Katedra Biochemii i Immunochemii

Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu

Ocena produktów zaawansowanej glikacji i mikrobiomu u pacjentów nadużywających alkoholu

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ROZPRAWA DOKTORSKA

Cykl publikacji powiązanych tematycznie

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1. Wykaz publikacji stanowiących pracę doktorską

1. **<u>Kamil Litwinowicz</u>**, Ewa Waszczuk, Andrzej Gamian

   Advanced glycation end-products in common non-infectious liver diseases: systematic review and meta-analysis.

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2. **<u>Kamil Litwinowicz</u>**, Ewa Waszczuk, Aleksandra Kuzan, Agnieszka Bronowicka-Szydełko, Kinga Gostomska-Pampuch, Piotr Naporowski, Andrzej Gamian

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3. **<u>Kamil Litwinowicz</u>**, Andrzej Gamian

   Microbiome Alterations in Alcohol Use Disorder and Alcoholic Liver Disease

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2. Wykaz zastosowanych skrótów

Skrót	Określenie	Polski odpowiednik
AUD	*Alcohol use disorder*	Zaburzenie używania alkoholu
ALD	*Alcoholic liver disease*	Alkoholowa choroba wątroby
AGE	*Advanced glycation end-products*;	Produkty zaawansowanej glikacji
RAGE	*AGE receptor*	Receptor AGE
MAGE	*Melibiose-derived AGE*	Melibiozopochodne AGE
NGS	*Next generation sequencing*;	Sekwencjonowanie nowej generacji
FDR	*False discovery rate*;	Odsetek fałszywych odkryć
CML	*Nε-(Carboxymethyl)lysine*	Nε-karboksymetylolizyna
ALT	*Alanine aminotransferase*	Aminotransferaza alaninowa
AST	*Aspartate aminotransferase*	Aminotransferaza asparaginianowa
GGTP	*Gamma-glutamyl transpeptidase*	Gamma-glutamylotranspeptydaza

3. Streszczenie

Wstęp: Zaburzenie używania alkoholu (AUD) pozostaje istotną przyczyną śmiertelności na świecie. Jest ono bezpośrednią przyczyną ponad 40 jednostek chorobowych, spośród których jedną z najważniejszych jest alkoholowa choroba wątroby (ALD). Uszkodzenie wątroby w ALD przebiega etapami – od stłuszczenia, przez zapalenie aż po marskość. W patofizjologii progresji ALD odgrywają rolę liczne mechanizmy molekularne, w tym produkty zaawansowanej glikacji (AGE) oraz zaburzenia mikrobiomu. Niedawno odkryty został nowy podtyp AGE (nazwany AGE10) reagujący z przeciwciałami przeciwko syntetycznemu, melibiozopochodnemu AGE.

Cel pracy: Określenie powiązania klasycznych AGE z alkoholową chorobą wątroby. Ocena powiązania AGE10 z alkoholowym zapaleniem wątroby. Określenie dokładności diagnostycznej AGE10 w alkoholowym zapaleniu wątroby. Zbadanie zaburzeń mikrobiomu w AUD i ALD.

Materiały i metody: Na rozprawę składa się cykl 3 publikacji powiązanych tematycznie. Pierwsza publikacja to przegląd systematyczny i metaanaliza opracowane zgodnie z wytycznymi PRISMA. Włączono do niej 11 artykułów. Druga publikacja opiera się na porównaniu stężenia nowoodkrytego podtypu AGE (AGE10) u 65 pacjentów z alkoholowym zapaleniem wątroby i grupy kontrolnej składającej się z 65 osób bez istotnych schorzeń. Poziom AGE10 określono z wykorzystaniem kompetycyjnego testu ELISA. Trzecia praca opiera się na analizie sekwencji 16S rRNA. Uzyskano dane dla 122 pacjentów z AUD, 75 z alkoholową chorobą wątroby, 54 z chorobami wątroby o innej etiologii oraz grupę kontrolną składającą się z 260 osób bez istotnych schorzeń. Kontrolę jakości i wstępną obróbkę bioinformatyczną sekwencji przeprowadzono z wykorzystaniem USEARCH. Do uzyskania wariantów sekwencji amplikonu użyto algorytmu UNOISE3. Taksonomię przypisano z użyciem IDTAXA i bazy GTDB 07-RS207. Potencjał funkcjonalny mikrobiomu uzyskano przez PICRUSt2 z Metacyc jako bazą referencyjną. Różnicę w proporcjach występowania poszczególnych taksonów i w potencjalne funkcjonalnym określono z wykorzystaniem ANCOM-BC.

Wyniki: ALD jest powiązane z wyższym stężeniem klasycznych podtypów AGE (frakcja fluorescencyjna i CML). Żadne z badań zakwalifikowanych do przeglądu systematycznego i metaanalizy nie raportowało ich dokładności diagnostycznej w ALD. Pacjenci z alkoholowym zapaleniem wątroby mieli istotnie wyższe stężenia AGE10 w porównaniu z grupą kontrolną

(odpowiednio 184.5 ± 71.1 µg/mL i 123.5 ± 44.9 µg/mL, $p < 0.001$). Dokładność diagnostyczna AGE10 wyrażona jako AUC wynosiła 0.778. Dla wartości odcięcia 147.25 µg/mL czułość wynosiła 75% a swoistość 72%. Wykazano bardzo liczne zmiany mikrobiomu związane z AUD i ALD. Należą do nich między innymi proporcjonalny spadek typu *Firmicutes* oraz wzrost w typie *Proteobacteria*. Do spadku w *Firmicutes* w największym stopniu przyczyniły się rodziny *Ruminococcaceae, Lachnospiraceae, Oscillospiraceae* i *Butyricicoccaceae*. Za wzrost *Proteobacteria* odpowiadały głównie rodziny *Enterobacteriaceae i Burkholderiaceae*. Zmiany te były bardziej zaznaczone u pacjentów z ALD w porównaniu z grupą AUD bez uszkodzenia wątroby. W profilu funkcjonalnym zaobserwowano proporcjonalne zwiększenie szlaku związanego z syntezą lipopolisacharydu.

