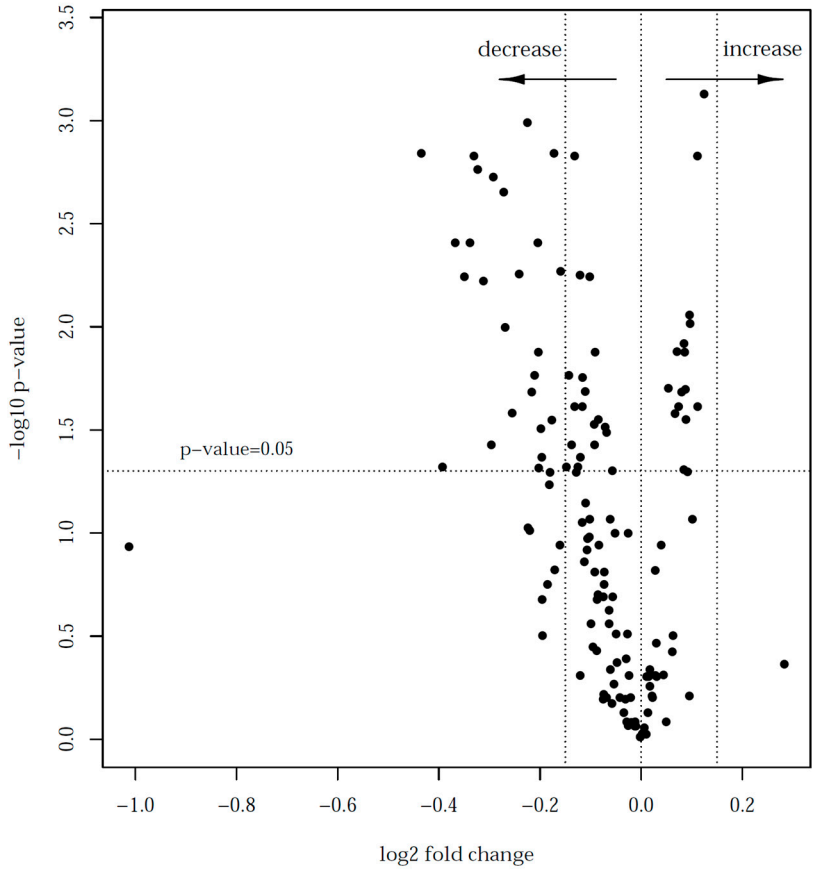

Figure 5. Volcano plot for the comparison of proteins in low- versus high-heat treated milk samples. The log two-fold change of protein expression between low- and high-heat treated milk samples is plotted against the corresponding *p*-values from a *t*-test given as negative decadic logarithm. A negative log two-fold change indicates a decrease in LFQ levels.

Table 3. Significantly differing proteins between high and no/low heat treated milk-types with a change of ≥10%.

Protein Code	Number of Peptides	*p*-Value *	Log2 Fold Change (95% CI)	Protein Name	Protein Function
P80457	67	0.001	−0.44 (−0.56; −0.31)	Xanthine dehydrogenase/oxidase	immunity
P24627	71	0.004	−0.37 (−0.51; −0.22)	Lactoferrin	immunity
G3X6N3	57	0.006	−0.35 (−0.50; −0.20)	Serotransferrin	transport
F1MR22	42	0.004	−0.34 (−0.47; −0.21)	Polymeric immunoglobulin receptor	immunity
P80025	37	0.001	−0.33 (−0.43; −0.23)	Lactoperoxidase	immunity
G3N1R1	4	0.002	−0.32 (−0.44; −0.21)	Uncharacterized protein	unknown
F1MGU7	7	0.04	−0.30 (−0.52; −0.07)	Fibrinogen gamma-B chain	Blood coagulation
G3X7A5	80	0.002	−0.29 (−0.41; −0.18)	Complement C3	immunity
F1MZ96	10	0.002	−0.27 (−0.36; −0.18)	Uncharacterized protein	unknown
F1MX50	4	0.01	−0.27 (−0.40; −0.13)	Uncharacterized protein	cell
F1MM32	8	0.026	−0.26 (−0.43; −0.08)	Sulfhydryl oxidase	enzyme
P81265	42	0.006	−0.24 (−0.35; −0.14)	Polymeric immunoglobulin receptor	immunity
F1N076	12	0.001	−0.23 (−0.30; −0.15)	Ceruloplasmin	cell
F1MXX6	26	0.02	−0.22 (−0.35; −0.08)	Lactadherin	cell
Q08DQ0	6	0.017	−0.21 (−0.34; −0.08)	Plakophilin-3	cell
P07589	6	0.004	−0.20 (−0.30; −0.11)	Fibronectin	immunity
A6QNL0	6	0.01	−0.20 (−0.32; −0.09)	Monocyte differentiation antigen CD 14	immunity
P10152	11	0.048	−0.20 (−0.37; −0.04)	Angiogenin-1 (ribonuclease 5)	cell
F1MMD7	5	0.031	−0.20 (−0.34; −0.06)	Inter-alpha-trypsin inhibitor heavy chain H4	Protease inhibitor
Q3MHN2	6	0.043	−0.20 (−0.35; −0.04)	Complement component C9	immunity
P00735	7	0.028	−0.18 (−0.30; −0.05)	Prothrombin	immunity
F1MCF8	9	0.001	−0.17 (−0.22; −0.12)	Uncharacterized protein	immunity
P17690	9	0.005	−0.16 (−0.23; −0.09)	Beta-2-glycoprotein 1	Blood coagulation

* *p*-values are adjusted for multiple testing.

4. Discussion

Heat treatment of milk led to a considerable decrease in number of detectable proteins and their levels of quantification with a clear relationship to the applied heat load. The most intensive treatment, i.e., boiling, reduced the number of proteins that could be detected by about 50% compared to raw milk, with the other heating types ranging in between. The various processing methods led to specific proteomic patterns covering 140 individual proteins as demonstrated by a cluster analysis. Of these, 23 distinct proteins were found to be substantially diminished in high heat treated milks. The majority of these heat-sensitive proteins were related to immune functions.

Typically, people in Westernized countries consume industrially processed milk and, increasingly, milk types with an extended shelf life. In addition, UHT milk with its very long storage duration of three months or more is nowadays very popular. Traditionally, commercially available milk had been pasteurized, i.e., heated at 72 °C for 20 s to inactivate potential hazardous microorganisms with only small gain in shelf life.

Despite the potential risk of life-threatening infections, a minority of people still consume raw cow's milk, which has repeatedly been reported to protect against asthma, allergies, and respiratory infections in childhood [1,3,21,22]. The wide consumption of cow's milk thus renders it an attractive strategy for prevention if the risk of infections were to be overcome. An option might be the isolation and purification of the protective milk ingredients, and various studies have focused on the impact of industrial processing on the potentially beneficial molecules. At the same time, reducing heat load of commercially available dairy products may already lead to an increase in the availability of potentially immunoactive proteins.

Of the industrially applied processing steps, predominantly fat separation for adjusting milk fat levels, and homogenization for preventing fat creaming, affect the milk lipid fraction. However, homogenization also leads to a massive increase in fat globule surface, which will be covered by milk proteins, leading to a reduction of milk proteins in serum.

Waser at al., 2007 [23] found an asthma and wheeze protective effect of milk fat containing products such as full cream milk and butter. In addition, Brick et al., 2016 [2] implied the higher fat content and more precisely the higher content of anti-inflammatory omega-3 fatty acids in raw milk in the asthma protective effect of full cream milk obtained directly from a farm. Despite mild heating to

55 °C, high pressure treatment of milk (250 bar) used during the homogenization process has been found to profoundly rearrange protein quantity and structure [22,24,25]. In addition, in the present study homogenization reduced total protein LFQ levels and specific protein detectability markedly.

Another major processing step is heating for destroying hazardous microorganisms and increasing shelf life. Thermo-labile milk components such as miRNAs [13] and proteins may thus be involved in the protective effect of raw milk. Particularly protein functionality, solubility and quantity are all affected by intensity of heat treatment [7,8,18,26]. Previously specific miRNA species were identified as possible contributors to the asthma-protective effect of farm milk [11]. This notion is not in conflict with our current findings; rather both molecule classes might add to the effect or might even interact.

Loss et al., 2011 [1] found inverse associations of asthma with higher levels of several milk whey proteins, i.e., bovine serum albumin, alpha-lactalbumin and beta-lactoglobulin. However, it remains unclear whether these specific proteins confer the effect themselves or whether they are proxies of heat labile proteins in general. Therefore, we quantified heat-induced alterations of the entire milk proteome by a comprehensive, standardized, and unbiased approach, i.e., without preselection of proteins.

First, we observed a considerable decrease of detectable proteins after heat treatment in a dose-dependent manner. Boiled cow's milk contained the lowest number of detectable proteins, which is explained by the high heat load applied. The lower temperature of boiling compared to ESL or UHT is more than compensated by the much longer duration of the heating (Table 2). In addition, the long heating time of boiling may also lead to more extensive chemical modification compared to industrial processes [11], further reducing protein levels in these samples.

For further investigation in the impact of heating on the protein quantity and heat sensitivity, milk samples were categorized into high heat and no/low heat treated milk groups according to the clusters presented in the heat map (Figure 3). This dichotomization was in line with findings on the first marginal transition of bovine whey proteins at about 81 °C [20]. Actually, the difference between high and no/low heat treated samples was more than 25 °C with pasteurization not exceeding 72 °C and high heat treatment starting with 100 °C.

Figures 1 and 4 describe some variance within milk types between the three farms, e.g., one of the UHT samples had a higher percentage of detectable proteins and a higher summed LFQ value than the respective other two UHT samples. Nevertheless, the heat map (Figure 3) still groups all the high-heated samples together. Even though this UHT sample contains a higher overall protein intensity, and a higher number of identified proteins, the proteome profile still reflects a high heated sample. This might be due to a similar pattern in decrease of individual, heat sensitive proteins. The exact underlying mechanisms for these individual variations however cannot be explained in this study. Further investigations on a larger scale are needed to better understand the variability in proteome profile after heat processing.

The sum over all proteins, and more specifically the levels of 23 individual proteins were substantially lower in high heat treated samples as expected by previous work from Zhang et al., 2016 [7]. Interestingly, most of these 23 particularly heat-sensitive proteins were related to immune functions (Table 3), and several proteins have already been mentioned in the context of asthma and allergies. Under the assumption that some proteins withstand the acidity of the stomach milieu, they may be resorbed in the gut and exert physiologic functions. At least this has been suggested for e.g., lactoferrin (LTF) [27], protease inhibitors [28] and IgG [29].

Among the most promising candidates was lactoferrin, which is known to stimulate the immune system by counteracting pathogenic invaders and injuries and preventing harmful overreactions of the immune system [30,31].

Lactoperoxidase is a peroxidase enzyme secreted from the mammary gland that operates as a natural antibacterial agent [32]. Asthmatic patients who were treated with lactoperoxidase aerosol showed lower disease activity and reduced damaging effects of hydrogen peroxide (H_2O_2), which is mainly generated by neutrophils and eosinophils in asthma and contributes to airway damages and inflammation [33].

Xanthine dehydrogenase/oxidase (XOR) might contribute to the formation of NO in the intestinal lumen and thereby exert antimicrobial properties [34]. In our study we were unable to differentiate the rather similar variants, dehydrogenase and oxidase, as the only difference is an intramolecular change of two cysteines in the disulfide bond, whereas the amino acid chain, analyzed with the LC-MS/MS analysis, is identical.

In addition, a number of acute phase proteins such as fibrinogen, prothrombin, complement C3 and C9 were found to be highly heat-sensitive. How they may be involved in the anti-inflammatory effects ascribed to raw milk remains unclear, although the complement pathway, and specifically C3, has been implied in the development of allergy and asthma [35–37].

Plakophilin-3 acts protective in both local and systemic inflammatory diseases [38] and inter-alpha-trypsin inhibitor has anti-inflammatory, anti-scarring and anti-angiogenic properties [39]. Protease inhibitors, including several inter alpha-trypsin inhibitors, have been found to be upregulated in the breast milk of allergic mothers and have been related to the pathogenesis of allergy and asthma [40,41].

Lactadherin expression is found to be markedly reduced in asthmatic patients compared to healthy subjects, and suppresses airway smooth muscle hypercontractility [42].

Polymeric immunoglobulin receptor may influence eosinophilic inflammation by binding secretory immunoglobulins [43]. In addition, secretory components, which are part of the polymeric immunoglobulin receptor that can be cleaved off, have shown individual effects in mucosal immunity [44].

Ultimately, the discovery of the CD14 molecule, a receptor of bacterial endotoxin, is interesting as gene–environment interactions of raw milk consumption and polymorphisms associated with this gene have been discussed controversially for childhood onset asthma [45,46]. Similar to the human CD14 molecule, its bovine counterpart might transmit signals elicited by endotoxin, and thereby have an effect on the development or prevention of allergy and asthma.

Despite the plausible involvement of several proteins in the beneficial health effects we have to acknowledge that we cannot provide a direct link to disease status in this study. However, the palette of immune-active milk components detected in the present study can be seen as an extension to the findings by Loss et al., 2011 [1], which explicitly linked protein levels to disease. In addition, this study only shows a decrease in native proteins, due to either denaturation or heat-induced chemical modification, without direct evidence for a loss-of-function. However, heating of milk has been shown to reduce biological activity of milk, including antibacterial capacity [6] and previous studies showed a loss-of-function of milk immune proteins upon denaturation (e.g., Paulson, 1993 [47]; Marin et al., 2003 [48]). However, future studies are needed to investigate in the biological function of milk's immunoactive proteins after applying heat treatments.

Another limitation of this analysis is the omission of the milk fat globule membrane (MFGM) fraction [49]; their relatively low abundance in cow's milk, however, precludes a major contribution to the effects by the entirety of immunoactive proteins present in milk. Our analyses were made after one freezing cycle; resulting alterations, however, seem to be very limited [7,50].

5. Conclusions

Taken together, we have performed a comprehensive search for proteins most likely to be affected by industrial processing methods. Their higher abundance in native cow's milk as compared to industrially processed milks renders them potential candidates for protection from asthma, allergies, and respiratory infections. However, in this study, we solely analyzed protein patterns of differently processed milks, thus associations of found potential protein candidates with disease status have to be investigated in population based studies.

Supplementary Materials: The supplementary file is available online at www.mdpi.com/2072-6643/9/9/963/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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nutrients

MDPI

Supplemental Table S1: Differentiation of Table 3.

Protein	p-Value	Difference logscale	Full protein name	Protein function	farm
A6QNL0	0.081671945	**-0.23802192**	**Monocyte differentiation antigen CD14**	immunity	**1**
F1MCF8	0.034750355	**-0.17906498**	**Uncharacterized protein**	immunity	**1**
F1MGU7	0.256887543	**-0.29739410**	**Fibrinogen gamma-B chain**	blood coagulation	**1**
F1MM32	0.114634543	**-0.37410274**	**Sulfhydryl oxidase**	enzyme	**1**
F1MMD7	0.490982158	**-0.13948175**	**Inter-alpha-trypsin inhibitor heavy chain H4**	protease inhibitor	**1**
F1MR22	0.084272485	**-0.40955884**	**Uncharacterized protein**	immunity	**1**
F1MUT3	0.138322440	**-0.23642798**	**Xanthine dehydrogenase/oxidase**	enzyme	**1**
F1MX50	0.155685884	**-0.23493682**	**Uncharacterized protein**	cell	**1**
F1MXX6	0.196097111	**-0.21122983**	**Lactadherin**	cell	**1**
F1MZ96	0.028505851	**-0.29591851**	**Uncharacterized protein**	unknown	**1**
F1N076	0.050156461	**-0.23059976**	**Uncharacterized protein**	cell	**1**
G3N1R1	0.081971360	**-0.35570269**	**Uncharacterized protein**	unknown	**1**
G3X6N3	0.093323121	**-0.41136014**	**Serotransferrin**	transport	**1**
G3X7A5	0.074969423	**-0.32985784**	**Complement C3**	immunity	**1**
P00735	0.246247618	**-0.20028194**	**Prothrombin**	immunity	**1**
P07589	0.249664417	**-0.13446460**	**Fibronectin**	immunity	**1**
P10152	0.262017558	**-0.24632455**	**Angiogenin-1**	cell	**1**
P14923	0.442842564	**-0.23557883**	**Junction plakoglobin**	contaminant	**1**
P17690	0.067252125	**-0.19795256**	**Beta-2-glycoprotein 1**	blood	**1**

				coagulation	
P24627	0.618396262	**-0.05641995**	**Lactotransferrin**	immunity	**1**
P80025	0.056354987	**-0.31864315**	**Lactoperoxidase**	immunity	**1**
P80457	0.024660244	**-0.48824513**	**Xanthine dehydrogenase/oxidase**	enzyme	**1**
P81265	0.030342765	**-0.23337220**	**Polymeric immunoglobulin receptor**	immunity	**1**
Q08DQ0	0.408093065	**-0.18463537**	**Plakophilin-3**	cell	**1**
Q3MHN2	0.161391410	**-0.13610006**	**Complement component C9**	immunity	**1**
A6QNL0	0.069013882	**-0.23518111**	**Monocyte differentiation antigen CD14**	immunity	**2**
F1MCF8	0.015784629	**-0.20428409**	**Uncharacterized protein**	immunity	**2**
F1MGU7	0.083850004	**-0.30868066**	**Fibrinogen gamma-B chain**	blood coagulation	**2**
F1MM32	0.006709007	**-0.39112003**	**Sulfhydryl oxidase**	enzyme	**2**
F1MMD7	0.105977817	**-0.28763775**	**Inter-alpha-trypsin inhibitor heavy chain H4**	protease inhibitor	**2**
F1MR22	0.051009420	**-0.43844310**	**Uncharacterized protein**	immunity	**2**
F1MUT3	0.008728533	**-0.16801522**	**Xanthine dehydrogenase/oxidase**	enzyme	**2**
F1MX50	0.039875202	**-0.36328489**	**Uncharacterized protein**	cell	**2**
F1MXX6	0.067107745	**-0.28572580**	**Lactadherin**	cell	**2**
F1MZ96	0.033714785	**-0.29583862**	**Uncharacterized protein**	unknown	**2**
F1N076	0.014556345	**-0.23929233**	**Uncharacterized protein**	cell	**2**
G3N1R1	0.079212935	**-0.24260691**	**Uncharacterized protein**	unknown	**2**
G3X6N3	0.058801881	**-0.36820290**	**Serotransferrin**	transport	**2**
G3X7A5	0.025457293	**-0.33343274**	**Complement C3**	immunity	**2**
P00735	0.189244691	**-0.18823088**	**Prothrombin**	immunity	**2**
P07589	0.001882570	**-0.16339849**	**Fibronectin**	immunity	**2**