Wnioski: Alkoholowa choroba wątroby jest powiązana z istotnie wyższymi stężeniami klasycznych AGE oraz nowoodkrytego AGE10. Dokładność diagnostyczna AGE10 w alkoholowym zapaleniu wątroby jest obiecująca. Zarówno ALD jak i AUD wykazują liczne, negatywne zmiany w mikrobiomie; nasilenie tych zmian jest większe w ALD w porównaniu z AUD bez uszkodzenia wątroby.

4. Summary

Introduction: Alcohol use disorder (AUD) remain a significant cause of mortality worldwide. Alcohol abuse is a direct cause of over 40 diseases, including alcoholic liver disease (ALD). The progression of liver injury in ALD occurs in stages, from steatosis to inflammation and cirrhosis. Numerous molecular mechanisms play a role in the pathophysiology of ALD progression, including advanced glycation end products (AGE) and microbiome disturbances. Recently, a new subtype of AGE (named AGE10) that reacts with antibodies against the synthetic melibiose-derived AGE has been discovered.

Aims: To determine the association of classic AGE with alcoholic liver disease. To evaluate the association of AGE10 with alcoholic hepatitis. To determine the diagnostic accuracy of AGE10 in alcoholic hepatitis. To investigate microbiome disturbances in AUD and ALD.

Materials and methods: The thesis consist of a cycle of 3 thematically related publications. The first publication is a systematic review and meta-analysis conducted in accordance with the PRISMA guidelines. 11 articles were included. The second publication is based on a comparison of the plasma levels of the newly discovered AGE subtype (AGE10) in 65 patients with alcoholic hepatitis and a control group of 65 individuals without significant illnesses. The level of AGE10 was determined using a competitive ELISA test. The third study is based on 16S rRNA sequencing analysis. Data was obtained for 122 patients with AUD, 75 with alcoholic liver disease, 54 with liver diseases of other etiologies and a control group of 260 individuals without significant illnesses. Quality control and bioinformatics preprocessing of the sequences was performed using USEARCH. The UNOISE3 algorithm was used to obtain amplicon sequence variants. Taxonomy was assigned using IDTAXA and the GTDB 07-RS207 database. Microbiome functional potential was obtained using PICRUSt2 with Metacyc as a reference database. The differential abundance was determined using ANCOM-BC.

Results: ALD is associated with higher levels of classic AGE subtypes (fluorescent fraction and CML). None of the studies included in the systematic review and meta-analysis reported their diagnostic accuracy in ALD. Patients with alcoholic liver disease had significantly higher AGE10 levels compared to the control group (184.5 ± 71.1 μg/mL and 123.5 ± 44.9 μg/mL, respectively, $p < 0.001$). The diagnostic accuracy of AGE10 expressed as AUC was 0.778. For the cut-off value of 147.25 μg/mL, the sensitivity was 75% and specificity 72%. Numerous microbiome changes related to AUD and ALD were observed, including a proportional decrease in *Firmicutes* phylum and an increase in *Proteobacteria* phylum. The families *Ruminococcaceae, Lachnospiraceae, Oscillospiraceae*, and *Butyricicoccaceae* contributed the most to the decrease in *Firmicutes*, while *Enterobacteriaceae* and *Burkholderiaceae* were mainly responsible for the increase in *Proteobacteria*. These changes were more pronounced in patients with ALD compared to the AUD without liver damage group. A proportional increase in the lipopolysaccharide synthesis pathway was observed in the functional profile.

Conclusions: Alcoholic liver disease is associated with significantly higher levels of classic AGE and newly discovered AGE10. The diagnostic accuracy of AGE10 in alcoholic liver disease is promising. Both ALD and AUD show numerous negative microbiome changes, with more pronounced changes in ALD compared to AUD without liver damage.

5. Wstęp

Zaburzenie używania alkoholu (AUD, tłumaczenie zgodne z polskim wydaniem kryteriów diagnostycznych DSM-5 [1]) jest bezpośrednią przyczyną ponad 40 jednostek chorobowych [2]. Spośród nich, alkoholowa choroba wątroby (ALD) stanowi jeden z najważniejszych czynników przyczyniających się do globalnego obciążenia chorobami [3]. Uszkodzenie wątroby w ALD przebiega w kilku etapach. Niemal każdy chory z AUD rozwinie alkoholowe stłuszczenie wątroby, jednak jedynie 10 do 35% z nich rozwinie bardziej zaawansowane stadium – alkoholowe zapalenie wątroby – oraz 8 do 20% alkoholową marskość wątroby [4]. Identyfikacja mechanizmów molekularnych odpowiedzialnych za progresję wczesnego ALD do bardziej zaawansowanych postaci uszkodzenia wątroby jest niezwykle istotna. Wyodrębnienie takich czynników ryzyka umożliwiłaby wyselekcjonowanie grupy pacjentów szczególnie narażonych na rozwój zapalenia i marskości wątroby, u których wdrożenie działań profilaktycznych byłoby szczególnie korzystne. Dotychczas zidentyfikowano wiele mechanizmów powiązanych z uszkodzeniem wątroby w AUD, do których należą między innymi produkty zaawansowanej glikacji (AGE) [5] i zaburzenia mikrobiomu [6].

Podwyższony poziom AGE jest czynnikiem ryzyka licznych stanów chorobowych, w tym rozwoju powikłań cukrzycowych, choroby Alzheimera oraz uszkodzenia wątroby [5], [7], [8]. Najważniejszy szlak formowania AGE (zwany reakcjami Maillarda) rozpoczyna się od reakcji między redukującym cukrem i wolną grupą aminową białka. Wynikiem tej reakcji jest powstanie niestabilnej zasady Schiffa, która ulega dalszej reorganizacji do stabilnego produktu Amadori. W wyniku tych reakcji powstają tak zwane wczesne produkty glikacji. Ich najważniejszym przedstawicielem jest hemoglobina glikowana (HbA1c), wykorzystywana w praktyce klinicznej jako wskaźnik wielomiesięcznej średniej glikemii [9]. Produkt Amadori podlega dalszym reakcjom utlenienia i glikacji prowadząc do powstania AGE [10]. AGE stanowią heterogenną grupę związków o zróżnicowanych właściwościach. Poszczególne typy AGE różnią się między innymi siłą wiązania ze swoistym receptorem (RAGE) [11]. Interakcje AGE z tym receptorem wywołują aktywację szeregu szlaków molekularnych powodujących między innymi uwalnianie cytokin prozapalnych oraz wytwarzanie reaktywnych form tlenu [12]. W związku z tym ocena stężenia poszczególnych typów AGE (w odróżnieniu od klasycznie stosowanego zbiorczego oznaczania frakcji fluorescencyjnej) ma duże znaczenie patofizjologiczne.

Niedawno uzyskany został nowy, syntetyczny AGE (MAGE), powstający w wyniku reakcji między białkami i melibiozą. W tkankach człowieka wykryty został epitop AGE reagujący z przeciwciałami przeciwko syntetycznemu MAGE [13]. Przeciwciała anty-MAGE nie reagują z żadną z klasycznych frakcji AGE, w związku z czym wykryty epitop stanowi nowy, niezbadany do tej pory typ AGE (nazwany – zgodnie z aktualną nomenklaturą opisującą typy AGE od 1 do 9 – AGE10).

Postęp technologiczny umożliwił znaczne obniżenie kosztów sekwencjonowania DNA. Dzięki opracowaniu technik sekwencjonowania nowej generacji (NGS) cena sekwencjonowania miliona par zasad spadła z 10 milionów dolarów w 2001 roku do 1 centa w 2021 [14]. Tak znacząca redukcja kosztów umożliwiła zastosowanie NGS na szeroką skalę w praktyce klinicznej oraz w badaniach naukowych, w tym w analizie mikrobiomu. Sekwencjonowanie metagenomu identyfikuje znacznie większą ilość bakterii obecnych w danej niszy ekologicznej w porównaniu z klasycznymi metodami opartymi na namnażaniu w kulturach hodowlanych [15]. Ze względu na swój niski koszt, wymaganie jedynie śladowych ilości materiału genetycznego oraz mniejsze wymagania dotyczące mocy obliczeniowej, sekwencjonowanie 16S rRNA stanowi obecnie najpopularniejszą metodę badania mikrobiomu. Polega ono na sekwencjonowaniu fragmentu 16S rRNA małej podjednostki rybosomów prokariontów. Gen ten zawiera wysoko konserwowane sekwencje (tj. różniące się w niewielkim stopniu u większości bakterii) poprzedzielane wysoce zmiennymi sekwencjami (tj. różniącymi się u nawet blisko spokrewnionych bakterii). Zastosowanie primerów komplementarnych do konserwowanych sekwencji umożliwia tanie sekwencjonowanie wysoce zmiennych sekwencji, na podstawie których możliwe jest odróżnienie od siebie nawet blisko spokrewnionych bakterii. Postępowi w zakresie technik sekwencjonowania towarzyszy dynamiczny rozwój bioinformatycznych algorytmów i narzędzi analitycznych. Powstały algorytmy umożliwiające ocenę profilu funkcjonalnego mikrobiomu na podstawie wyników sekwencjonowanie 16S rRNA. Z punktu widzenia tematu przewodu doktorskiego szczególnie interesujące jest określenie aktywności mikrobowej α-galaktozydazy. Jest to enzym odpowiedzialny między innymi za hydrolizę melibiozy. Zmiany w jego aktywności mogłyby prowadzić do zwiększenia lub zmniejszenia puli melibiozy w ustroju, potencjalnie zwiększając dostępność substratu do generowania AGE10/MAGE. Przy analizie 16S rRNA wybór odpowiednich narzędzi bioinformatycznych ma kluczowe znaczenie dla uzyskania wiarygodnych wyników. Badania analizujące wpływ

zastosowanych algorytmów na otrzymane wyniki wykazały, że niektóre powszechnie stosowane metody mogą mieć odsetek fałszywych odkryć (FDR) wynoszący nawet 70% [16]. Oznacza to, że znaczny odsetek wyników uzyskanych w badaniach analizujących mikrobiom może być fałszywie dodatni. Wiele zespołów badawczych udostępnia surowe sekwencje 16S rRNA w ogólnodostępnych bazach danych jak Sequence Read Archive (SRA) czy European Nucleotide Archive (ENA). Umożliwia to powtórną ich analizę z wykorzystaniem nowszych metod charakteryzujących się niższym wskaźnikiem FDR, dzięki czemu mogą zostać uzyskane bardziej wiarygodne wyniki.

6. Cele

1. Ocena powiązania alkoholowego zapalenia wątroby ze stężeniem AGE10 w osoczu.
2. Określenie zależności pomiędzy wiekiem i płcią a stężeniem AGE10 w osoczu
3. Określenie korelacji pomiędzy wykładnikami uszkodzenia wątroby (AST, ALT, bilirubina i GGTP) a stężeniem AGE10 w osoczu
4. Ocena dokładności diagnostycznej AGE10 w odróżnianiu pacjentów z alkoholowym zapaleniem wątroby od zdrowych
5. Ocena powiązania i dokładności diagnostycznej klasycznych typów AGE z ALD.
6. Ocena zaburzeń mikrobiomu związanych z AUD i ALD.
7. Określenie powiązania mikrobowej α-galaktozydazy z ALD.

7. Materiały i metody

Rozprawę doktorską stanowi cykl trzech spójnych tematycznie artykułów.

6.1. Publikacja *Advanced glycation end-products in common non-infectious liver diseases: systematic review and meta-analysis.*

Pierwszy artykuł stanowi przegląd systematyczny oraz metaanaliza oceniającą powiązanie i skuteczność diagnostyczną różnych typów AGE z niealkoholową oraz alkoholową chorobą wątroby. Badanie wykonane zostało zgodnie z wytycznymi PRISMA. Z wstępnie uzyskanych 892 artykułów wyselekcjonowano 11 kwalifikujących się do przeglądu systematycznego oraz 8 kwalifikujących się do metaanalizy. Spośród 11 zakwalifikowanych prac 8 odnosiło się do niealkoholowej choroby wątroby oraz 3 do alkoholowej choroby wątroby. Wyniki opracowano wykorzystując pakiet meta dostępny dla języka R (wersja 4.03), wykorzystując model efektów losowych. Wyniki przedstawiono jako standaryzowane różnice średnich z towarzyszącymi 95% przedziałami ufności.