P10152	0.265167328	**-0.16016051**	**Angiogenin-1**	cell	**2**
P14923	0.066201298	**-0.26988851**	**Junction plakoglobin**	contaminant	**2**
P17690	0.034045350	**-0.17825876**	**Beta-2-glycoprotein 1**	blood coagulation	**2**
P24627	0.606428473	**-0.10691213**	**Lactotransferrin**	immunity	**2**
P80025	0.015727966	**-0.40803462**	**Lactoperoxidase**	immunity	**2**
P80457	0.017164248	**-0.45127591**	**Xanthine dehydrogenase/oxidase**	enzyme	**2**
P81265	0.076082396	**-0.31250532**	**Polymeric immunoglobulin receptor**	immunity	**2**
Q08DQ0	0.568689620	**-0.07368748**	**Plakophilin-3**	cell	**2**
Q3MHN2	0.038221029	**-0.32713635**	**Complement component C9**	immunity	**2**
A6QNL0	0.808810787	**-0.04043559**	**Monocyte differentiation antigen CD14**	immunity	**3**
F1MCF8	0.102303854	**-0.11513041**	**Uncharacterized protein**	immunity	**3**
F1MGU7	0.530105296	**-0.26148465**	**Fibrinogen gamma-B chain**	blood coagulation	**3**
F1MM32	0.366806380	**-0.30728700**	**Sulfhydryl oxidase**	enzyme	**3**
F1MMD7	0.706563764	**-0.03510671**	**Inter-alpha-trypsin inhibitor heavy chain H4**	protease inhibitor	**3**
F1MR22	0.290188981	**-0.28563547**	**Uncharacterized protein**	immunity	**3**
F1MUT3	0.053002049	**-0.56981654**	**Xanthine dehydrogenase/oxidase**	enzyme	**3**
F1MX50	0.324407131	**-0.19072733**	**Uncharacterized protein**	cell	**3**
F1MXX6	0.382993501	**-0.11720980**	**Lactadherin**	cell	**3**
F1MZ96	0.317901367	**-0.19368184**	**Uncharacterized protein**	unknown	**3**
F1N076	0.082822215	**-0.19684531**	**Uncharacterized protein**	cell	**3**
G3N1R1	0.237873253	**-0.28592347**	**Uncharacterized protein**	unknown	**3**
G3X6N3	0.238346830	**-0.23144136**	**Serotransferrin**	transport	**3**

G3X7A5	0.203309875	**-0.18251452**	**Complement C3**	immunity	**3**
P00735	0.435981155	**-0.07472569**	**Prothrombin**	immunity	**3**
P07589	0.074917993	**-0.25812729**	**Fibronectin**	immunity	**3**
P10152	0.329023124	**-0.22825551**	**Angiogenin-1**	cell	**3**
P14923	0.717283262	**-0.17966716**	**Junction plakoglobin**	contaminant	**3**
P17690	0.142082287	**-0.07824901**	**Beta-2-glycoprotein 1**	blood coagulation	**3**
P24627	0.891718223	**-0.02615023**	**Lactotransferrin**	immunity	**3**
P80025	0.146780262	**-0.23888622**	**Lactoperoxidase**	immunity	**3**
P80457	0.226280758	**-0.32739478**	**Xanthine dehydrogenase/oxidase**	enzyme	**3**
P81265	0.147299243	**-0.15649395**	**Polymeric immunoglobulin receptor**	immunity	**3**
Q08DQ0	0.482109041	**-0.20910686**	**Plakophilin-3**	cell	**3**
Q3MHN2	0.465662798	**-0.19406143**	**Complement component C9**	immunity	**3**

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ORIGINAL ARTICLE

WILEY

Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes

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Abstract

Background: Childhood asthma is often preceded by early wheeze. Usually, wheezing episodes are recorded retrospectively, which may induce recall bias.

Aims and objectives: The aim of this study was to investigate true-positive recall of parent-reported wheeze at 1 year of age, its determinants, and its implications for asthma and lung function at 6 years of age.

Methods: The PASTURE (Protection Against Allergy—Study in Rural Environments) study followed 880 children from rural areas in 5 European countries from birth to age 6 years. Wheeze symptoms in the first year were asked weekly. At age 6, parent-reported asthma diagnosis was ascertained and lung function measurements were conducted. Correct parental recall of wheeze episodes at the end of the first year was assessed for associations with lung function, asthma, and the asthma risk locus on chromosome 17q21.

Abbreviations: ALEX, Allergy and Endotoxin Study; AMICS, Asthma Multicenter Infants Cohort Study; AT, Austria; ATS, American Thoracic Society; CH, Switzerland; Chr, chromosome; CI, confidence interval; C-section, cesarean section; DE, Germany; FEV1, forced expiratory volume in one second; FL, Finland; FR, France; FVC, forced vital capacity; GLI, Global Lung Function Initiative; HR, hazard ratios; IFN-γ, interferon-γ; IgE, immunoglobulin E; ISAAC, International Study of Allergy and Asthma in Childhood; LPS, lipopolysaccharides; OR, odds ratio; PARSIFAL, Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle study; PASTURE, Protection Against Allergy—Study in Rural Environments; SNP, single nucleotide polymorphism; B, beta estimates (linear regression).

*See Appendix 1 for authors in The PASTURE/EFRAIM study group.

Results: Parents correctly recalled wheeze after the first year in 54% of wheezers. This true-positive recall was determined by number of episodes, timing of the last wheeze episode, and parental asthma. Independently from these determinants, true-positive recall predicted asthma at age 6 years (odds ratio 4.54, 95% confidence interval (CI) [1.75-14.16]) and impaired lung function (β = −0.62, 95% CI [−1.12; −0.13], *P*-value = .02). Associations were stronger in children with asthma risk SNPs on chromosome 17q21.

Conclusion: Correct parental recall of wheezing episodes may reflect clinical relevance of early wheeze and its impact on subsequent asthma and lung function impairment. Questions tailored to parental perception of wheezing episodes may further enhance asthma prediction.

KEYWORDS

asthma risk, childhood asthma, chromosome 17q21, early wheeze, genetic asthma risk, parental wheeze recall

1 | INTRODUCTION

With about one in three children affected in the first 3 years of life, wheeze is a common phenomenon in early childhood, implying a substantial burden on children, their caregivers, and healthcare resources (EuroPrevall).[1,2]

Suffering from recurrent or persistent early wheeze and exacerbations implies a higher risk for later asthma, airway hyper-responsiveness, and decreased expiratory flow.[3] Particularly during the first year of life, wheeze exacerbations might set the stage for impaired airway function and persistent wheeze later in life,[4,5] whereas early diagnosis and intervention might prevent from detrimental lung sequelae.[4,6] Low interferon-γ (IFN-γ) levels have been shown to predict subsequent wheeze.[7]

Wheeze phenotype classifications are often based on parental reports of age of onset, intensity, and frequency of symptoms combined with suspected trigger factors.[8] Awareness of symptoms, however, varies among parents. Likewise, physicians may interpret reports of wheezing episodes differently, particularly when devices to conduct confirmatory lung function testing in young children are not available.[9-11]

The PASTURE study offered the opportunity to analyze wheeze episodes pro- and retrospectively during the first year of life and to relate them to subsequent development of asthma. The aims of the present analysis were (a) to compare the parent-reported wheeze prevalence at 12 months against the cumulative wheeze prevalence derived from weekly diaries from 2 to 12 months without assumption on over- or underestimation, (b) to find determinants affecting parental recall of wheeze episodes, and (c) to estimate the implications of true-positive recall at age 12 months on long-term respiratory health outcomes, that is, asthma and impaired lung function at age 6 years.

Key Message

In early childhood, physicians rely on parental report of physical symptoms, such as wheezing, to estimate the risk for a future asthma development in the children. We found that the parental tendency to recall or forget wheeze episodes in early childhood carries important information on severity and clinical relevance of early wheeze for a later asthma development. Thus to identify children with a high risk for later asthma, physicians should ask parents in detail about their perception of wheeze episodes, e.g. how threatening they experienced the situation.

2 | METHODS

Protection Against Allergy—Study in Rural Environments is a prospective birth cohort study conducted in five European countries.[12] The study was approved by local research ethics committees, and written informed consent was obtained from the children's parents. Pregnant women living in rural areas were contacted during the last trimester of pregnancy, and 1133 children were recruited shortly after birth.

Questionnaires at 2, 12, and 72 months were based on previously validated questionnaires from the International Study of Allergy and Asthma in Childhood (ISAAC),[13] the Allergy and Endotoxin Study (ALEX),[14] the Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle study (PARSIFAL),[15] and the American Thoracic Society (ATS).[16]

Information on wheezing episodes was collected via weekly diaries from the 8th until the 53rd week of life using the question "Within the last seven days, has your child had wheezing or whistling

sounds while breathing?" meaning a "sound coming out of the breast, not from the nose".[17] In the 12-month questionnaire, parents were asked how often their child had wheezed since the last home visit at age 2 months. Possible answer categories were *"never," "less than once a month," "once a month,"* and *"at least twice a month."* The four answer categories were dichotomized; children with any wheeze were classified as wheezers, and children whose parents answered "never" were classified as non-wheezers.

Because of prospective and timely collection of data, we used wheeze in the diaries as reference. The retrospectively collected information on wheeze using the 12-month questionnaires was then categorized for true-positive, true-negative, false-positive, and false-negative recall (Table S1).

Asthma was defined as either parent-reported physician's diagnosis of asthma once or parent-reported physician's diagnosis of obstructive bronchitis more than once when retrospectively asked in the 6-year follow-up questionnaire.

Atopy was defined as inhalant sensitization as previously classified by a latent-class approach over the first 6 years.[18]

Lung function measurements were conducted at age 6 years as previously validated.[19] FEV1/FVC z-score transformation was based on GLI (Global Lung Function Initiative) equations.[20,21] Genotyping included previously reported asthma risk alleles on chromosome 17q21.[17] Interferon-γ (IFN-γ) production at 12 months was measured after 24 hours of incubation of peripheral blood mononuclear cells with lipopolysaccharides.[22]

Statistical analyses were performed using R 3.3.2 (R Core Team, 2016). Mutually adjusted logistic regression models for true-positive recall (vs false-negative recall) and asthma, respectively, were established via backward elimination with level of entry < 0.2 and level of stay < 0.05.

After log transformation and subtraction from the maximum value, IFN-γ levels were left-censored. Therefore, associations with true-positive recall were assessed by Kaplan-Meier estimation and Cox proportional hazards models after confirmation of the proportional hazards assumption by the Schoenfeld residuals test. Estimates are reported as odds ratios (ORs), hazard ratios (HRs), or beta estimates (β) with corresponding 95% confidence intervals (CIs).

3 | RESULTS

Of all 1133 children primarily included in the PASTURE birth cohort, 880 children (78%) had diary data on wheeze events from the 8th until the 53rd week of life and information on asthma status until six years of age and were thus included in the analysis population (Figure S1). Among the included children (n = 880), there was a slight predominance of farm exposure, longer duration of breast-feeding, and parental education, whereas smoking during pregnancy was less common (Table S2). Within the analysis population, 611 children completed lung function measurements (Table S3).

The weekly prevalence of wheeze ranged between 1% and 3% and peaked at about 42 weeks; the proportion of concomitant rhinitis increased marginally (Figure 1). The cumulative incidence of wheeze until the first birthday was 35%. In 167 of all 307 children wheezing ever during the first year (54%), parents recalled wheeze symptoms at the end of the first year correctly. Parents of 140 children (46%) did not recall wheeze episodes in the preceding year in the questionnaire at 12 months though they had reported episodes in the diaries (Figure 2A).

Determinants of true-positive recall were parental asthma, total number of wheeze episodes within the first year of life, and a short

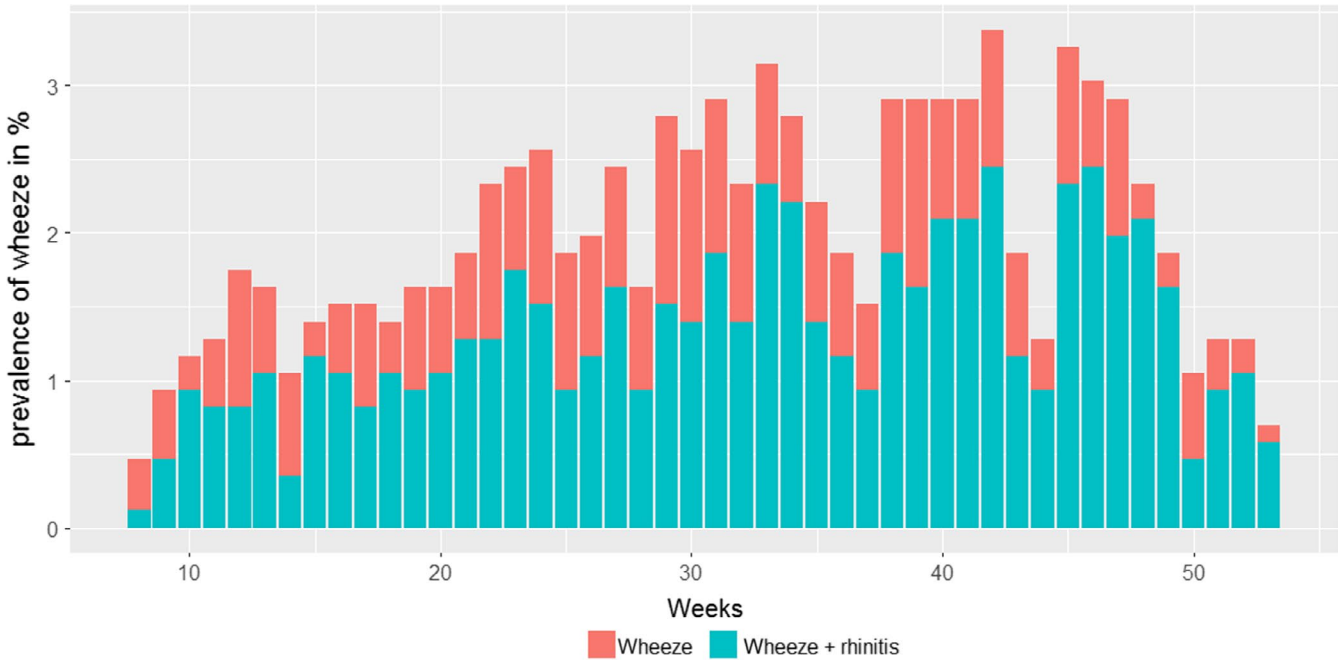

FIGURE 1 Wheeze weekly in the 1st y of life

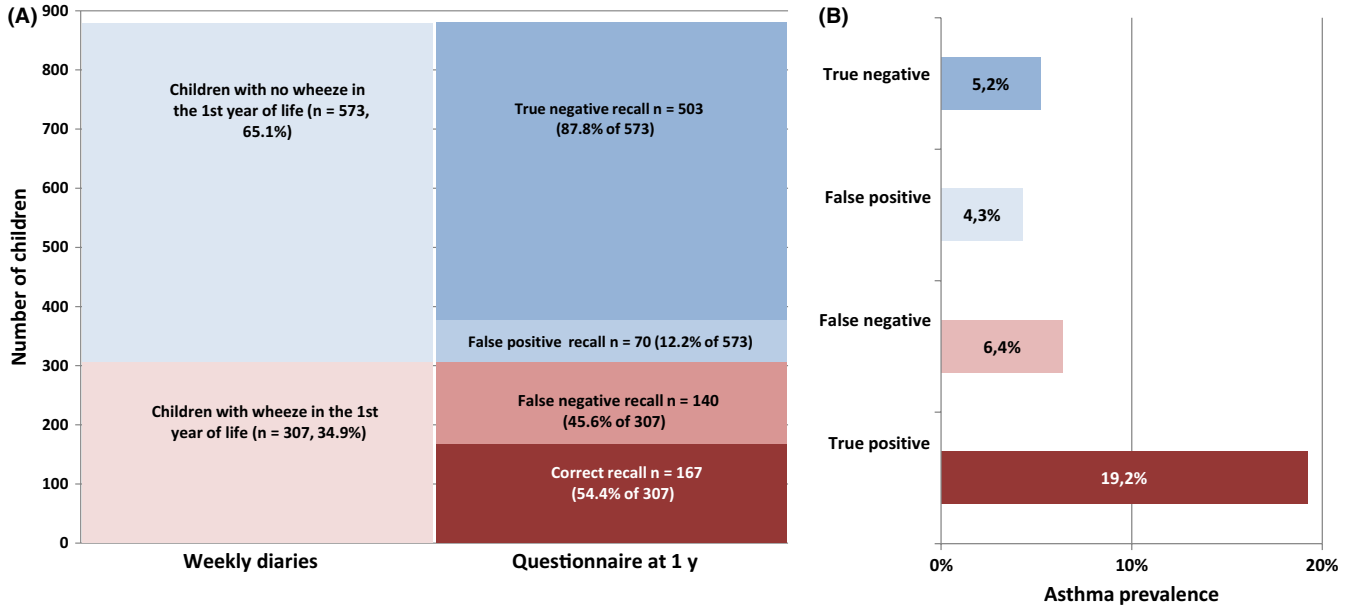

FIGURE 2 True-positive recall of wheeze episodes and subsequent asthma. A, Absolute numbers and percentages of children meeting the respective definitions. B, Prevalence of asthma within groups of children meeting the respective definitions. Information on wheeze based on weekly diary questionnaires or on the questionnaire at 1 y. True negatives are defined as no wheeze reported by both instruments. False positive means wheeze reported in the questionnaire at 1 y only. False-negative recall means wheeze reported only in weekly diaries but not recalled in the questionnaire at 1 y. True-positive recall is defined as wheeze reported by both instruments. The total number of children with data provided by both instruments was 880

time lag between completion of the one-year questionnaire and the last wheeze episode (Table 1). Average duration of wheeze episodes was 4.2 days with no significant difference between children with true-positive recall and children with false-negative recall (Wilcoxon test, P = .37). Sensitivity analyses stratifying for sex and for concomitant rhinitis or excluding Austria (because of no case of asthma) supported the determinants of true-positive recall albeit at varying effect size (Table S4). Medication for wheeze was reported only occasionally (2.7%) including antibiotics (n = 15), mucolytics (n = 9), inhalant corticosteroids (n = 4), and other drugs (n = 2) with a non-significant association with true-positive recall (OR = 1.77, CI 95% [0.59-5.32]).