6.2. Publikacja *Alcoholic Liver Disease Is Associated with Elevated Plasma Levels of Novel Advanced Glycation End-Products: A Preliminary Study*

Do badania włączono 65 pacjentów z alkoholową chorobą wątroby. Kryteria włączenia stanowiło nadmierne spożycie alkoholu (zdefiniowane jako konsumpcja powyżej 3 jednostek alkoholu odpowiadających po 14g czystego etanolu dla mężczyzn oraz 2 jednostek dla kobiet), spełnienie kryteriów klinicznej diagnozy alkoholowego zapalenia wątroby (żółtaczka o nagłym początku, zwiększony poziom ALT lub AST, z ALT i AST poniżej 400 jednostek/l oraz stosunkiem AST/ALT powyżej 1.5). Kryteria wyłączające stanowiło współwystępowanie innych, poważnych współistniejących zaburzeń jak sepsa, HCC lub niewydolność wielonarządowa. Grupę kontrolną stanowiło 65 zdrowych krwiodawców z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa im. prof. dr hab. Tadeusza Dorobisza.

Krew została pobrana na podłożę z EDTA, odwirowana i przechowywana w temperaturze –80 st. C do wykonania oznaczeń miana AGE10.

Stężenie AGE10 oznaczano z wykorzystaniem kompetycyjnego testu ELISA. W tym celu, na 96-dołkową płytkę MaxiSorp (Nunc®, Sigma-Aldrich, Darmstadt, Germany) nałożono syntetyczne MAGE o wysokiej masie cząsteczkowej i pozostawiono na 5 godzin w temperaturze

pokojowej celem związania. Płytkę następnie przepłukano i zablokowano przez noc roztworem 10% mleka odtłuszczonego w temperaturze 4 st. C. Po rozmrożeniu próbki zostały rozpipetowane do probówek Eppendorfa, gdzie przeprowadzona została inkubacja z niekomercyjnymi przeciwciałami monoklonalnymi IgE przeciwko MAGE. Jednocześnie przygotowano standard składający się z seryjnie rozcieńczonych MAGE o niskiej masie cząsteczkowej o znanych stężeniach, który również inkubowano z przeciwciałami IgE przeciwko MAGE. Po 45-minutowej inkubacji próbki oraz standard z przeciwciałami został przetransferowany na płytki MaxiSorp. Po kolejnych dwóch godzinach inkubacji na płytki dodano przeciwciała skoniugowane z peroksydazą chrzanową (Acris Antibodies GmbH, Herford, Germany). W następnym kroku dodany został dichlorowodorek o-fenylenodiaminy (Sigma-Aldrich, Darmstadt, Germany) stanowiący substrat dla peroksydazy chrzanowej. Po 5 minutach inkubacji odczytano absorbancję przy długości fali 450 nm z użyciem czytnika Enspire (Perkin Elmer, Waltham, MA, USA). Stężenia AGE10 obliczono na podstawie krzywej standardowej.

Analiza statystyczna wykonana została z wykorzystaniem R w wersji 4.0.3. Grupy porównywano z wykorzystaniem testu U Manna-Whitneya. Korelację określono z wykorzystaniem statystyki tau Kendalla. W celu określenia dokładności diagnostycznej obliczono czułość i swoistość oraz skonstruowano krzywe ROC.

6.3. Publikacja *Microbiome alterations in alcohol use disorder and alcoholic liver disease*

W trzeciej publikacji wykorzystano sekwencje 16S rRNA dostępne w bazach danych Sequence Read Archive oraz European Nucleotide Archive dla następujących porównań: 1) pacjenci z AUD i zdrowa grupa kontrolna, 2) pacjenci z AUD bez alkoholowej choroby wątroby oraz pacjenci z alkoholową chorobą wątroby, 3) pacjenci z alkoholową chorobą wątroby oraz pacjenci chorobami wątroby o innej etiologii. Łącznie uzyskano dane dla 122 pacjentów z AUD, 75 z alkoholową chorobą wątroby, 54 z chorobami wątroby o innej etiologii i 260 zdrowych. Całość analiz bioinformatycznych przeprowadzono z wykorzystaniem systemu zarządzania przepływem pracy Snakemake [17]. Kontrolę jakości oraz wstępną obróbkę bioinformatyczną próbek przeprowadzono z wykorzystaniem oprogramowania USEARCH. Warianty sekwencji amplikonu uzyskano za pomocą algorytmu UNOISE3 [18]. Sekwencje zostały przypisane do odpowiednich taksonów z wykorzystaniem algorytmu IDTAXA [19] i bazy taksonomicznej

GTDB w wersji 07-RS207 [20]. Potencjał funkcjonalny mikrobiomu (w tym stężenie α-galaktozydazy) został określony wykorzystując PICRUSt 2 używając Metacyc jako referencyjnej bazy szlaków metabolicznych [21]. Różnice w proporcjach występowania poszczególnych mikrobów oraz w potencjalne funkcjonalnym między grupami określano przy użyciu narzędzia ANCOM-BC [16].

8. Wyniki

Przegląd systematyczny i metaanaliza wykazały, że stężenia klasycznych podtypów AGE (frakcji fluorescencyjnej oraz CML) są istotnie wyższe u Pacjentów z alkoholową chorobą wątroby w porównaniu ze zdrową grupą kontrolną. Dwa badania wykazały istotnie wyższe stężenie CML u Pacjentów z alkoholową marskością wątroby w porównaniu z grupą kontrolną. Jedno badanie wykazało istotnie wyższe stężenie frakcji fluorescencyjnej AGE u pacjentów z alkoholową marskością wątroby i alkoholowym zapaleniem wątroby w porównaniu do zdrowej grupy kontrolnej. Nie wykazano istotnej różnicy w stężeniu tej frakcji pomiędzy pacjentami z alkoholową marskością i alkoholowym zapaleniem wątroby. Żadna z zakwalifikowanych prac nie uwzględniła w swoich wynikach dokładności diagnostycznej dla alkoholowej choroby wątroby.

Drugie badanie wykazało istotnie wyższe stężenie AGE10 w osoczu pacjentów z alkoholowym zapaleniem wątroby w porównaniu do zdrowej grupy kontrolnej (odpowiednio 184.5 ± 71.1 µg/mL i 123.5 ± 44.9 µg/mL, $p < 0.001$). Nie wykazano istotnej korelacji pomiędzy stężeniem AGE10 a ALT, AST, GGTP, bilirubiną, wiekiem lub płcią uczestników badania. Dokładność diagnostyczna AGE10 w odróżnianiu alkoholowego zapalenia wątroby od zdrowej grupy kontrolnej oceniona przez AUC wynosiła 0.778. Dla wartości odcięcia 147.25 µg/mL czułość wynosiła 75% a swoistość 72%.

W trzecim badaniu wykazano bardzo liczne zaburzenia składu mikrobiomu powiązane z AUD i ALD. Do najważniejszych zmian należy spadek *Firmicutes* u pacjentów z AUD i ALD. Nie wykazano istotnych różnic w poziomie *Bacteroidetes*, ani w stosunku *Firmicutes/Bacteroidetes,* stanowiącego często stosowany wykładnik zdrowej mikroflory. Zmniejszenie proporcji *Firmicutes* w największym stopniu spowodowane było przez redukcję w rodzinach *Ruminococcaceae, Lachnospiraceae, Oscillospiraceae* i *Butyricicoccaceae,* które zawierają liczne bakterie produkujące maślan, krótkołańcuchowy kwas tłuszczowy (SCFA) o właściwościach przeciwzapalnych i działający protekcyjnie na barierę jelitową. Redukcja tych korzystnie działających taksonów była większa u pacjentów z ALD w porównaniu z pacjentami z AUD bez choroby wątroby. Oprócz tego w AUD i ALD wykazano proporcjonalnie wyższy udział typu *Proteobacteria*, zawierającego bakterie wytwarzające silnie immunogenny lipopolisacharyd. Zaobserwowany wzrost był większy u pacjentów z ALD w porównaniu z AUD

bez choroby wątroby. Na poziomie rodziny najistotniejszy wzrost bakterii z *Proteobacteria* zaobserwowano w *Enterobacteriaceae* i *Burkholderiaceae*, które powiązane są z rozwojem ogólnoustrojowego stanu zapalnego. Pacjenci z ALD mieli istotnie niższą alfa-różnorodność w porównaniu z grupą AUD bez choroby wątroby. Nie wykazano jednak istotnych różnic w alfa-różnorodności pomiędzy pacjentami z AUD a zdrową grupą kontrolną. Analiza profilu funkcjonalnego wykazała między innymi zwiększenie aktywności szlaków związanych z syntezą lipopolisacharydu u pacjentów z ALD w porównaniu z grupą AUD bez choroby wątroby. Nie wykazano istotnych różnic w aktywności α-galaktozydazy pomiędzy AUD, ALD i grupą kontrolną.

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10. Publikacje wchodzące w skład cyklu

Systematic Review

Advanced Glycation End-Products in Common Non-Infectious Liver Diseases: Systematic Review and Meta-Analysis

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Abstract: Background: Excessive intake of fructose, glucose and alcohol is associated with the development of non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD). At the same time, these dietetic factors create an environment favorable for the generation of advanced glycation end-products. For this reason, advanced glycation end-products (AGEs) are hypothesized to play role in the development of NAFLD and ALD. In this systematic review and meta-analysis, we explore the relationship between NAFLD and ALD with AGE levels, including their diagnostic accuracy. Methods: The systematic review and meta-analysis has been pre-registered with PROSPERO (CRD42021240954) and was performed in accordance with the PRISMA guidelines. Meta-analyses were performed using the meta R package. Results: We have obtained 11 studies meeting our inclusion criteria, reporting data on 1844 participants (909 with NAFLD, 169 with ALD and 766 healthy controls). NAFLD was associated with significantly higher AGE fluorescence and serum N-(carboxyethyl)lysine (CEL) levels. Patients with alcoholic cirrhosis had significantly higher levels of N-(carboxymethyl)lysine (CML). Only individual studies examined AGEs in the context of their diagnostic accuracy. AGE fluorescence distinguished low and moderate steatosis with an AUC of 0.76. The ratio of CML, CEL and pentosidine to a soluble variant of the AGE receptor differentiated patients with NAFLD from healthy controls with high AUC (0.83–0.85). Glyceraldehyde-derived AGE separated non-alcoholic fatty liver (NAFL) from non-alcoholic steatohepatitis (NASH) with acceptable performance (AUC 0.78). Conclusions: In conclusion, NAFLD and ALD are associated with significantly higher levels of several AGEs. More research is needed to examine the diagnostic accuracy of AGEs, however individual studies show that AGEs perform well in distinguishing NAFL from NASH.

Keywords: serum levels of AGE; advanced glycation end-products; liver disease; alcohol; fructose

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1. Introduction

Chronic liver diseases account for over 2% of the global total deaths [1]. The percentage contribution of different etiologies is constantly shifting, with non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) increasing in incidence worldwide. NAFLD is estimated to affect 24% of the global population and is currently one of the main causes of the increase in the incidence of cirrhosis [2]. ALD is responsible for 27% of deaths related to liver disease and 30% of cases of liver cancer worldwide [3].

NAFLD is defined as the presence of at least 5% hepatic steatosis in biopsy specimens. It includes non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). Currently, the only diagnostic tool which reliably differentiates NAFL from NASH is a biopsy. In the case of NAFL, a biopsy must show one of the following appearances: (1) simple steatosis; (2) steatosis with lobular/portal inflammation, without ballooning; (3) steatosis with ballooning but without inflammation. Diagnosis of NASH requires

steatosis with both ballooning and lobular inflammation [4]. NAFLD commonly coexists with insulin resistance (IR) and other disorders associated with it, including metabolic syndrome (MetS) [5]. The lifestyle risk factors of NAFLD are similar to those of MetS and include obesity, excessive intake of saturated fats, refined carbohydrates and fructose [6].