Asthma was reported in 70 children at age 6 (8.0%). When used as a cumulative measure, the yearly asked question on asthma led to an overestimation of the asthma prevalence with 21.7% and was not considered for further analyses.

In the group with true-positive recall, the risk of asthma was about 4-fold increased as compared to children without wheeze or false-negative recall (Figure 2B). In general, the association of asthma with true-positive recall (OR = 3.45 [1.59-7.51]) was stronger as the association of asthma with any wheeze in the first year in the diaries, that is, true-positive and false-negative recall combined (OR = 2.89 [1.76-4.79]).

The association of true-positive recall with asthma was neither explained by number of wheeze episodes (OR = 1.06 [0.95-1.20]) nor by time lag from the last wheeze episode (OR = 0.98 [0.94-1.03]). Besides true-positive recall, only parental hay fever and being carrier of the asthma risk allele on chromosome 17q21 represented by the SNP rs7216389 emerged as additional asthma-predicting factors in our analysis population (Table 2). Associations were similar across the study centers (Table S5).

The association of true-positive recall with subsequent asthma was restricted to carriers of the 17q21 risk allele (Figure 3). When considering this interaction from a different perspective, association of asthma risk with risk genotype was only seen in children with true-positive recall of wheeze (OR = 2.46 [0.95-6.39]), whereas asthma risk was unrelated to genotype in children with false-negative recall (OR = 1.07 [0.12-9.75]).

Moreover, true-positive recall was strongly associated with impaired lung function as determined by the FEV1/FVC ratio at age 6 years (β = −0.62 [−1.12; −0.13]; Figure 4) independently from parental history of atopy (Table S6).

Children with true-positive recall produced about 50% higher IFN-γ levels upon in vitro stimulation with LPS as compared to children with false-negative recall (P = .04; Figure S2).

4 | DISCUSSION

When asked prospectively by weekly diaries, about one-third of the parents reported at least one wheeze episode in their children during the first year of life; when asked retrospectively at 12 months, however, only half of them remembered those episodes at all. The parental ability to recall those wheezing episodes in their infant's first year of life correctly was related to parental history of asthma, number of wheeze events during that one-year period, and only a

TABLE 1 Determinants of true-positive recall of wheeze episodes in children with wheeze in the first year as recorded in diaries (n = 307)

Determinants	Crude odds ratio	Adjusted odds ratio
Concomitant rhinitis	0.91 [0.50-1.64]	-
Timing of the last wheeze episode (in weeks of age)	1.05 [1.03-1.07]	1.04 [1.01-1.06]
Number of wheeze episodes	1.49 [1.26-1.76]	1.38 [1.17-1.65]
Average duration of wheeze episodes (in days)	0.91 [0.74-1.12]	-
Number of cough episodes	1.09 [1.03-1.16]	-
Number of rhinitis episodes	1.03 [0.98-1.09]	-
Sex	1.56 [0.99-2.47]	-
Farming status	0.75 [0.48-1.18]	-
Mode of birth	1.61 [0.80-3.23]	-
Breast-feeding (>6 mo)	1.12 [0.72-1.76]	-
Smoking in pregnancy	1.01 [0.53-1.91]	-
Older siblings	1.18 [0.73-1.91]	-
Parental hay fever	1.09 [0.69-1.71]	-
Parental asthma	2.30 [1.24-4.27]	2.46 [1.27-4.95]
Parental atopic eczema	1.10 [0.50-2.40]	-
Parental atopy	1.32 [0.83-2.08]	-
Parental education	1.06 [0.67-1.67]	-
Having pets in the 4th y	0.71 [0.43-1.19]	-
Day care attendance in the first 3 y	1.44 [0.46-4.55]	-

Note: Odds ratios are given with 95% confidence intervals. For crude models, only determinants with *P*-values < .2 are listed. Adjusted models were established by backward elimination.

TABLE 2 Associations of true-positive recall of wheeze with an asthma diagnosis (n = 880)

Determinants	Crude odds ratio	Adjusted odds ratio
True-positive recall	3.45 [1.59-7.51]	4.54 [1.75-14.16]
Sex	2.21 [1.31-3.74]	-
Farmer	0.56 [0.34-0.93]	-
Parental asthma	3.94 [2.30-6.73]	-
Parental hay fever	3.11 [1.83-5.30]	2.86 [1.23-7.09]
Parental atopic eczema	4.08 [1.50-11.13]	-
Parental atopy	2.83 [1.61-4.97]	-
Chr 17q21 (rs7216389)	1.68 [1.12-2.52]	1.92 [1.05-3.61]

Note: Odds ratios are given with 95% confidence intervals. For crude models, only determinants with *P*-values < .05 are listed. Adjusted models were established by backward elimination.

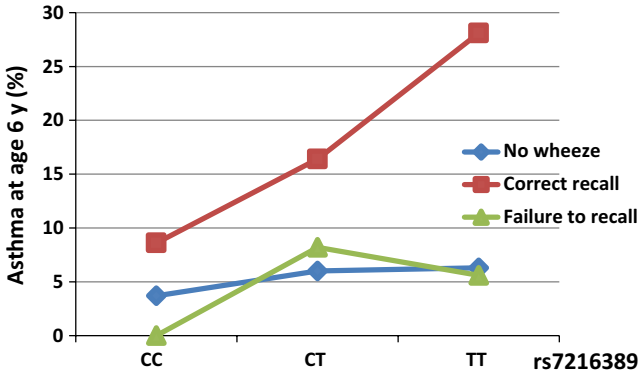

FIGURE 3 Asthma risk stratified for true-positive recall and genotypes (CC, CT, TT) of asthma risk allele (rs7216389) on chromosome 17q21

short time lag since the last wheeze episode. In turn, true-positive recall was associated with a higher risk of subsequent asthma and impaired lung function as compared to false-negative recall. The association of true-positive recall with asthma was restricted to the asthma risk genotype encoded on 17q21.

Obviously, parental observations on wheeze are susceptible to recall bias and vary with respect to disease severity, compliance, and socioeconomic background of the study participants.[3,13,23] In the current setting, however, we demonstrate that recall bias can be informative in itself.

The reasons why parents recall wheeze episodes in their children differently may include previous knowledge and experience, cautiousness, health perception, and possibly the impressiveness or severity of the episode. Evidently, parents suffering from asthma themselves are more attentive to wheeze events in their children and might more likely recall wheezing episodes. Moreover, children with a family history of asthma might be affected by more serious wheeze events, which may present more impressively to any observer.[24-26] Likewise, a higher number of total wheeze events during the first year of life and a short time lag from the last episode may enhance parental recall.

Despite the rather unspecific questions on wheeze episodes, a strong association of true-positive recall with subsequent asthma emerged as the main result of this analysis. This association was independent of all the above determinants for true-positive recall, thereby suggesting that parents anticipate subsequent chronic disease from the way the wheeze episodes present. Unfortunately, more detailed information on wheeze episodes was not collected; thus, we do not know why these episodes were kept in memory. At least, the mere duration of episodes and usage of wheeze medication within the first year were unrelated to true-positive recall though reporting of medication was rather low and possibly incomplete.

The predictive value of wheeze episodes in the first year of life has previously been attributed to the asthma risk genotype encoded on chromosome 17q21, whereas in the non-risk genotype, early wheeze was unrelated to subsequent asthma.[17,27] The current analysis refines this finding insofar as the interaction of genotype

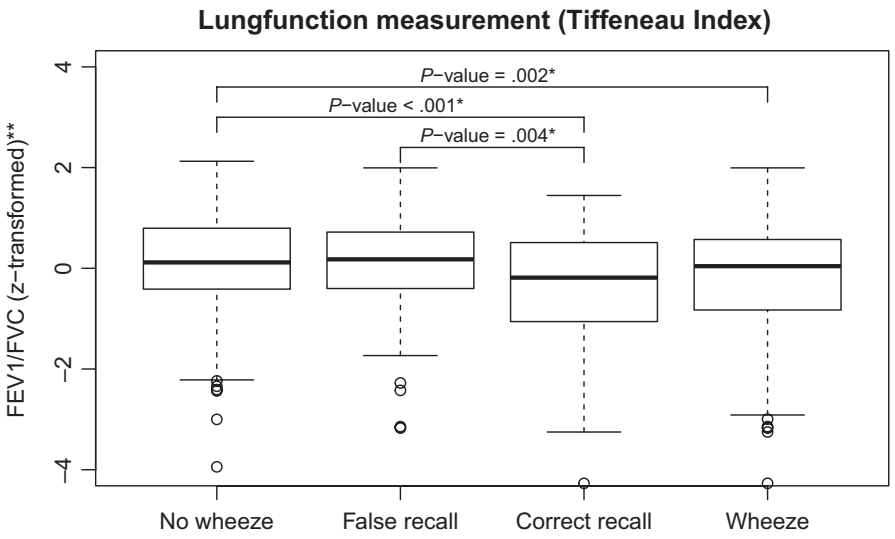

FIGURE 4 Lung function (FEV1/FVC) in children with false-negative recall and true-positive recall. Shown is a standard box-and-whisker plot with boxes representing the interquartile range and whiskers extending to maximum and minimum values within 1.5 times the interquartile range. **P*-values are based on an ANOVA and a post hoc *t* test. ***FEV1/FVC z-score based on GLI (Global Lung Function Initiative) equations

with early wheeze is restricted to wheeze episodes that parents recall. Children whose parents forgot wheeze episodes resembled non-wheezing children in their absent association of asthma with genotype. Vice versa, genetically determined more severe forms of asthma may manifest early in life with conspicuous symptoms. Accordingly, genetic testing for the asthma risk genotype on chromosome 17q21 might be advisable in children presenting with remarkable wheezing episodes.

Functionally, the asthma risk genotype on chromosome 17q21 has been implied in enhanced susceptibility to virus infections and impaired antiviral immunity.[28] The latter might also be reflected by lower IFN-γ levels upon stimulation with lipopolysaccharides as seen in children with true-positive recall. Moreover, wheeze often occurred concomitantly with rhinitis; thus, also the frequency of wheeze episodes may indicate a partially compromised immune system.[7,29]

The strong association of true-positive recall with asthma was paralleled by the association of true-positive recall with a decreased FEV1/FVC ratio measured at age 6 years. This may indicate that children with more severe wheeze symptoms are already prone to deteriorating lung function later in life through incompletely healed injuries of pulmonary tissue or alteration of lung epithelial cell growth.[4-6] Alternatively, causation might be reverse, and children born with compromised airway function might present with more severe symptoms.[30]

Current scientific knowledge favors early diagnosis and early medical treatment of asthma symptoms. This may enhance lung growth from the beginning, mitigate progression toward asthma, prevent from lung function impairment, and thus improve quality of life in the long run.[6,31] However, assessment of lung function in very young children is limited or can only be conducted in an incomplete manner [6]; thus, clinicians often have to rely on basic physical examination, family history, and parental report of symptoms.

Though the quality of data collected via weekly diary questionnaires filled by parents is considered rather high and has been validated against the gold standard of medical records,[32] we admit that the mere question on wheeze episodes as asked in the PASTURE study seems rather unspecific. Furthermore, a proper distinction between separate and ongoing wheeze episodes over several weeks was not possible based on the way of questioning within the weekly diary reports. These drawbacks might have led to an underestimation of the predictive value of parental report. Thus, in the future more detailed questions should be included in research questionnaires and standardized history taking.

Another strength of the PASTURE study besides its longitudinal study design is the involvement of children from 5 different European countries, well depicting a profile of distinct European lifestyles. The associations of parental wheeze recall with later asthma were rather consistent over all study centers (I^2 < 10%) and thus demonstrate reliability and generalizability of our results across various regions of Europe.

This is remarkable since cross-cultural validity and comparability are hampered by language differences, and the terminology of wheeze in non-Anglophone countries might be difficult. In the German language, for example, there is not even a colloquial term for "wheezing".[33] Likewise, German-speaking physicians are somewhat reluctant to diagnose "asthma"; among others, they prefer the diagnosis "obstructive bronchitis." This led to the definition of asthma as a physician's diagnosis of asthma or recurrent obstructive bronchitis in the ISAAC study.[13] Originally this definition was established and validated against cold-air provocation in schoolchildren,[34] whereas in a prospective study such as PASTURE, it would lead to overestimation of the asthma prevalence when used as a cumulative variable. This phenomenon again suggests that forgetting early episodes of obstructive bronchitis until school age seems justified; oblivion may transport unconscious information.

In conclusion, our findings suggest that parents intuitively notice clinically relevant wheeze episodes in their children. This perception might contribute to a better prognosis of asthma and impaired lung function later on. Conversely, wheeze episodes without long-lasting consequences are more likely forgotten. Parents might therefore be advised not to bother much about mild wheeze episodes.

Concurrently, continuous monitoring may run the risk of capturing irrelevant episodes and thus unsettling parents unnecessarily. Nevertheless, history taking should include more detailed questions on alarming wheeze episodes, as it could help identifying children who might benefit from immediate treatment, reduce need for emergency treatment, and enhance quality of life. Ultimately, this analysis demonstrates the value of skillful listening to parents reporting their children's symptoms.

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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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APPENDIX 1

The PASTURE/EFRAIM study group: Marjut Roponen, Maija Riitta Hirvonen, Anne Hyvärinen, Pirkka V. Kirjavainen, Pekka Tittanen, and Sami Remes (Finland); Marie-Laure Dalphin (France); Sabina Illi, Bianca Schaub, Jon Genuneit (Germany); Remo Frei (Switzerland); Gert Doekes (The Netherlands); Urs Frey and Oliver Fuchs (lung function measurements); Michael Kabesch, Michel Sven, and Jörg Tost (DNA analysis); Harald Renz (cytokine measurements); and Elisabeth Schmaußer-Hechfellner (data management).

Table E1: Definition of true and false recall

Wheeze prospectively recorded in diaries	Wheeze retrospectively recorded at 12 months	Recall	Coding for analysis
present	present	True positive	1
present	absent	False negative	0
absent	present	False positive	NA
absent	absent	True negative	NA

Individuals with wheeze episodes absent in the diaries were coded as ‘not available’ (NA) in statistical analyses.

Table E2: Individuals included and not included in the analysis population

Variable	Included (n = 880)[#]	Not included (n = 253)[#]	p-value[°]
Asthma	70 (8.0%)	8 (16.7)	0.06
Atopy	154 (21.6%)	9 (16.7%)	0.49
Male sex	450 (51.1%)	110 (52.4%)	0.81
Living on a farm	429 (48.8%)	101 (39.9%)	0.02
High parental education	460 (52.8%)	102 (45.9%)	0.08
Parental asthma	131 (15.0%)	34 (16.1%)	0.76
Parental hay fever	390 (44.5%)	101 (45.7%)	0.80
Parental atopy	472 (53.8%)	119 (53.6%)	1.0
Older siblings	557 (63.3%)	163 (64.4%)	0.80
Breastfeeding at least 6 months	495 (56.2%)	74 (45.1%)	0.01
Mode of birth (C-section)	164 (18.8%)	28 (13.4%)	0.08
Smoking during pregnancy	105 (12.0%)	53 (21.0%)	<0.001
Day care	33 (4.9%)	5 (4.9%)	0.85
Pets at home	240 (28.1%)	38 (28.1%)	0.59

[#]Variable comparison among those with information on the respective variable

°p-value from fisher's exact test

Table E3: Individuals included and not included in the lung-function measurements at age 6 years among all 880 children of the analysis population

Variable	Included (n = 611)[#]	Not included (n = 269)[#]	p-value°
Asthma	54 (8.8%)	16 (5.9%)	0.19
Atopy	121 (19.8%)	33 (12.3%)	0.01
Male sex	327 (53.5%)	123 (45.7%)	0.04
Living on a farm	298 (48.8%)	131 (48.7%)	1.0
High parental education	328 (54.0%)	132 (50.0%)	0.31
Parental asthma	100 (16.5%)	31 (11.6%)	0.08
Parental hay fever	280 (46.0%)	110 (41.0%)	0.2
Parental atopy	341 (56.0%)	131 (48.9%)	0.06
Older siblings	398 (65.1%)	159 (59.1%)	0.1
Breastfeeding at least 6 months	332 (54.3%)	163 (60.6%)	0.1
Mode of birth (C-section)	109 (18.0%)	55 (20.6%)	0.41
Smoking during pregnancy	77 (12.6%)	28 (10.4%)	0.42
Day care	23 (5.0%)	10 (4.6%)	0.96
Pets at home	161 (27.1%)	79 (30.5%)	0.34

[#]Variable comparison among those with information on the respective variable

°p-value from fisher's exact test

Table E4: Determinants of true positive recall of wheeze (stratified for sex, wheeze with and without concomitant rhinitis and all centers and all centers but Austria)

Determinants	OR [95%CI]	OR [95%CI]
	Boys (n=174)	**Girls (n=133)**
Parental asthma	1.85 [0.74-4.89]	3.64 [1.34-11.04]
Timing of last wheeze episode (in weeks of age)	1.05 [1.01-1.09]	1.02 [0.98-1.05]
Number of wheeze episodes	1.65 [1.27-2.28]	1.18 [0.97-1.51]
	Children with wheeze and concomitant rhinitis (n=243)	**Children with wheeze only (n=54)**
Parental asthma	3.02 [1.46-6.55]	1.11 [0.11-7.40]
Timing of last wheeze episode (in weeks of age)	1.04 [1.01-1.07]	1.05 [1.00-1.12]
Number of wheeze episodes	1.42 [1.20-1.73]	1.18 [0.75-1.01]
	All centers (n=307)	**All centers but Austria (n=257)**
Parental asthma	2.46 [1.27-4.95]	2.30 [1.15-4.77]
Timing of last wheeze episode (in weeks of age)	1.04 [1.01-1.06]	1.04 [1.01-1.06]
Number of wheeze episodes	1.38 [1.17-1.65]	1.35 [1.15-1.63]

Multiple logistic regression models were used to calculate sex-specific associations between true positive recall and asthma.