ALD refers to liver damage caused by excessive intake of alcohol (typically defined as regular consumption of >30 g of ethanol a day for males and >20 g for females). Analogous to NAFLD, it encompasses a wide range of disease severity, including steatosis, steatohepatitis (ASH) and cirrhosis (AC). Even though biopsy remains the mainstay of the ASH diagnosis, the American Association for the Study of Liver Diseases states that probable diagnosis can be established using clinical criteria [7]. The risk factors for the development and progression of ALD consist of various lifestyle (for example, drinking patterns—daily heavy drinking results in greater risk than binge drinking [8]) and genetic factors (e.g., disturbances in circadian clock genes [9,10]).

Although NAFLD and ALD are caused by different factors, they share similar clinical courses. The initial stage for both diseases is simple steatosis, which progresses further to steatohepatitis, fibrosis or cirrhosis in a relatively small fraction of patients with ALD and an even smaller proportion of patients with NAFLD. In the case of patients with alcohol use disorder (AUD), over 90% develop hepatic steatosis, 20–40% fibrosis and 8–20% cirrhosis [11]. In the case of NASH, progression to cirrhosis occurs in less than 2.5% of patients [12]. While multiple risk factors for the development and progression of NAFLD and ALD have been already identified, it is still challenging to identify patients at the highest risk of progression with high certainty and reliability.

Similarities in the clinical course are mirrored by multiple pathophysiological features shared between NAFLD and ALD. These include—but are not limited to—disturbances in circadian clock genes, alterations in the composition of the microbiome and increased intestinal permeability [9,13–16]. Recently advanced glycation end-products (AGEs) have been identified as an additional potential factor associated with the pathophysiology of both NAFLD and ALD [17].

AGEs are generated by non-enzymatic Maillard reactions between the carbonyl group of reducing sugar (or other carbonyl compounds) and a free amino group of a protein, aminophospholipid or nucleic acid. The first stage results in the generation of Schiff bases, which are then rearranged to stable Amadori products. Further oxidation and glycation result in the generation of AGEs [17–19]. Early research on AGEs focused mainly on the fluorescent fraction of AGEs; however, advances in biochemistry allowed detection of non-fluorescent fractions, with N-(carboxymethyl)lysine (CML) and pentosidine being the most widely studied. Novel approaches have allowed for the detection of even trace amounts of non-standard AGE subtypes [20].

Currently, the leading hypothesis for the involvement of AGEs in NAFLD and ALD is the toxic AGE (TAGE) theory, which focuses on the role of glyceraldehyde- and acetaldehyde-derived AGEs (GA- and AA-AGEs). GA and AA are produced in the liver through three pathways—fructolysis, glycolysis and alcoholysis. As increased consumption of fructose is associated with NAFLD and overconsumption of alcohol is necessary for the development of ALD, GA and AA provide a convenient link between risk factors and pathophysiology of NAFLD and ALD. For a comprehensive overview of TAGE theory, please refer to the manuscript by Takeuchi et al. [17].

Although recent advances in the understanding of the AGEs' role in the development and progression of NAFLD and ALD are promising, AGE levels assessment and characterization are not yet incorporated into the clinical practice. Estimation of their clinical usefulness is complicated by an extensive range of studied AGE subtypes. The goal of this systematic review and meta-analysis was to evaluate if NAFLD and ALD are associated with increased levels of serum AGE. In addition, we have compared serum concentrations of AGEs and their diagnostic accuracy in patients with simple steatosis and more advanced stages of NAFLD and ALD.

2. Materials and Methods

The study was performed under the PRISMA guidelines [21]. The review protocol has been prospectively registered with PROSPERO, CRD42021240954 [22].

2.1. Search Strategy

We have searched MEDLINE, EMBASE and Cochrane Register of Controlled Trials (CENTRAL) from their inception to the final search date (4 March 2021) using terms related to liver diseases and advanced glycation end-products. In addition, we have scanned references of the full-text manuscripts. The full search strategy has been described in the Supplementary File.

2.2. Inclusion and Exclusion Criteria

We have included studies reporting serum concentrations of AGEs of patients with NAFLD and ALD (including simple steatosis, steatohepatitis, hepatitis and cirrhosis). The control groups were people with liver diseases excluded by normal ultrasound and aminotransferases levels. No study type restrictions were applied. We have excluded interventional studies that did not provide baseline comparison between patients with liver diseases and healthy individuals. The outcomes we sought for meta-analysis were means and standard deviations (SDs). If the publication reported medians and interquartile ranges (IQR), we checked the results for skewness [23], and if no significant skewness was found, we used the approach proposed by Shi et al. [24] and Luo et al. [25] to estimate means and SDs. If significant skewness was detected, we have reported medians and IQR obtained from the paper, without including it in the meta-analysis.

2.3. Assessment of Eligibility, Data Extraction, and Quality Assessment

After the removal of duplicates, two authors (KL and EW) screened abstracts and titles obtained from the search. Studies qualified after initial screening were read in full text and assessed using our exclusion and inclusion criteria. For data extraction, we have devised a datasheet with fields relating to study characteristics (title, author, study design), participants (including the type of liver disease, age, BMI, aminotransferases level) and outcomes. The risk of bias was assessed using the QUADAS-2 tool [26].

2.4. Synthesis of Results

Meta-analysis was conducted using the meta package [27] in R, version 4.03 [28]. Pairwise analyses were performed for each comparison where at least two studies were available. A random-effects model with the inverse-variance method was used. To detect heterogeneity, we have employed Cochran's Q test and I^2 inconsistency statistic. For τ^2 we have used the Empirical Bayes estimator. Results were presented as standardized mean differences (SMD) with 95% confidence intervals. Due to a low number of papers obtained for meta-analysis, we did not perform meta-regression or sensitivity analyses.

3. Results

Our search yielded 11 papers meeting our inclusion criteria, out of which eight qualified for the meta-analysis. A flowchart of the study selection process is provided in Figure 1. Obtained results and characteristics of the included studies are presented in Table 1. Seven of the included studies exhibited moderate and four high risk of bias (Figure 2). Due to significant clinical heterogeneity and a low number of obtained papers, we did not attempt to perform meta-analytical synthesis of data regarding the diagnostic accuracy of AGEs, which is instead presented as narrative synthesis.