Table E5: Associations of true positive recall with asthma and lung-function

Associations of true positive recall of wheeze with an asthma diagnosis – whole study population and separately in study centers			
	OR [CI 95%]	P-value	I^2 (Inconsistency)
Total study population	3.45 [1.59-7.51]	0.0018	0.0%
Germany	2.48 [0.6-10.21]	0.208	
Austria[#]	(>5.22)	-	
Switzerland	1.85 [0.33-10.42]	0.483	
France	2.06 [0.35-12.02]	0.422	
Finland	6.07 [1.18-31.24]	0.031	
Associations of true positive recall of wheeze with lung-function parameters (Tiffenau Index FEV1/FVC) – whole study population and separately in study centers			
	ß-Estimate [CI 95%]	P-value	I^2 (Inconsistency)
Total study population	-0.41 [-0.68; -0.13]	0.005	6.74%
Germany	-0.24 [-1.04; 0.29]	0.412	
Austria	-0.95 [-1.60; -0.31]	0.017	
Switzerland	-0.59 [-1.41; 0.14]	0.075	
France	-0.25 [-0.79; 0.17]	0.255	
Finland	-0.10 [-0.71; 0.54]	0.737	

Logistic regression models were used to calculate center-specific associations between wheeze recall and asthma.

[#]in Austria no case of asthma was observed among children with false negative recall; if there were one case, the OR would be 5.22

Table E6: Associations of true positive recall of wheeze with lung-function parameters (Tiffenau Index FEV1/FVC z-score)

Determinants	Crude β-Estimate [95% CI]	Adjusted β-Estimate [95% CI]
True positive recall	-0.41 [-0.68; -0.12]	-0.62 [-1.12; -0.13]
Sex	-0.38 [-0.47; -0.16]	-
Parental asthma	-0.28 [-0.49; -0.07]	-
Parental hay fever	-0.12 [-0.28; 0.04]	-
Parental atopic eczema	-0.5 [-0.80; -0.21]	-0.51 [-1.01; -0.01]
Parental atopy	-0.18 [-0.34; -0.02]	-

Only determinants with crude p-values <0.2 are listed. β-estimates are given with 95%-confidence intervals (CI). Adjusted models were established by backward elimination. FEV1/FVC z-score based on GLI (Global Lung-function Initiative) equations.

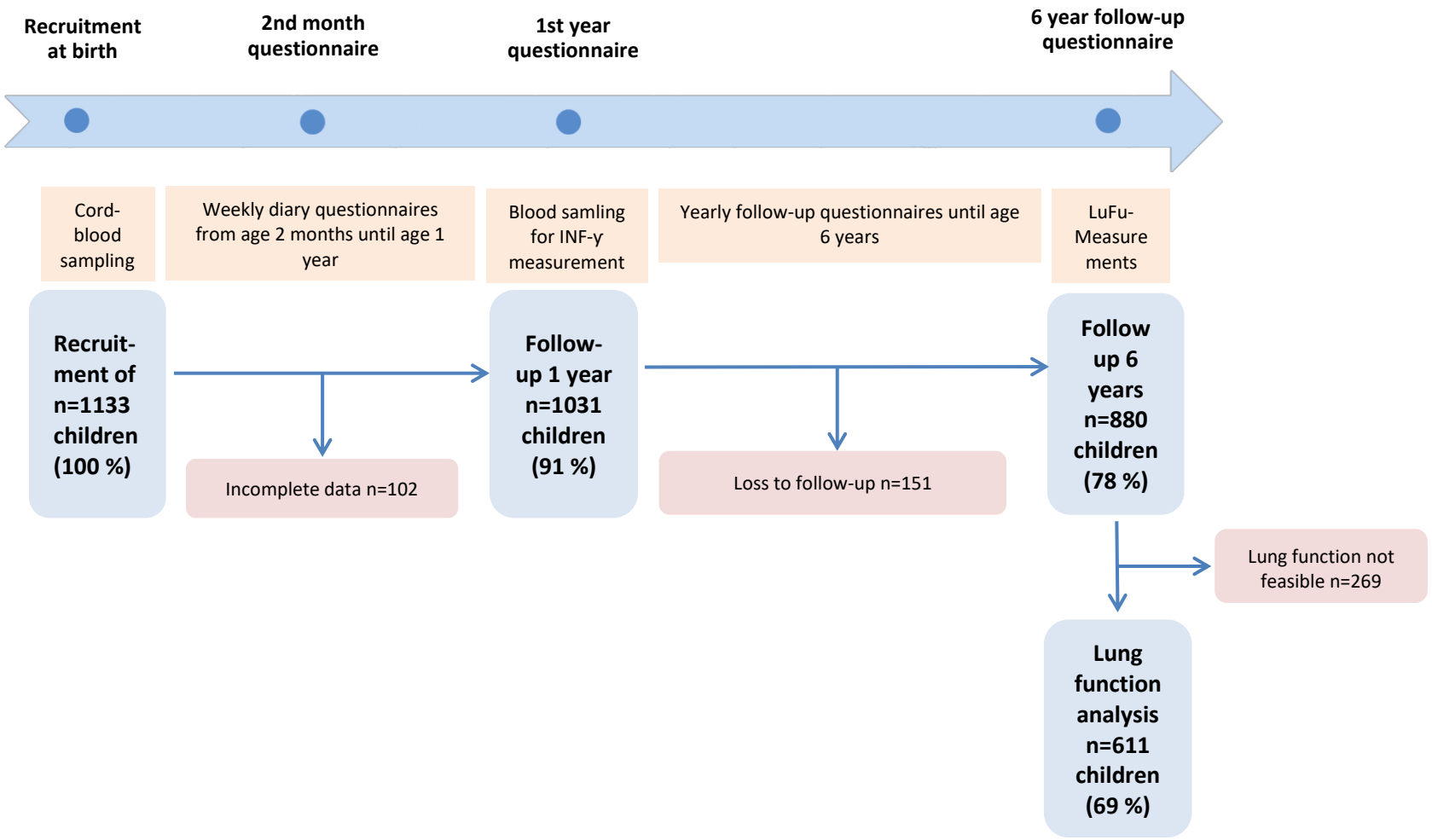

Figure E1: Selection of the study population and timeline of the PASTURE study up to age 6 years

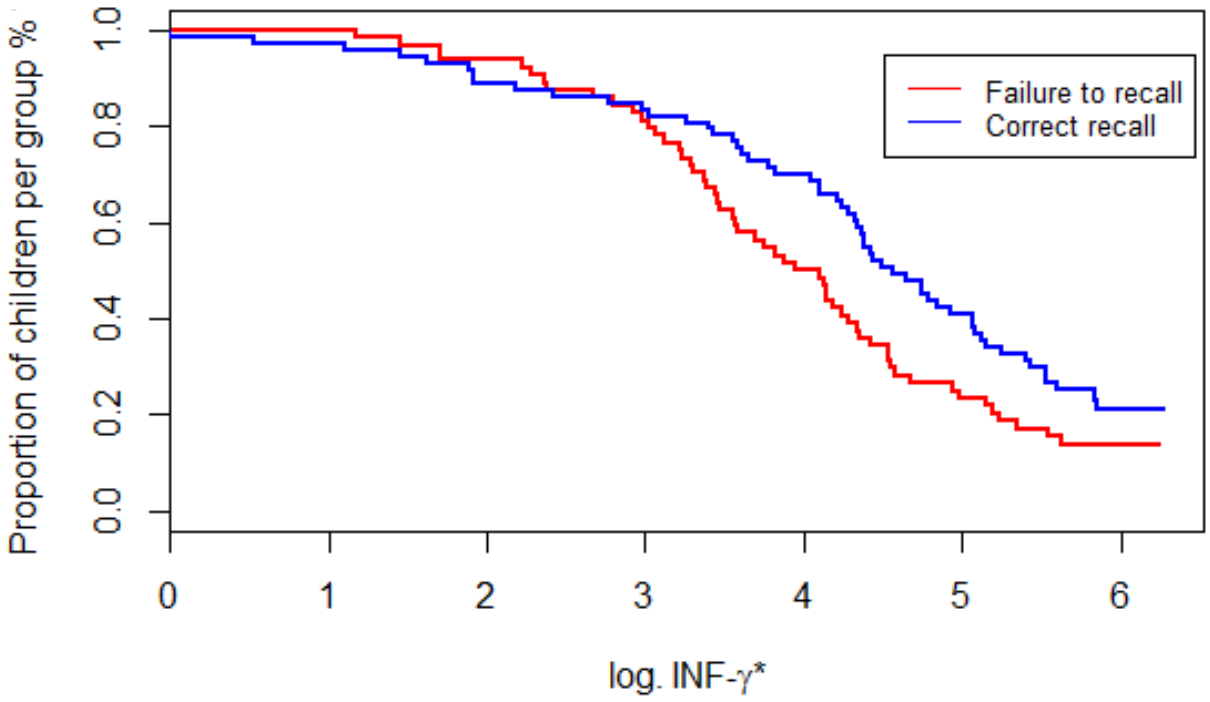

Figure E2: INF-γ levels in children with correct recall and failure to recall
*to achieve normal distributed, left censored INF-y values, original values were first log-tranformed and then subtracted from max. INF-y.
HR=0.68, CI 95% [0.46-0.98] Proportional hazards assumption was previously confirmed (Schönefeld Residuals Test, p-value= 0.51)

Online supplement

Methods

Study design and population

The PASTURE project is a prospective birth cohort study which started in 2002. The prior study aim was to assess influencing factors of rural and farming environment on the development of asthma and allergies in children. The study was conducted in five European countries; Austria, Finland, France, Germany and Switzerland. Local research ethics committees in each country approved the study; written informed consent was obtained from the children's parents. Detailed information on study design is described elsewhere [1].

Initially pregnant women living in rural areas were contacted during the last trimester of pregnancy. 1133 children matching the inclusion criteria were recruited shortly after birth. Detailed information on the study design are described elsewhere[1]. Children without information on wheeze events during their first year of life (n=104) and who did not complete the follow-up period of 6 years (n=214) were excluded; thus 880 children with sufficient background and health information were included in the following analysis. Lung function measurements were performed in a subgroup of 611 children (Figure 1).

Questionnaires

The questionnaires of the PASTURE study based on items of the International Study of Allergy and Asthma in Childhood (ISAAC) [2], the Asthma Multicenter Infants Cohort Study (AMICS), the Allergy and Endotoxin Study (ALEX) [3], the Prevention of Allergy Risk factors for Sensitization In children relating to Farming and Anthroposophic Lifestyle study (PARSIFAL) [4] and the American Thoracic Society (ATS) [5] questionnaire.

Parents were initially asked to complete a questionnaire at two months age of their child. Thereafter additional information on farm-related exposures and feeding practices, occurring infections, medication and wheezing episodes of the infant was collected via weekly diary questionnaires from the 8th until the 53rd week of life[1]. In the one-year-

questionnaire, parents were asked how often their child had wheezed since the last home visit at age two months. Possible answer categories were *"never"*, *"less than once a month"*, *"once a month"* and *"at least twice a month"*. The four answer categories were dichotomized; children with any wheeze were classified as wheezers, children whose parents answered "never" were classified as non-wheezers.

Thereupon regular questionnaires were handed out the parents up to six years of age. These questionnaires comprised detailed information about current socioeconomic and lifestyle, agricultural exposures and atopic symptoms and further health issues of the child and the family of origin. A physician's diagnosed asthma was enquired in the six-year-questionnaire.

Reference standards and definition of true positive and false negative reporting

Report of wheeze episodes via weekly questionnaires between the 8th and 53rd week of life was regarded as reference standard. Correct recall and failure to recall were defined as followed.

True positive recall: at least one wheezing episode during the child's first year of life reported in the weekly diary questionnaires plus retrospectively correct recall of the wheezing episodes when asked "Since we last visited you, how often has your child had wheezing or whistling" in the one-year questionnaire.

False negative recall: at least one wheezing episode during the child's first year of life reported in the weekly diary questionnaires but no recall of the wheezing episodes when asked "Since we last visited you, how often has your child had wheezing or whistling" in the one-year questionnaire.

Children whose parents did not report any wheeze in the diary questionnaires were subdivided into true negative recall (children with no wheeze ever until the age of 6 years) and false positive recall (children whose parents reported wheeze in the one-year questionnaire).

Asthma was defined as either parent-reported physician's diagnosis of asthma once or parent-reported physician's diagnosis of obstructive bronchitis more than once when retrospectively asked in the 6 year follow-up questionnaire.

Atopy was defined as inhalant sensitization as previously classified by a latent-class approach over the first 6 years [6].

Measurements

Lung function measurements were conducted at 6 years age as previously validated [7]. Forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were measured. Hence based on GLI (Global lungfunction initiative) [8, 9] equation the z-transformed Tiffeneau Index (ratio of FEV1 and FVC) was calculated for every child. Cord blood was collected and genotyped for previously reported asthma risk alleles on chromosome 17q21 [10] to investigate in possible associations of genetic predisposition and parental ability to recall wheezing episodes. Peripheral blood mononuclear cells were isolated at age 1 year and incubated with LPS (lipopolysaccharides) for 24 hours. Interferon- γ (IFN- γ) production was measured via multiplexed cytometric bead array according to the manufacturer's instructions (BD) in Marburg, Germany [11]. Resulting IFN-γ values were standardized on leukocyte counts.

Statistical Analysis

Comparison of background characteristics between children in the recruitment, the one-year follow-up and the six-year follow-up population were calculated via Fisher's exact test. Mutually adjusted logistic regression models for true positive recall (versus false negative recall) and asthma, respectively, were established via backward elimination with level-of-entry<0.2 and level-of-stay<0.05.

After log-transformation and subtraction from the maximum value, IFN-γ levels were left-censored. Then IFN-γ levels between children with correct recall and failure to recall were compared via Kaplan-Meier-Estimation and Cox proportional hazards models after confirmation of the proportional hazards-assumption by the Schönefeld Residuals-test. Estimates are reported as odds ratios (ORs), hazards ratios (HR), or beta-estimates (β) with corresponding 95% confidence intervals (CIs), respectively. All reported logistic regression models were considered statistically significant with a p-value < 0.05. Statistical analyses were performed using R 3.3.2 (R Core Team, 2016).

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13. Publikation 4 (Metaanalyse)

The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial

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The low prevalence of asthma and allergies in farm children has partially been ascribed to the consumption of raw cow's milk. A literature search identified 12 publications on 8 pertinent studies. A meta-analysis corroborated the protective effect of raw milk consumption early in life (<1 to 5 years, according to study) on asthma (odds ratio [OR], 0.58; 95% CI, 0.49-0.69), current wheeze (OR, 0.66; 95% CI, 0.55-0.78), hay fever or allergic rhinitis (OR, 0.68; 95% CI, 0.57-0.82), and atopic sensitization (OR, 0.76; 95% CI, 0.62-0.95). The effect particularly on asthma was observed not only in children raised on farms (OR, 0.62; 95% CI, 0.58-0.82) but also in children living in rural areas but not on a farm (OR, 0.60; 95% CI, 0.48-0.74). This demonstrates that the effect of farm milk consumption is independent of other farm exposures and that children not living on a farm can theoretically profit from this effect. Because of the minimal but real risk of life-threatening infections, however, consumption of raw milk and products thereof is strongly discouraged. Raw farm milk and industrially processed milk differ in many instances including removal of cellular components, manipulation of the fat fraction, and various degrees of heating. Preliminary evidence attributes the effect to heat-labile molecules and components residing in the fat fraction. The Milk Against Respiratory Tract Infections and Asthma (MARTHA) trial is currently testing the protective effect of microbiologically safe, minimally processed cow's milk against standard ultra-heat-treated milk in children from 6 months to 3 years with the primary outcome of an asthma diagnosis until age 5 years. If successful, this approach might provide a simple but effective prevention strategy. © 2019 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2020;8:878-89)

Key words: *Asthma; Allergies; Respiratory infections; Farm exposures; Farm milk; Raw cow's milk; Milk components*

INTRODUCTION

The "farm effect" relates to the phenomenon that children growing up on traditionally husbanded farms are less affected by atopic sensitization, asthma, allergic rhinitis, and hay fever compared with children who are raised in rural areas but not on farms.[1-9] Initially observed in central Europe, similar protective effects were found in other parts of Europe, the United States, South America, and New Zealand, pointing toward a global phenomenon.[10-13] In distinct settings, however, increased risk of asthma and wheeze has been described, for example, by exposure to more industrialized farms, hog farming, extensive usage of antibiotics, or in occupational exposure.[14,15] However, the beneficial farm effect observed in children living on traditional farms has been attributed to 2 independent factors, that is, contact to straw and cows, and the consumption of unprocessed cow's milk directly obtained from a farm.[16,17]

Consumption of unprocessed cow's milk is particularly interesting for future preventive measures, because it exerts asthma- and allergy-protective effects also in children otherwise not exposed to farming.[3,18,19] Although the consumption of raw cow's milk bears serious health risk and is strongly discouraged by health authorities, consumption of raw cow's milk and derived raw products including fermented dairy is still common practice in farm families and is often introduced in the children's diet before the first birthday.[13,20] Health and food authorities require raw milk to be heated before consumption, but this cannot be controlled and is often ignored.