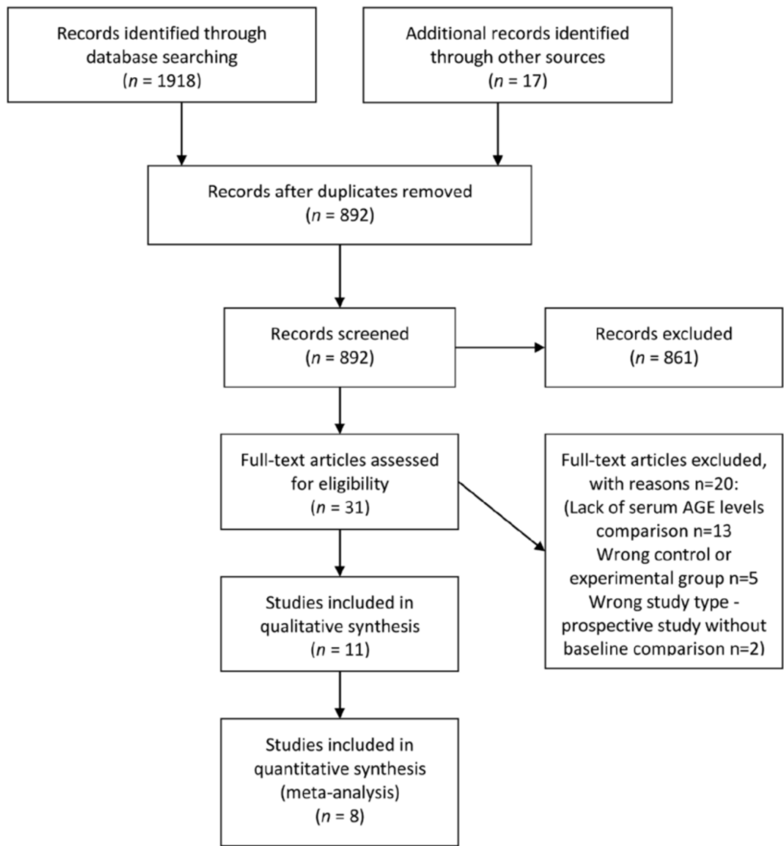

Figure 1. Diagram of the study selection process (adapted from PRISMA). AGE—advanced glycation end-product.

Table 1. Characteristics of the included studies.

Study	Study Population	Which AGE Was Measured? What Method Was Used?	How Is the Outcome Reported?	Outcome
Bronowicka-Szydelko et al. [29]	46 NAFLD 170 Control	AGE-1; Slot-blot	Median with 95% CI	Control vs NAFLD: 0 (0;9093) vs. 14580 (1049;29852); $p < 0.05$
Swiderska et al. [30]	67 NAFLD 40 Control	AGE fluorescence; Measured at 350/440 nm	Mean and SD *	Control vs NAFLD: 1.76 (0.93) vs. 3.77 (2.32); $p < 0.001$ Early NAFLD vs. advanced NAFLD: 3.36 (1.22) vs. 4.08 (1.47); $p = 0.02$
Mehta et al. [31]	103 NASH 143 NAFL 93 Control	AGE; ELISA	Mean and SD	Control vs. NAFL vs. NASH: 10.37 (4.68) vs. 9.32 (4.74) vs. 10.14 (5.31); non-significant
Palma-Duran et al. [32]	58 NAFLD 58 Control	AGE fluorescence, CML, CEL, Pentosidine; Fluorescence measured at 370/440 nm, AGEs quantification by HPLC-fluorescence-MS	Mean and SD * Median and IQR for pentosidine	Control vs NAFLD: AGE fluorescence—410.08 (51.69) vs. 502.88 (139.73); $p < 0.001$ CML—9.9 (2.5) vs. 10.6 (6.8); non-significant CEL—93.9 (30.7) vs. 122.9 (34.7); $p < 0.001$ Pentosidine—1.5 (1.4–1.7) vs. 1.6 (1.5–1.9); $p < 0.02$
Kan et al. [33]	NASH 56 Control 27	Non-CML AGE; ELISA	Mean and SD	Control vs. NASH: 3.5 (1.2) vs. 5.2 (1.7); $p < 0.05$

Table 1. *Cont.*

Study	Study Population	Which AGE Was Measured? What Method Was Used?	How Is the Outcome Reported?	Outcome
Butscheid et al. [34]	10 NASH 13 NAFL 20 Control	Imidazolone, CML; ELISA	Mean and SD	Control vs non-NASH NAFLD vs. NASH: Imidazolone—3.4 (1.6) vs. 3.6 (2.3) vs. 3.3 (1.4); non-significant CML—893 (212) vs. 913 (170) vs. 916 (92); non-significant
Hyogo et al. [35]	30 Control 10 NAFLD 66 NASH	GA-AGE, Glucose-AGE, CML; ELISA	Mean and SD	Control vs. NASH: Glucose-AGE—44 (22.5) vs. 72.9 (52.8); $p < 0.005$ GA-AGE—6.96 (2.36) vs. 9.78 (3.73); $p < 0.005$ CML—11.8 (7.6) vs. 16.3 (10.2); $p = 0.054$ NAFL vs. NASH: GA-AGE—7.17 (2.28) vs. 9.78 (3.73); $p < 0.05$; no glucose-AGE and CML analysis was performed for this comparison
Bijnen et al. [36]	337 NAFLD 168 Control	CML, CEL; Liquid chromatography-tandem MS	Mean and SD *	Control vs. NAFLD: CML—76.42 (24.38) vs. 81.73 (50.13); non-significant CEL—43.33 (13.16) vs. 48.93 (29.4); $p < 0.001$
Das et al. [37]	100 SAH 20 AC 20 Control	AGE fluorescence; Measured at 350/440 nm	Mean and SD	Control vs. SAH vs. AC: 8457 (2500) vs. 14574 (2670) vs. 15691 (3138); $p < 0.01$ for control vs. SAH and AC; non-significant for SAH vs. AC
Sebekova et al. [38]	19 AC 19 Control	CML; ELISA	Mean and SD	Control vs. AC: 432 (16) vs. 811 (100); $p < 0.01$
Yagmur et al. [39]	30 AC 121 Control	CML; ELISA	Mean and SD *	Control vs. AC: 466.84 (659.32) vs. 1182.77 (1418.27); $p < 0.05$

*—calculated from median and IQR. AGE—advanced glycation end-product, NAFLD—non-alcoholic fatty liver, NASH—non-alcoholic steatohepatitis, NAFL—non-alcoholic fatty-liver, SAH—severe alcoholic hepatitis, AC—alcoholic cirrhosis, CML—N-(carboxymethyl)lysine, CEL— N-(carboxyethyl)lysine, GA-AGE—glyceraldehyde-derived AGE.