In contrast, children not living on farms mainly consume milk from supermarkets ("shop milk"). Some European countries allow the sale of raw milk in special vending machines where it is subjected to strict regulations and permanent controls; however, additional boiling before consumption must be explicitly

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Conflicts of interest: K. Hettinga has received research support from FrieslandCampina; has received research support, personal fees, and nonfinancial support from Mead Johnson Nutrition; and has a patent pending (WO2018108883). M. J. Ege has received research support from the German Federal Ministry of Research for funding of the German Center for Lung Research, the Bavarian State Ministry of Health and Care, and Dutch Longfonds for funding of the Milk Against Respiratory Tract Infections and Asthma (MARTHA) trial; has received nonfinancial support from FrieslandCampina (which provides milk powder for the MARTHA trial); and has patents US000009950017B2/EP000001964570B1 and EP000002361632B1, the latter of which is with royalties paid to Ludwig Maximilians University/Technical University Munich. The rest of the authors declare that they have no relevant conflicts of interest.

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Abbreviations used
ALEX- Allergy and Endotoxin
GABRIEL- Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community
HR- Hazard ratio
microRNA- miRNA
PARSIFAL- Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style
PASTURE- Protection Against Allergy Study in Rural Environments
UHT- Ultra-heat treatment

recommended to consumers. In most countries, pasteurization at 72°C is the minimal requirement for microbial safety.[21] For extended shelf life, heat treatment is performed at high or ultra-high temperature. Besides heat treatment, shop milk usually passes several other processing steps including centrifugation, filtering, and homogenization.

The most profound differences between raw milk and shop milk result from manipulation of the fat fraction and heating. These procedures affect the various components of the complex liquid cow's milk specifically. Moreover, composition and quantity of the components depend on species, feeding, lactation stage, milking frequency, and other environmental factors.[22] Although quantity and functionality of potential health-promoting milk constituents are difficult to disentangle, several candidate molecules have been suggested: whey proteins, microRNA (miRNA), polyunsaturated fatty acids, and oligosaccharides.[18,19,22-30]

In this review, we provide a meta-analysis of all studies worldwide addressing the asthma- and allergy-protective effect of unprocessed cow's milk. In addition, we present an in-depth meta-analysis of the associations between raw cow's milk consumption in early childhood and asthma, current wheeze, atopy, and hay fever in the 4 large Central European studies, whose data were directly available (Allergy and Endotoxin [ALEX] Study; Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style [PARSIFAL]; Protection Against Allergy Study in Rural Environments [PASTURE]; Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL]). Subsequently, we review the evidence for candidate molecules possibly involved in the protective effect. Finally, we present an approach to demonstrate the farm milk effect in an interventional setting.

META-ANALYSIS OF THE ASTHMA- AND ALLERGY-PROTECTIVE EFFECT OF UNPROCESSED COW'S MILK

The present meta-analysis was conducted in accordance to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA). A comprehensive literature search was performed within the electronic databases PubMed and ClinicalTrias.gov. The inclusion criteria for the meta-analysis were (1) longitudinal, cross-sectional, or case-control studies, (2) human studies, (3) investigation in the association of cow's milk consumption directly derived from a farm compared with shop milk or no milk consumption and at least 1 of the outcomes of interest (asthma, current asthma/current wheeze, atopy, hay fever), (4) early in life or current consumption of milk reported, (5) age of participants below 20 years, (6) reporting of the odds ratio with CI or P value, and (7) publication in English or German. All eligible articles published until June 30, 2019, were included. Meta-analyses, review articles, and case studies were excluded. Definitions of outcomes were based on questionnaire data (standardized International Study of Asthma and Allergies in Childhood [ISAAC] questions) primarily filled by the parents or the participants themselves and skin prick test or specific IgE measurements in the children. In the given age group of schoolchildren, the terms hay fever and seasonal/allergic rhinoconjunctivitis were considered equivalent. Consumption of raw or unprocessed farm milk was defined as reported consumption of either nonpasteurized milk or milk directly derived from a farm without any heating before consumption. The reference group included children either not drinking raw milk or less than once a week (ALEX, PARSIFAL, PASTURE, GABRIEL), or only drinking milk bought in a supermarket, which was at least pasteurized.

Multiple logistic regression models were used to estimate the associations between raw cow's milk consumption and asthma, hay fever, atopy, and eczema. Original study effects were reported as odds ratios and 95% CI or P values. Interstudy heterogeneity was studied via Thompson's and Higgins' I^2 criterion. Summary effect sizes were calculated with random-effects models to account for heterogeneity between study effects. DerSimonian-Laird method with inverse variance weighting was used to assign specific weights to the respective studies.

Similarly, an additional meta-analysis was performed using data from the 4 Central European studies (ALEX, PARSIFAL PASTURE, GABRIEL), which were homogeneous with respect to exposure and outcome definitions. Selection of potential confounder variables was based on literature or on the change-in-estimate criterion. Adjusted P values of less than .05 were considered significant. All calculations were done with R (R Core Team, Vienna, Austria).[31]

META-ANALYSIS OF ALL STUDIES WORLDWIDE

The database search resulted in 59 matching publications for all 4 outcomes; 12 publications met the inclusion criteria (Figure 1) and reported findings from 8 studies, 6 on early in life and 5 on current milk consumption (Table I). Associations between raw milk consumption in early childhood and asthma, current wheeze, and rhinoconjunctivitis later in childhood were similar across the different studies (Figure 2; $I^2 = 0.0$, 7.6, and 0.0, respectively), but less so for atopy ($I^2 = 45.4$).

Comparability was somewhat limited because periods and definition of exposure, outcome definitions, and study populations differed considerably between the studies. Barnes et al[34] investigated raw milk within the first 5 years, Wickens at al[12] surveyed the first 2 years, whereas the other studies focused on the first year of life. Sozanska et al[33] stratified for frequency of milk consumption and found relevant inverse associations only in children frequently drinking raw milk (Figure 2).

The 4 Central European studies and Perkin and Strachan[32] assessed *current* raw milk consumption and found consistent associations (Figure 3). In PASRIFAL, current raw milk consumption was inversely associated with sensitization to pollen but no other allergen specificities.

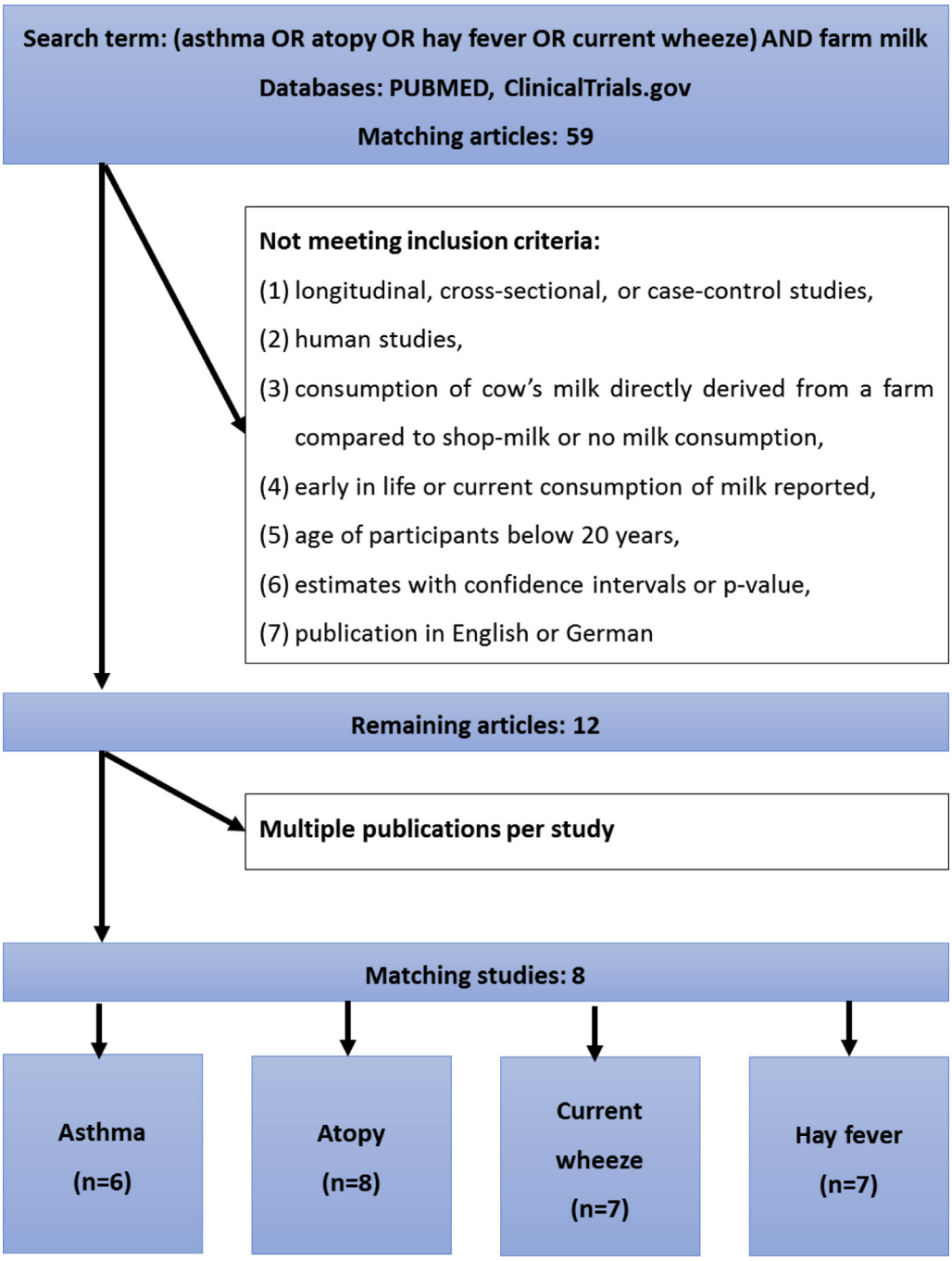

FIGURE 1. Flowchart search strategy.

IN-DEPTH META-ANALYSIS IN THE 4 LARGE CENTRAL EUROPEAN STUDIES

The higher comparability of the 4 Central European studies with respect to study populations, exposure and outcome definitions, and the availability of original data allowed for additional analyses. Stratification according to farming status showed similar effects of early milk consumption on asthma in farm and nonfarm children (Figure 4). Upon stratification, the effects on atopy were weaker in both groups and on hay fever in farm children (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). The effect of current consumption on wheeze was absent in nonfarm children and on atopy in farm children (see Figure E2 in this article's Online Repository at www.jaci-inpractice.org).

In conclusion, we found a consistent protective potential of early and current raw milk consumption for asthma in both farm and nonfarm children and with some limitations for the other outcomes.

TABLE I. Study characteristics

Name/origin	Study population	Exposure: Raw milk consumption	Outcome definition according to ISAAC questionnaire
New Zealand, Wickens et al,[12] 2002	Children (7-10-y-olds) from Dannevirke (New Zealand) and surrounding small towns and rural area (n = 293)	Unpasteurized milk consumption ever vs never in the first 2 y of life (dichotomized: yes vs no)	Asthma: Positive response to ''Has your child ever had asthma?''
			Wheeze: Positive response to ''Has your child had wheezing or whistling in the chest in the last 12 months?"
			Atopy: Positive SPT result (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, mold mix, cockroach, rye grass, timothy grass, cat, dog)
			AR: Positive response to the question ''Has your child ever had hay fever?''
The Study of Asthma and Allergy in Shropshire, England, Perkin and Strachan,[32] 2006	Children (5-10-y-olds) from the rural county of Shropshire (n = 4767)	Current unpasteurized milk consumption (dichotomized: yes vs no)	Asthma: Not assessed
	Categorized into 3 strata: children whose families live and work on a farm; children whose families do not live on a farm but whose parents work there; and children without any farm contact (reference group)		Wheeze: Reported current asthma symptoms
			Atopy: Positive SPT result (dog hair, cat hair, horse hair, cow hair, 6-grass mix, house-dust mite [*D pteronyssinus*], *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*)
			AR: Reported current seasonal rhinitis (ISAAC questionnaire)
Poland, Sozanska et al,[33] 2013	Children (5-18-y-olds) from Sobotka and nearby villages in southwest Poland (n = 450). Stratification into 2 strata: children whose families live on a farm and children whose families do not live on a farm (reference group)	Unpasteurized milk consumption (categorized: never, sometimes, regular) in the first year of life	Asthma: Reported doctor's diagnosis of asthma ever
			Wheeze: Reported current wheeze
			Atopy: Positive SPT result (house-dust mite [*D pteronyssinus*], mixed grass pollens, mixed tree pollens, and cat fur)
			AR: Reported doctor's diagnosis of hay fever
Crete, Greece, Barnes at al,[34] 2001	Children (11-19-y-olds) from 5 villages in the south of Crete (n = 997)	Consumption of unpasteurized milk straight from the farm in the first 5 y of life (dichotomized: yes vs no)	Asthma: Not assessed
			Wheeze: Not assessed
			Atopy: Positive SPT result (mixed grass pollen [Mediterranean], house-dust mite, cat, Parietaria, olive blossom, Altenaria and goat allergens)
			AR: Not assessed

(continued)

TABLE I. *(Continued)*

Name/origin	Study population	Exposure: Raw milk consumption	Outcome definition according to ISAAC questionnaire
ALEX study, cross-sectional,[3] 1999	Children (6-13-y-olds) from rural areas of Austria, Germany, and Switzerland (n = 2618)	Raw cow's milk directly derived from a farm (consumed at least weekly vs raw cow's milk directly derived from a farm consumed less than weekly, or boiled cow's milk directly bought from a farm or milk bought in a supermarket or no milk consumption at all) in the first year of life and currently (within the last 12 mo before study questionnaire)	Asthma: Reported doctor's diagnosis of asthma or reported doctor's diagnosis of spastic or asthmatic bronchitis at least twice
			Wheeze: Positive response to "In the last 12 months did your child have wheezing or whistling in the chest while breathing?"
			Atopy: Specific IgE (house-dust mite, storage mite, Derp1, timothy grass, cat, cow, hen's egg, cow's milk)
			AR: Reported doctor's diagnosis of hay fever or running nose and itchy eyes in the last 12 mo
PARSIFAL study,[35] cross-sectional, 2006	Children (5-13-y-olds) from Austria, Germany, the Netherlands, Sweden, and Switzerland (n = 15, 137)	Raw cow's milk directly derived from a farm (consumed at least weekly vs raw cow's milk directly derived from a farm consumed less than weekly, or boiled cow's milk directly bought from a farm or milk bought in a supermarket or no milk consumption at all) in the first year of life and currently (within the last 12 mo before study questionnaire)	Asthma: Reported doctor's diagnosis of asthma or reported doctor's diagnosis of obstructive, spastic, or asthmatic bronchitis at least twice
	Categorized into 3 strata: children from farm families; children from anthroposophic families (recruited from Steiner schools); and reference children		Wheeze: Positive response to "In the last 12 months did your child have wheezing or whistling in the chest while breathing?"
			Atopy: Specific IgE (grass pollen-mix, tree pollen-mix, horse, cat, *D pteronyssinus*, *L destructor*)
			AR: Reported doctor's diagnosis of hay fever or running nose and itchy eyes without a cold in the last 12 mo
GABRIEL study,[16] cross-sectional, 3 phases, 2006-2008	Children (6-12-y-olds) from rural areas of southern Germany (Bavaria and Baden-Württemberg), Switzerland, Austria, and Poland (phase I n = 34 491, phase II n = 9668, phase III n = 895) Categorized into 3 strata: children living on a farm run by the family; children not living on a farm but regularly exposed to stables, barns, or cow's milk produced on a farm; and nonexposed nonfarm children	Raw cow's milk directly derived from a farm (consumed at least weekly vs raw cow's milk directly derived from a farm consumed less than weekly, or boiled cow's milk directly bought from a farm or milk bought in a supermarket or no milk consumption at all) in the first year of life and currently (within the last 12 mo before study questionnaire)	Asthma: Doctor's diagnosis of asthma or obstructive bronchitis at least twice
			Wheeze: Positive response to "In the last 12 months did your child have wheezing or whistling in the chest while breathing?"
			Atopy: Specific IgE (*Dermatophagoides pteronyssinus*, cat, rye, timothy grass, birch, mugwort, Phadia gx3)
			AR: Reported doctor's diagnosis of hay fever or running nose and itchy eyes without a cold in the last 12 mo

PASTURE study,[26] birth cohort, started in 2002, follow-up ongoing	Inclusion of pregnant women from rural areas in 5 European countries: Germany, Austria, Switzerland, Finland, and France (n = 1133) Data included from age 1-6 y of the offspring	Raw cow's milk directly derived from a farm consumed at least weekly vs raw cow's milk directly derived from a farm consumed less than weekly, or boiled cow's milk directly bought from a farm or milk bought in a supermarket or no milk consumption at all in the first year of life and currently (within the last 12 mo before study questionnaire)	Asthma: Doctor's diagnosis of asthma or obstructive or spastic bronchitis at least twice
			Wheeze: Positive response to "In the last 12 months did your child have wheezing or whistling in the chest while breathing?" without simultaneously occurring cold
			Atopy: Specific IgE (inhalant allergens)
			AR: Reported doctor's diagnosis of hay fever or allergic rhinitis or sneezing or runny, blocked, or itchy nose without having a cold along with itchy or runny eyes in the last 12 mo

AR, Allergic rhinoconjunctivitis; *ISAAC*, International Study of Asthma and Allergies in Childhood; *SPT*, skin prick test.
Atopy was defined as a positive SPT result (mean wheal diameter of ≥3 mm) or specific IgE level ≥0.35 kU/L.

CANDIDATE MOLECULES POTENTIALLY UNDERLYING THE PROTECTIVE EFFECTS

Cow's milk is a complex lipid-in-water emulsion comprising more than 2000 constituents with 86% to 88% water as its main component.[36,37] Besides the main categories, lipids, proteins, and carbohydrates, there are many other low abundant components, such as vitamins, minerals, and miRNAs.[36,37] Although some of these components are simply dissolved in the water phase, milk also contains many complex structures. For example, the milk contains lipids in the form of droplets (0.1-10 μm) coated with a trilayer of phospholipids and proteins.

Both the milk components themselves and their surrounding structures can be sensitive to heat and pressure. So, they may be altered in quantity or functionality by industrial milk processing and thus be responsible for the protective effect of unprocessed cow's milk. In dairy processing (Table II), milk first undergoes centrifugation to precipitate foreign matter and separate the fat fraction, which allows subsequent adjustment of a specific fat content. After this adjustment of the fat content, milk is generally homogenized to prevent creaming. In this step, the fat globule structure is destroyed and the trilayer of phospholipids and proteins is mostly replaced by milk protein. Subsequently, most shop milk is heated, with the lowest heat treatment generally being pasteurization for inactivation of pathogenic microorganisms. To prolong shelf life and allow uncooled storage, many milks are heated more intensively (eg, extended shelf life or ultra-heat treatment [UHT] milk). Throughout Europe, there are many differences in the heating intensity according to customers' preferences of flavor and storage conditions. Some people prefer the flavor of sterilized over pasteurized milk, whereas others appreciate longer storage duration also at room temperature. In the populations of the 4 Central European studies, there was a strong preference of UHT milk, with a proportion of 30% to 80% of all children drinking predominantly shop milk (see Table E1 in this article's Online Repository at www.jaci-inpractice.org).

Carbohydrates

Carbohydrates are the most abundant constituents in milk, being present at a level of 4.7% to 4.8%. In bovine milk, lactose is by far the most abundant carbohydrate, and present at a very constant level due to its role in the osmotic pressure of milk. Its digestion differs from that of other carbohydrates, and it may serve as a conditional prebiotic.[39] Oligosaccharides are present at much more variable levels. Their prebiotic activity is probably related to the stimulation of the growth of beneficial bacteria in the intestine, which may modulate immune responses and thus protect from asthma and allergies.[40] Likewise, anti-inflammatory mechanisms of oligosaccharides have been described at least for human milk.[41] However, the scientific foundation for effects by carbohydrates from cow's milk is very limited, and the World Allergy Organization recommendations for prebiotic supplementation to prevent asthma and allergic diseases are conditional.[42,43]

Proteins

Proteins are another group of major components accounting for 3% to 4% of the milk.[44] Caseins (80%) are dispersed as a colloidal suspension encased in rather thermostable micelles (100-200 nm). The bioactive whey proteins (20%) are generally present as single globular proteins dissolved in the water phase and undergo profound changes upon heat exposure.[45] Besides α-lactalbumin, β-

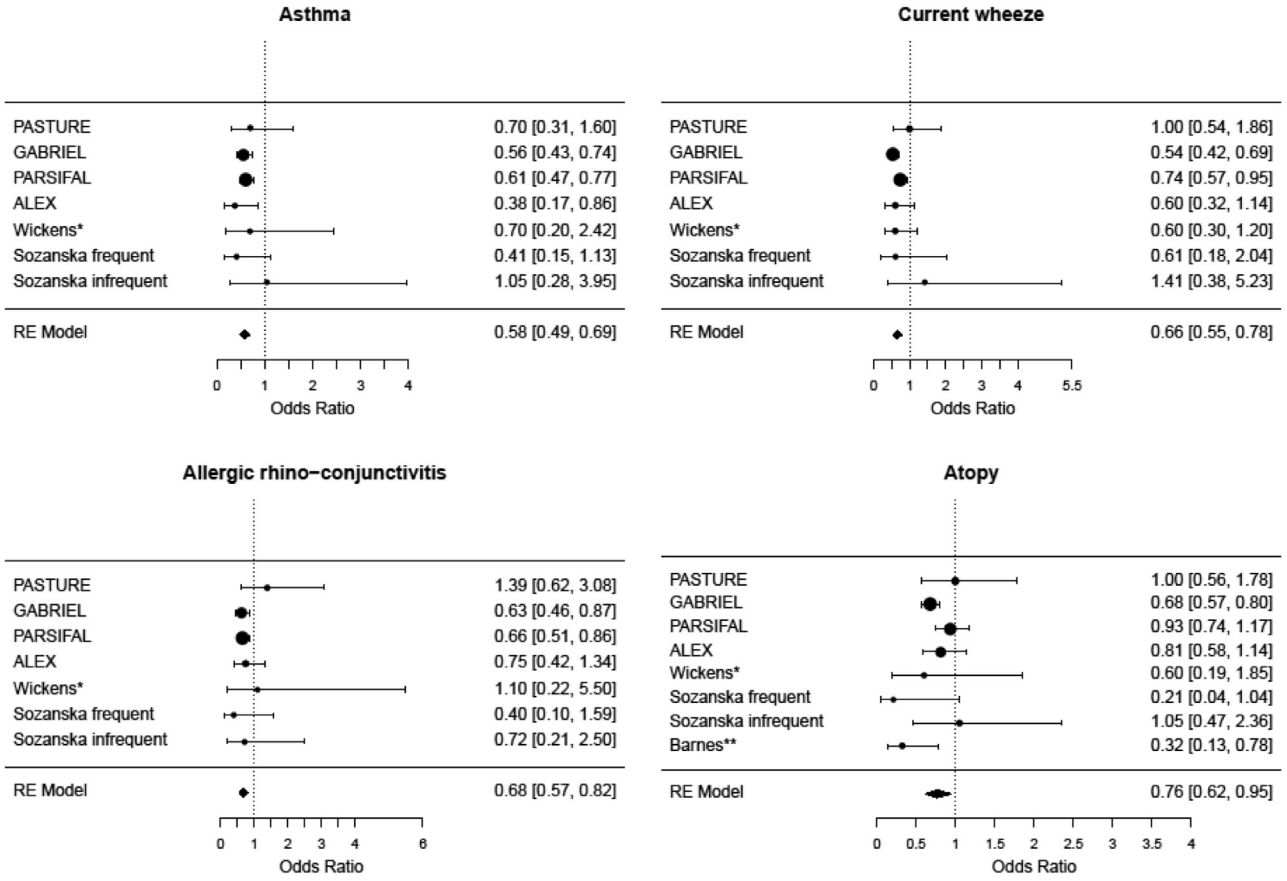

FIGURE 2. Association between raw milk consumption in the first years of life and outcome variables (OR [95% CI]). *OR*, Odds ratio; *RE*, random effects.

lactoglobulin, and the immunoglobulins,[46] low abundant whey proteins such as serum albumin, lactoferrin, lactoperoxidase, and different enzymes and cytokines are hypothesized to play a role in the protective effect.[25] One specific category of protein of interest is the enzymes (eg, alkaline phosphate and lipase), because most of them loose their bioactivity upon heat processing.[44] Although human milk enzymes have been well studied for their health benefits in infants,[47,48] hardly any research is done on the bioactivity of bovine milk enzymes. Generally, proteins are suggested to hold different properties altering development or expression of asthma or allergies, respectively, by modulating the gut microbial composition[49,50] or altering the maturation of the child's immune system toward allergen tolerance and thus reduce inflammatory reactions.[33,51,52]

Fatty acids

Of the main milk components, the lipid fraction is the most variable, because it can be affected by feeding, lactation status, animal breed, and season. In commercially available shop milk, the fat fraction is altered by several industrially applied processing steps. The fat content of unprocessed cow's milk naturally ranges from 3% to 6%,[37] whereas commercially available milk is generally standardized to a fat content of, for example, 3.5% or 1.5%. The fat globules are coated by a trilayer of membrane; these structures contain 400 different fatty acids and mono-, di-, and triglycerides, phospholipids, cholesterol, fat-soluble vitamins, and hundreds of different proteins. During homogenization, the structure of these fat globules is broken under high pressure to create smaller fat globules, aiming to prevent fat creaming in the final dairy product.[25] This alteration of both the fat content and the fat globule structure might contribute to the loss of the health-promoting effect of processed cow's milk as exemplified for milk fat globule membranes.[53] Because both homogenization and heating affect the composition of this membrane material, these beneficial effects may be lost upon industrial processing.[54,55]

With respect to quantitative fat content, the PARSIFAL study revealed a reduced asthma risk in children consuming full-cream milk or farm-produced butter.[35] In the PASTURE study, an asthma-protective effect of cow's milk holding a higher fat content emerged. This fat effect was attributable to higher n-3 polyunsaturated fatty acid levels and a lower n-6/n-3 (polyunsaturated fatty acid) ratio in raw cow's milk as compared with industrially processed milk.[26] Moreover, higher contents of short-chain fatty acids in milk and milk products tended to be associated with a lower prevalence of asthma, atopic sensitization, food allergy, and allergic rhinitis.[56] In addition, other milk constituents such as enzymes stored in the milk fat globules[57] might be diminished by reduction of the fat content of shop milk or their functionality might be altered under high-pressure treatment during homogenization.

Minerals, vitamins, and hormones

Milk meets all physiologic needs of a neonate and provides a large variety of micronutrients.[44] Because of their chemical properties, minerals are not influenced by the industrial

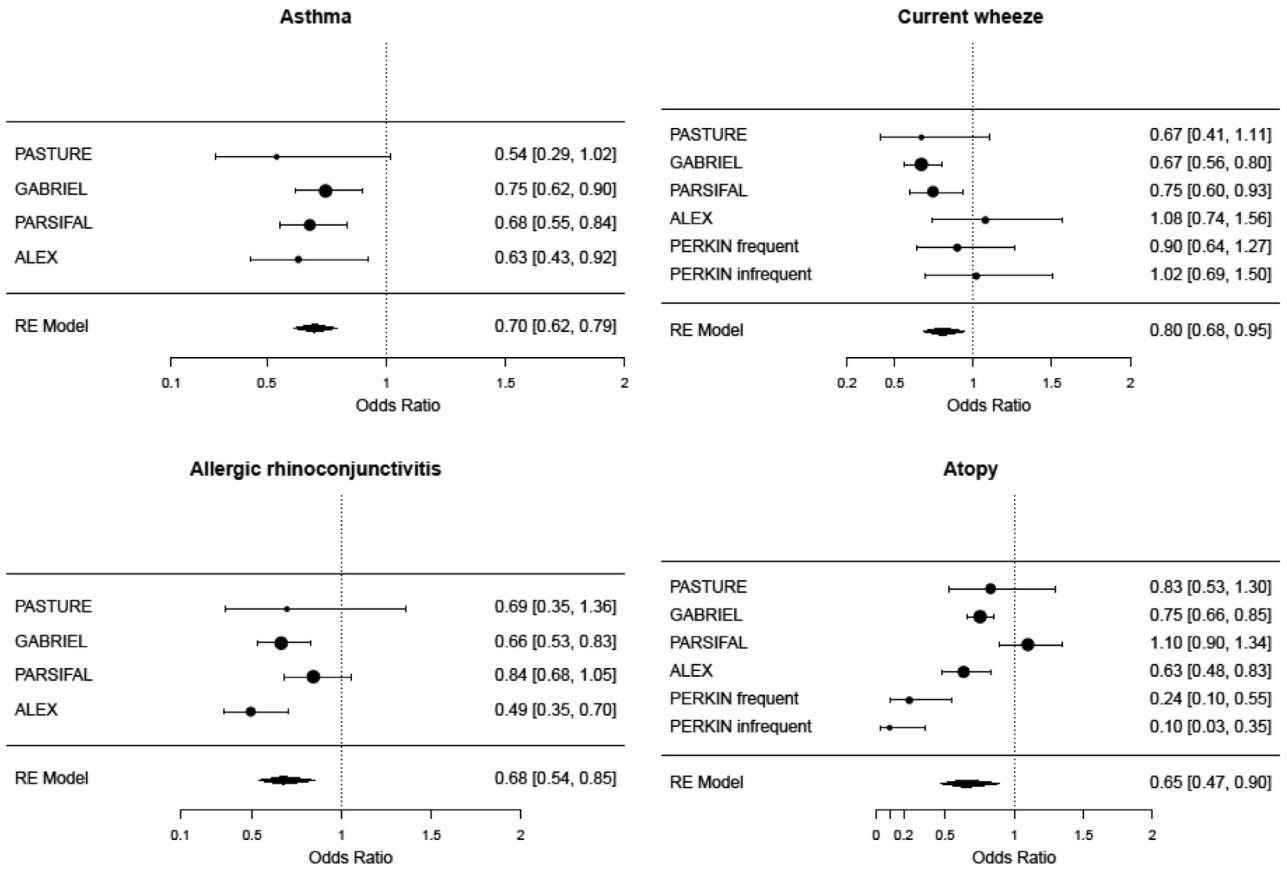

FIGURE 3. Association between current raw milk consumption and outcome variables (OR [95% CI]). *OR*, Odds ratio; *RE*, random effects.

processing. In contrast, many vitamins are heat-labile. Pasteurization generally does not cause a loss of vitamins,[58] whereas UHT sterilization and the resulting longer storage duration cause a limited loss of several vitamins.[59,60] However, the potential immunologic consequences of the loss of these vitamins upon UHT sterilization have not been studied.

Milk is a source of many different hormones, including among others growth factors and steroid and reproductive hormones.[61,62]

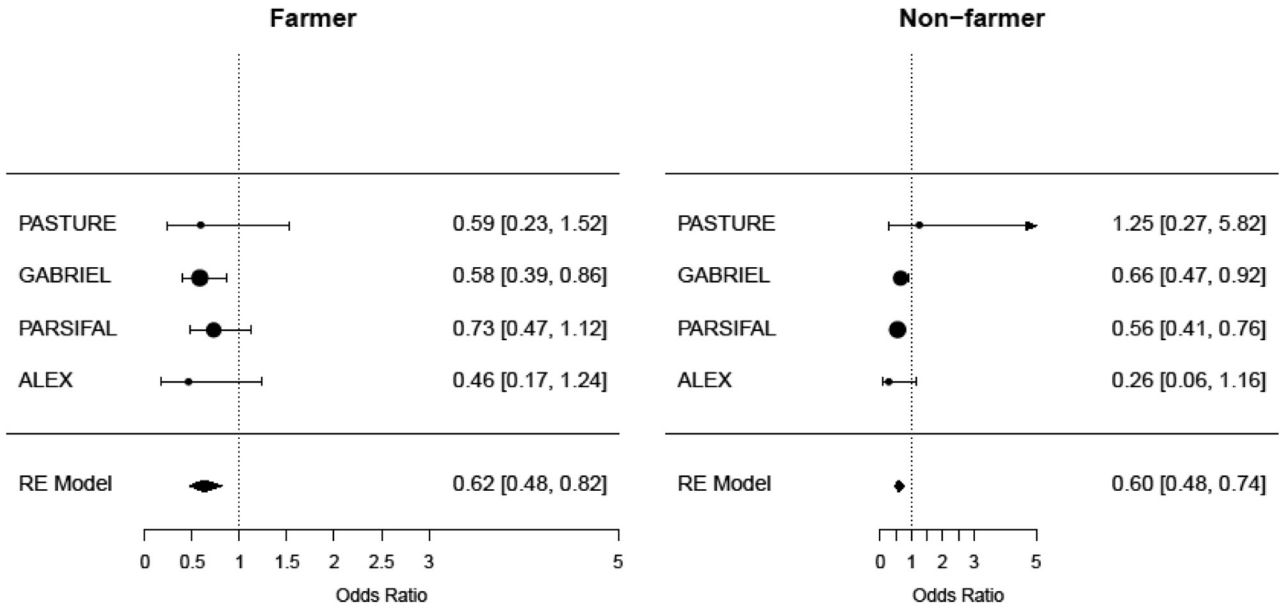

FIGURE 4. Raw cow's milk consumption in the first year of life and childhood asthma (OR [95% CI]). *OR*, Odds ratio; *RE*, random effects.

TABLE II. Industrially applied milk processing steps

Processing step	Comments
1. Centrifugation	Separation of dirt particles, somatic cells, and cream at 50°C
2. Adjustment of milk fat content	Commercially available milk is offered in 4 different categories:
	-natural full-cream milk (>3.5% fat)
	-full-cream milk (3.5% fat)
	-semiskimmed milk (1.5%-1.8% fat)
	-skimmed milk (≤0.3% fat)
3. Heat treatment (to kill potential pathogens and prolong shelf life)	
a. Pasteurization	Heating (72°C-75°C) for 20-30 s
b. High-heat treatment (extended shelf life)	Preheating at 95°C for 20 s, direct steam injection at 127°C for 5 s
c. UHT milk	Preheating at 93°C for 23 s, direct steam injection at 142°C for 5 s
d. Sterilization	Milk bottling, heating at 110°C -120°C for 10-30 min
4. Homogenization	2-stage homogenization* at 250/50 bar

Processing steps are listed in the order they are usually applied in dairy companies in Bavaria[28,38]; this order might slightly vary over countries.
*Milk is pressed through fine nozzles to reduce fat globule sizes and prevent creaming.

The heat sensitivity differs largely between individual hormones, from complete to no inactivation by industrial processing.[63-65] Health effects of milk-derived hormones are known,[66] but effects on the immune development have hardly been studied, and not all in the context of allergy and asthma.[62] However, knowing that levels of hormones derived from dairy product intake are relatively small compared with endogenous hormone production and the hormone levels in breast milk, a major role of bovine milk–derived hormone intake is not to be expected.[65]

microRNA

With recent advances in analytical methodology such as high-throughput sequencing, new milk components have been detected, among them miRNA, which are short noncoding RNA sequences. Cow's milk miRNAs strongly resemble human milk miRNAs and might thus be able to attach to human mRNA and thereby affect gene expression posttranscriptionally by regulating mRNA degradation and translation initiation at ribosomes. The possible effects on the human immune system are not yet clear; however, a reduced susceptibility to the development of asthma and allergies has been proposed.[28,67-69] miRNA species interfering with asthma genes were found to be affected by heat treatment as performed during industrial processing.[28] In milk samples of the GABRIEL study, we found substantial differences between total miRNA quantities in farm and shop milk, albeit not directly related to asthma status (Figure 5; see Table E2 in this article's Online Repository at www.jaci-inpractice.org). Furthermore, we detected specific miRNA levels at consistently lower levels in UHT milk as compared with raw milk (eg, miR_21_5p hazard ratio [HR] = 0.40, $P < .001$ in GABRIEL and HR = 0.15, $P < .001$ in PASTURE). Again, this discrepancy did not explain the protective effect of farm milk. For example, the significant association of miR_148a_3p with asthma status in GABRIEL (HR = 0.44; $P = .024$) was changed by adjustment for milk type to HR = 0.85 ($P = .667$) by about 80% and thereby largely explained. Consequently, miRNAs are less likely to carry a substantial share of the asthma-protective effect. In practical terms, however, they may serve as a proxy for the quality and the asthma-protective potential of a milk type.

Cellular structures

Besides the milk components, milk contains cells and cellular fragments of different origin. Exosomes, that is, extracellular vesicles, secreted among others by mammary gland epithelial cells, can transport different components, including proteins and miRNAs.[70,71] With these contents, milk-derived exosomes may affect the immune development of infants and their risk of allergy,[72,73] particularly because the exosomes and their cargo may be absorbed intestinally.[74] The effect of processing on exosomes as such has not been studied, but it has been shown that the miRNAs contained in exosomes are largely degraded upon heating similarly to miRNA itself.[75]

Milk also contains bovine somatic cells, predominantly leukocytes. The composition of the somatic cell fraction depends largely on the health status of the cow.[76] Animal studies suggest that somatic cells in unprocessed milk may be absorbed by the suckling neonate.[77] During industrial dairy processing, these somatic cells are generally removed by the initial centrifugation step.[78] Although their impact on human health has not been studied, xenogeneic pressure on the developing immune system is conceivable.[79]

An obvious difference between raw cow's milk and heat-treated shop milk is the microbial contamination. Heat treatment to at least pasteurization level (Table II) is essential to inactivate potential pathogens such as *Escherichia coli* or *Staphylococci*, thereby ensuring physiologically safe milk consumption.[80] Because pathogenic microorganisms are not selectively inactivated by heat or removed by centrifugation, total bacterial counts are substantially lower in industrially processed milk.[19,81] Involvement of microorganisms in the beneficial effect of raw milk seems obvious,[18,82] though the underlying mechanisms are not completely understood. Possibly bacterial endotoxin might induce tolerance toward allergens.[25,83-85] Moreover, microbiota may operate as probiotics and thus indirectly shape the microbial colonization of the gut early in infancy. Varying with study region, 30% to 50% of the children receive cow's milk, particularly farm milk within the first year of life.[20,86] During this period, the gut microbiome is primarily influenced by dietary factors, which may subsequently affect health conditions such as asthma and allergies.[22,87-90]

MILK CONSUMPTION HABITS

Besides processing differences, consumption habits might differ between children drinking raw cow's milk and those drinking shop milk, particularly with respect to storage time and

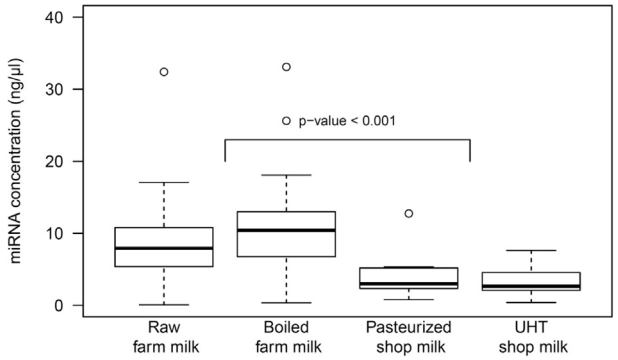

FIGURE 5. Concentration of miRNA in milk types.

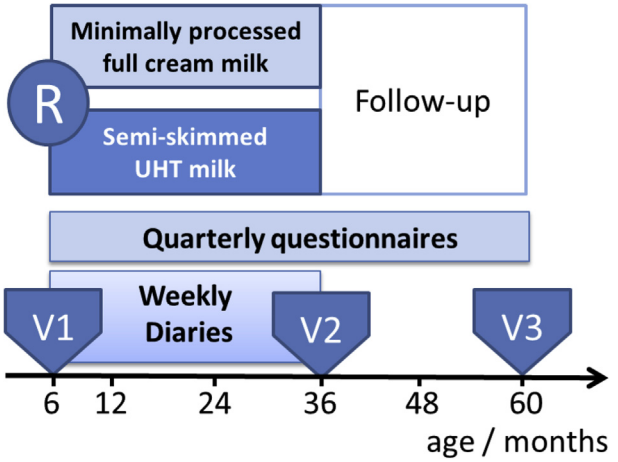

FIGURE 6. Design of the MARTHA trial. *R*, Randomization; *V*, clinical visit.

quantity of consumed milk. In GABRIEL and PASTURE, raw milk was mainly consumed within the day of milking (see Table E3 in this article's Online Repository at www.jaci-inpractice.org), whereas shop milk is often stored for 2 or more days between opening and consumption. As described above for vitamins, the levels of labile ingredients might decrease during prolonged storage, particularly in UHT milk. Because milk is a nutritious environment, heat-resistant microorganisms might proliferate and disintegrate health-promoting milk constituents.[91]

The frequency and overall amount of milk consumed by farm and shop milk drinkers might also vary. Among children drinking milk at least weekly, daily consumption was 20% more common for farm milk as compared with shop milk (see Table E4 in this article's Online Repository at www.jaci-inpractice.org). An unpublished analysis of a food frequency questionnaire from the ALEX study revealed that farm children consumed 207 g milk or milk products daily, whereas nonfarm children consumed on average 172 g/d ($P < .1$) corresponding to a means ratio of 1.20 (95% CI, 1.00-1.44; $P < .1$). In conclusion, quantitative differences in milk consumption may moderately contribute to the beneficial effect of raw milk on asthma and allergies.

INTERVENTION STUDY ASSESSING MINIMALLY PROCESSED MILK

As detailed above, we are far from understanding what actually drives the beneficial raw milk effect. Observational studies are limited in the range of the exposure; that is, there is no continuum of milk ingredients over the milk types. UHT milk, for example, has low levels of intact whey proteins, miRNA, vitamins, and other heat-susceptible components, which makes it nearly impossible to disentangle the health effects of the respective ingredient. Moreover, observational studies are hampered by the notorious difficulties with confounding and information bias.

A pragmatic and more promising approach would consist in an experimental setting directly comparing the effects of raw farm milk against shop milk. The low but existent risk of life-threatening infections, however, precludes any testing of raw milk in humans. The ideal milk for such a trial would be microbiologically safe but otherwise not exposed to any industrial processing.

The ongoing Milk Against Respiratory Tract Infections and Asthma (MARTHA) trial[92] now fills this gap. Supported by Dutch Longfonds, the University Children's Hospitals Munich and Regensburg have already started recruitment. The study has been set up to compare a minimally processed, safe full-cream milk against semiskimmed UHT milk. The latter was chosen as a comparator because it reflects "standard care": it is very common in infant nutrition and the basis for most infant formulas. Children receive a daily dose of 200 mL and, from the 11th month, 2 × 150 mL, conforming with national recommendations on nutrition of babies and infants.[93] Additional nutrition is ad libitum, which compensates for slight differences in energy value between the milk preparations. Irrespective of the family history of atopy, children are recruited and randomized to 1 of the 2 arms between age 6 and 12 months (Figure 6). The intervention starts as soon as children are no longer fully breast-fed and lasts until the third birthday. Thus, the intervention covers a period when cow's milk consumption is very common and has been shown to affect various health outcomes.[26] Physicians examine the children at inclusion, after intervention at age 3 years, and after follow-up at age 5 years. Parents complete weekly diaries for assessment of respiratory health and symptoms suggestive of adverse outcomes such as cow's milk allergy, and lactose or milk protein intolerance. The primary outcome "asthma as diagnosed by a physician" will be assessed at 5 years, which explains the long duration of the trial. Secondary outcomes are respiratory infections and wheeze during the intervention, low-grade inflammation, atopic sensitization, and eczema at 3 and 5 years. The MARTHA trial is registered with the German trial registry as DRKS00014781,[94] where more details on the trial can be found.

OUTLOOK

Regular consumption of minimally processed milk instead of industrially processed milk with long shelf life would be an attractive prevention because it is simple, acceptable, and easy to implement without major changes in lifestyle. From a scientific point of view, however, further research into the underlying mechanisms of the farm milk effect would be highly desirable.

Subsequent trials might assess the protective potential of the various components of cow's milk. Understanding the prevention might also foster understanding of the disease. Nevertheless, the example of John Snow's successful fight against the cholera epidemic in 19th-century London[95] teaches us that interventions can be effective even in the absence of a valid theory about pathomechanisms.

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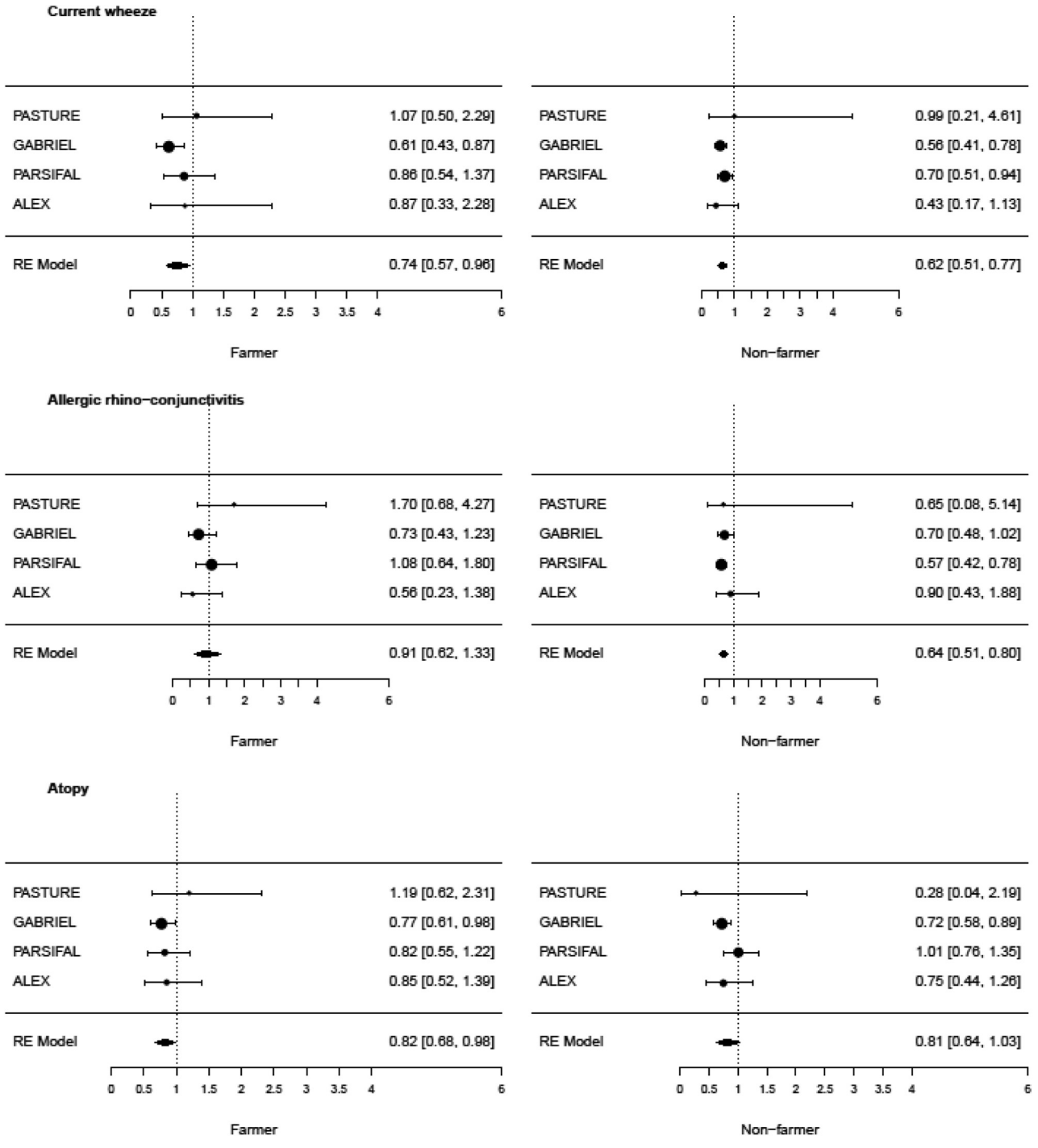

FIGURE E1. Raw cow's milk consumption in the first year of life and outcome variables. *RE*, Random effects.

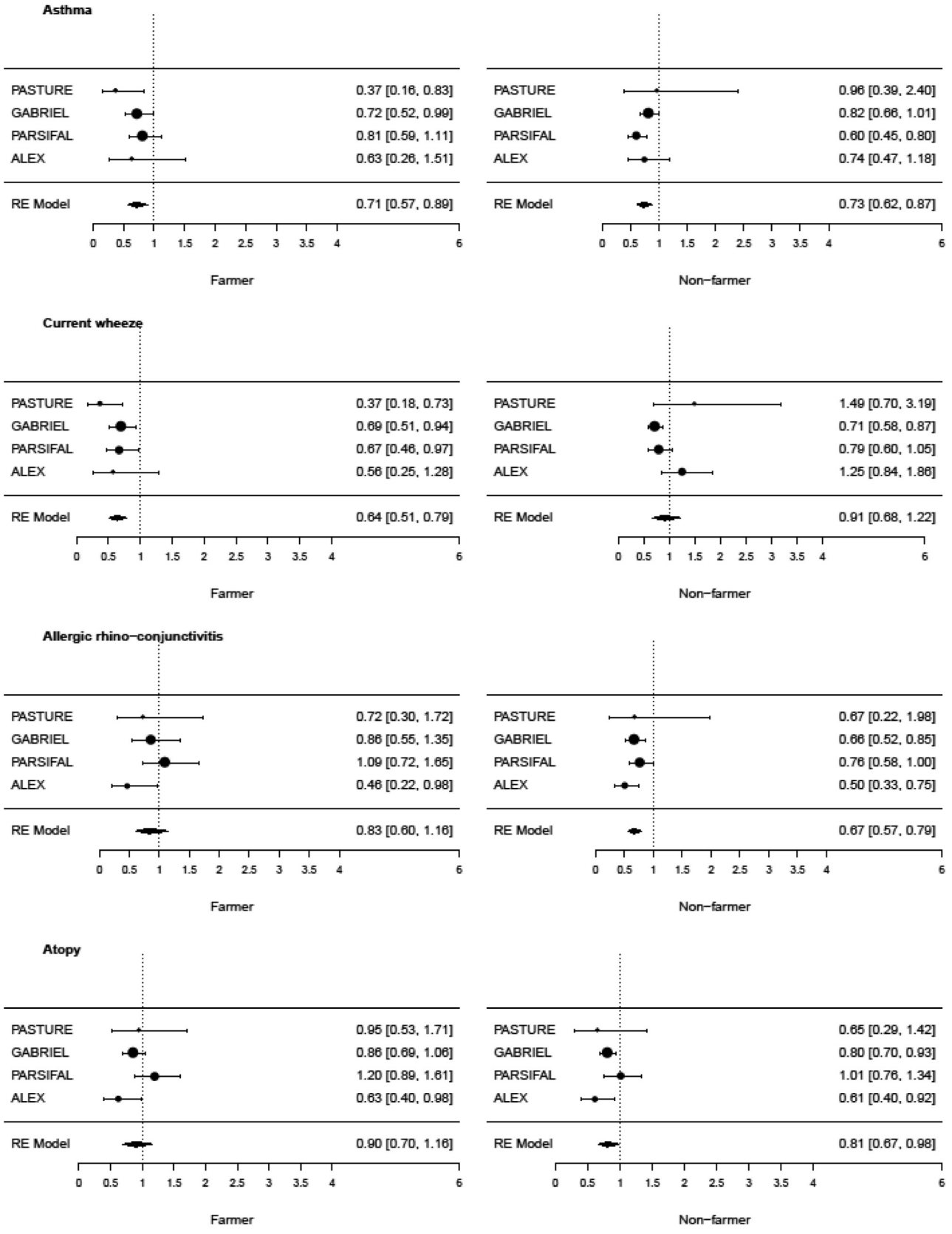

FIGURE E2. Current raw cow's milk consumption and outcome variables. *RE*, Random effects.

TABLE E1. Proportion of children drinking UHT milk among those drinking shop milk

Study	% of children drinking UHT milk*
GABRIEL	77.4% (429 of 554 children)
PASTURE	53.7% (267 of 497 children)
ALEX	44.8% (490 of 1093 children)
PARSIFAL	28.5% (2514 of 8816 children)

*Among those with information on specific milk type.

TABLE E2. Associations between total miRNA levels and milk type (linear regression)

Milk type	β estimate	SE	*P* value
Intercept	28.38		
Pasteurized vs raw cow's milk	1.87	0.58	.001
Boiled vs raw cow's milk	2.30	0.40	<.001
UHT vs raw cow's milk	5.29	0.39	<.001

TABLE E3. Proportion of milks consumed within 1 d after milking or after opening the milk bottle, respectively

Study	Age of assessment (y)	Raw farm milk	Shop milk
GABRIEL	6-12	55%	12%
PASTURE	6	94%	37%

TABLE E4. Daily consumption within children consuming milk at least weekly

Study	Age of assessment (y)	Farm milk	Shop milk	Ratio
GABRIEL	6-12	68%	56%	1.20
PASTURE	6	72%	60%	1.20

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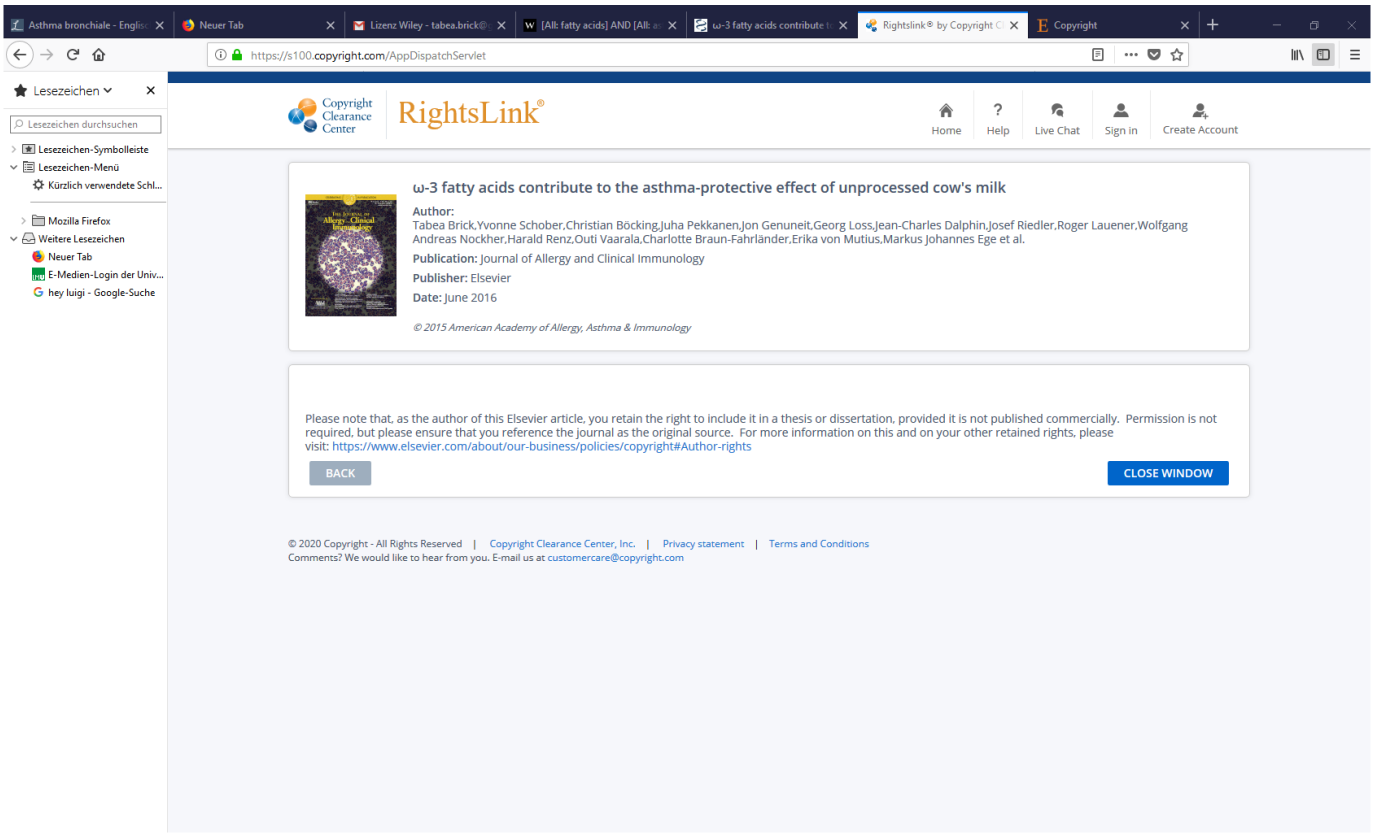
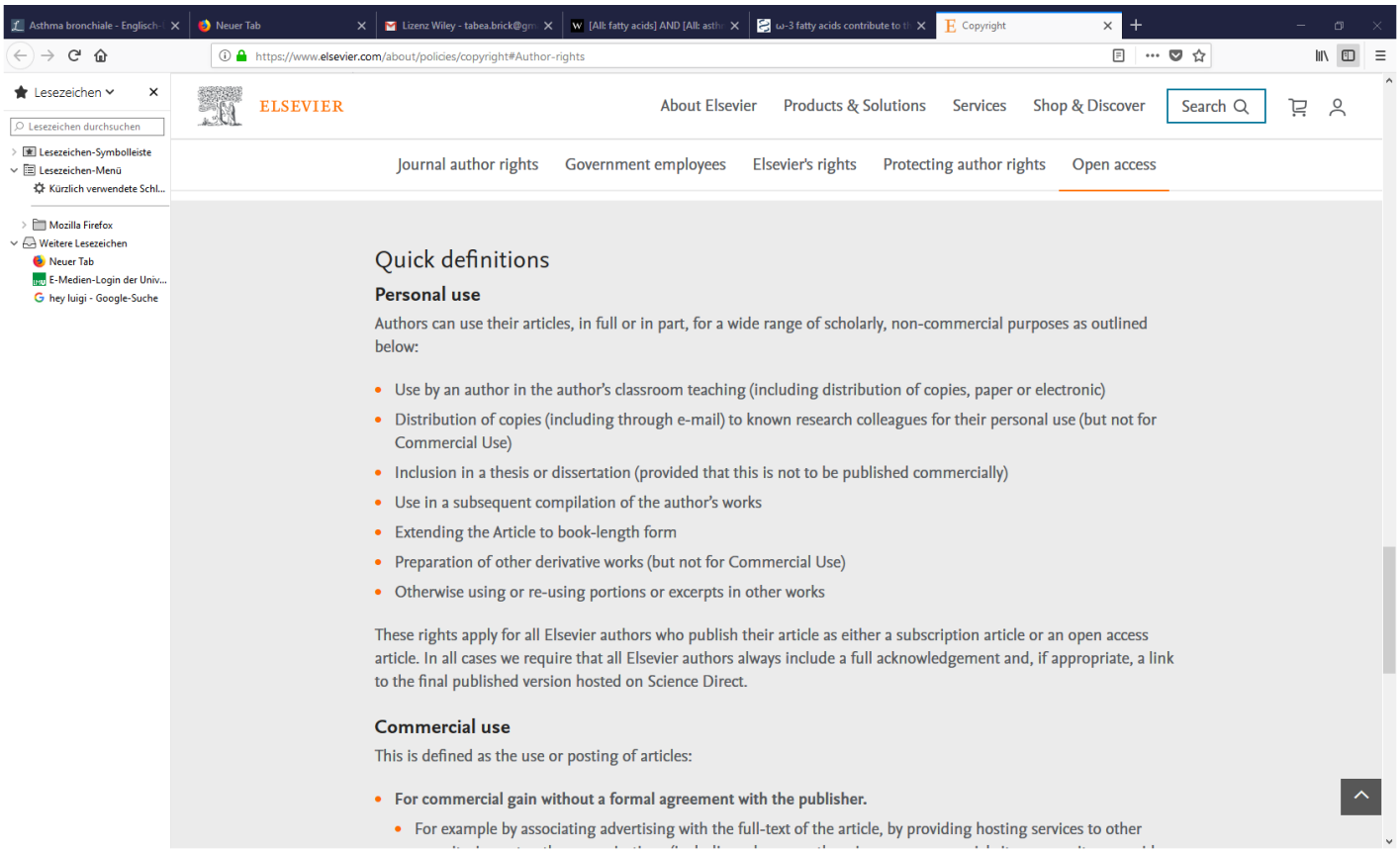
16. Lizenzen

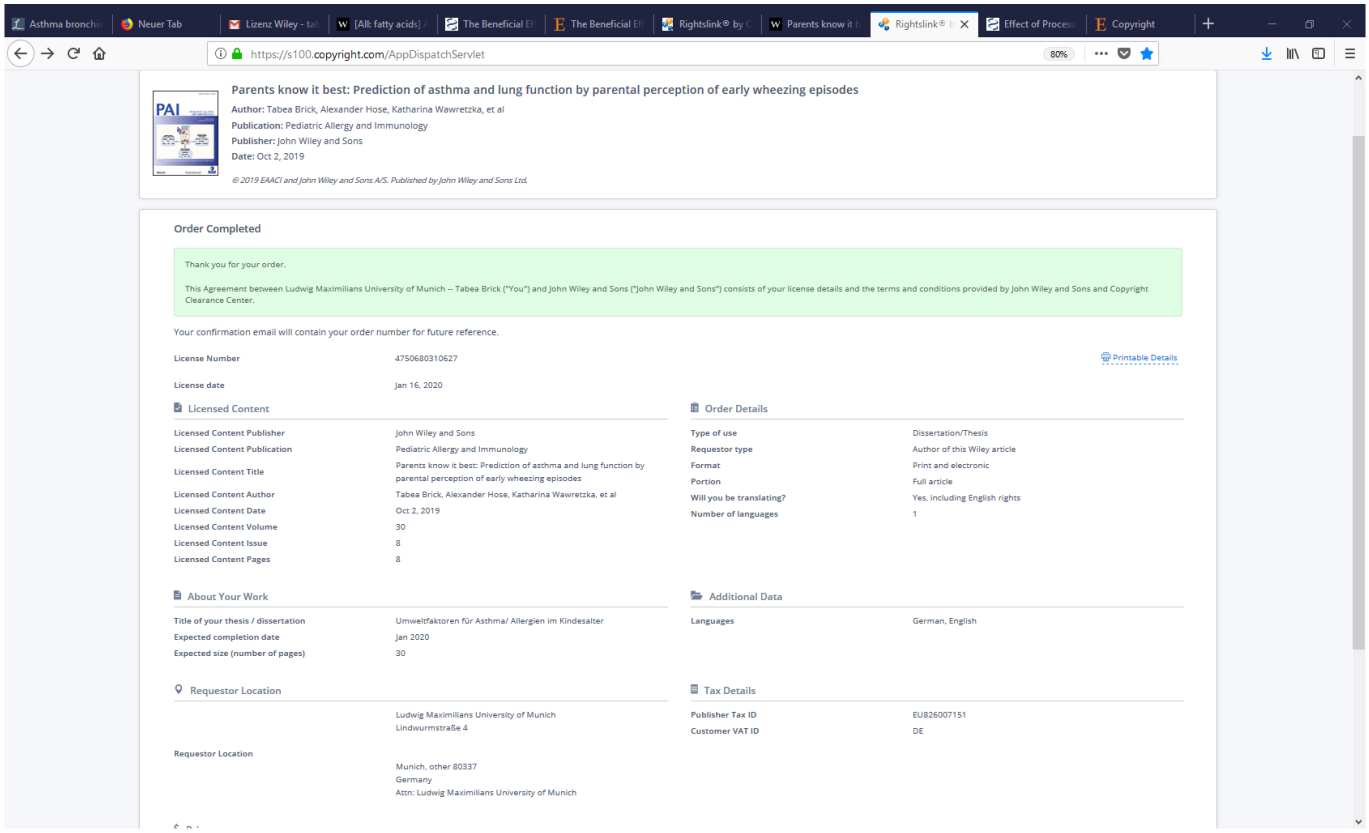
1. **Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk.** Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg Loss, Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group.



2. **Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins**
   Brick T, Ege M, Boeren S, Böck A, von Mutius E, Vervoort J, Hettinga K. (Open access Artikel)



3. **Parents know it best: Prediction of asthma and lung function by parental perception of early wheezing episodes**. Brick T, Hose A, Wawretzka K, von Mutius E, Roduit C, Lauener R, Riedler J, Karvonen AM, Pekkanen J, Divaret-Chauveau A, Dalphin JC, Ege MJ; PASTUREstudy group.



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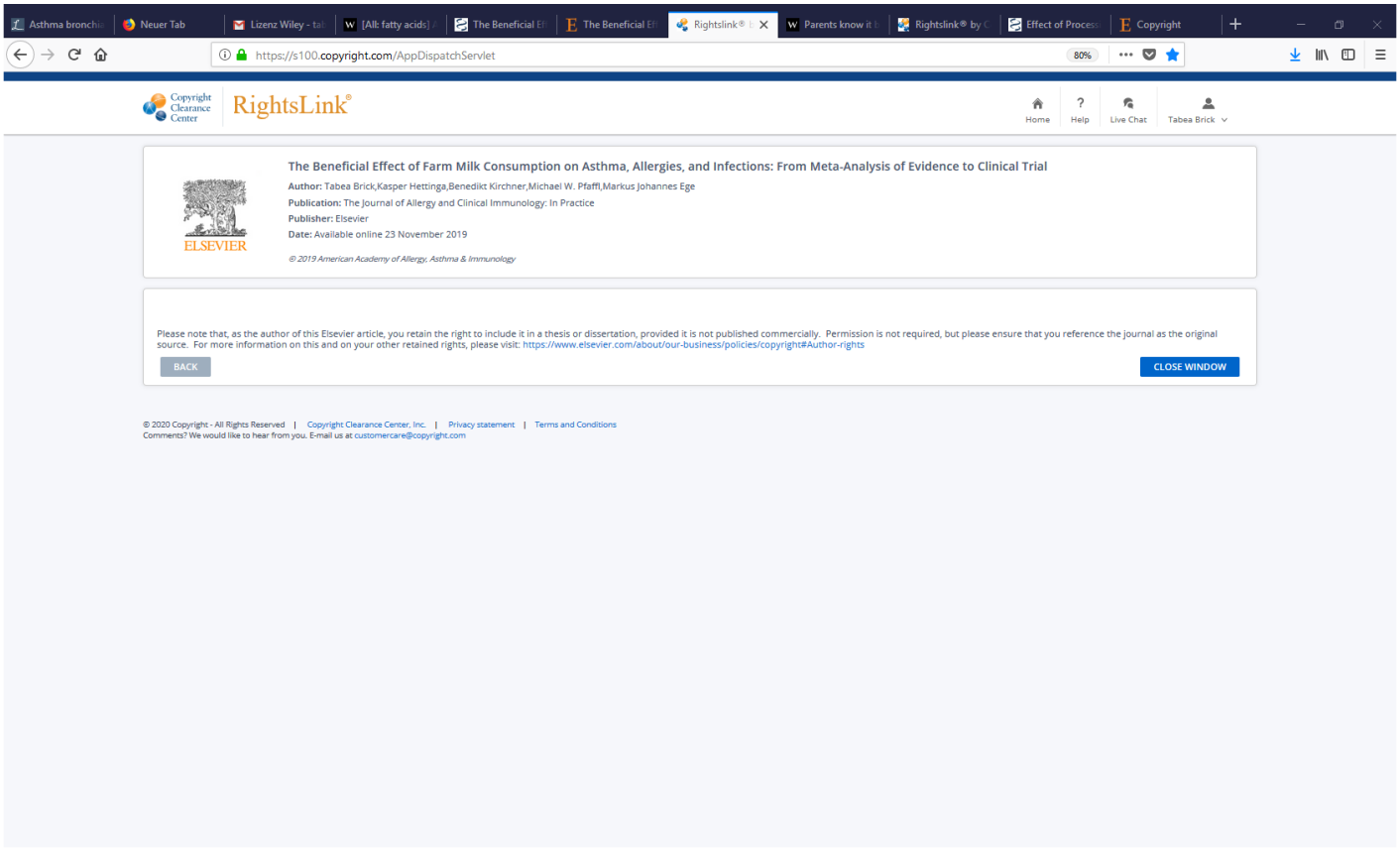

17. Lebenslauf

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Suaheli Grundkenntnisse

EDV-Kenntnisse

Gute MS Office Kenntnisse (Word, Excel, PowerPoint)

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Publikationen

Tabea Brick, Yvonne Schober, Christian Böcking, Juha Pekkanen, Jon Genuneit, Georg, Loss,Jean-Charles Dalphin, Josef Riedler, Roger Lauener, Wolfgang Andreas Nockher, Harald Renz, Outi Vaarala, Charlotte Braun-Fahrländer, Erika von Mutius, Markus Johannes Ege, Petra Ina Pfefferle, and the PASTURE study group. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol.* **2016** Jun;137(6):1699-1706.e13. doi: 10.1016/j.jaci.2015.10.042.

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