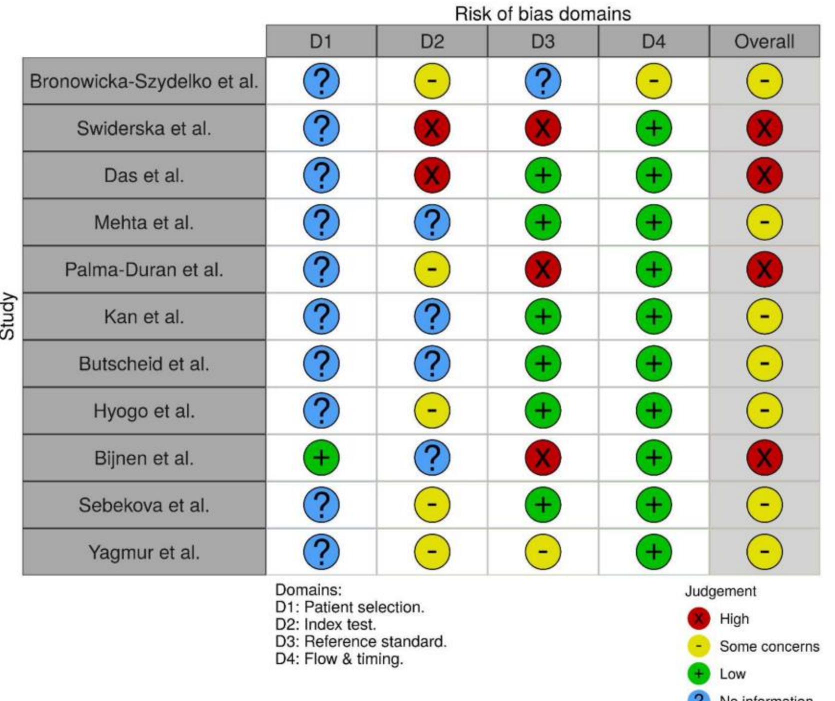

Figure 2. Risk of bias assessment [29–39].

3.1. Systematic Review

Studies included in the systematic review reported data on 1844 participants (909 with NAFLD, 169 with ALD and 766 healthy controls). Three studies focused on ALD [37–39] and eight studies on NAFLD [29–36]. In studies on ALD, the diagnosis was confirmed with a biopsy. For NAFLD, only four studies used biopsy for definitive diagnosis, the remaining four based the diagnosis on imaging (ultrasound or CT), elastography or validated equations for predicting the hepatic steatosis (using laboratory tests and other variables). Six of the studies on NAFLD reported that it is associated with higher levels of various AGEs. Out of four studies examining serum concentration of CML in NAFLD, none reported a significant difference from healthy controls. Interestingly, in the case of ALD, both studies examining CML showed that it had significantly higher serum levels in patients with alcoholic cirrhosis compared with healthy controls. One of the included studies showed, that AGE fluorescence was significantly higher in patients with advanced NAFLD (i.e., with fibrosis > 6.6 kPa measured by transient elastography) than in patients with early NAFLD (without fibrosis).

3.2. Meta-Analysis

We have performed pairwise comparisons of AGE fluorescence, CML and N-(carboxyethyl)lysine (CEL) serum concentrations in NAFLD, and CML serum concentration in ALD (Figures 3 and 4).

NAFLD, in comparison to healthy controls, was associated with significantly higher AGE fluorescence (SMD 0.95, CI 0.66; 1.24) and serum concentration of CEL (SMD 0.53, CI −0.09; 1.15). CML had a negligible effect size of 0.12 with −0.04;0.28 CI. The comparison of the CEL levels was the only one with high, statistically significant heterogeneity (I^2 = 83%, p < 0.01, Figure 3).

Patients with AC had higher levels of CML compared to healthy controls (SMD 2.95, CI −1.26; 7.16). However, the comparison had very high heterogeneity (I^2 = 97%, p < 0.01), which most likely stems from the extraordinarily high effect size reported by Sebekova et al. (5.18 SMD, Figure 4) [38]. The number of studies obtained for each comparison was too small to assess publication bias using statistical methods such as Egger's test.

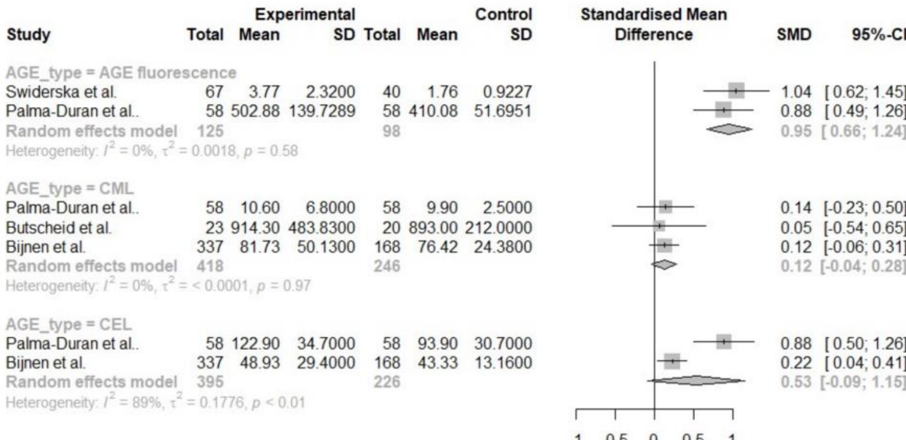

Figure 3. Pairwise comparisons of serum AGE levels between patients with NAFLD and healthy controls [30,32,34,36].

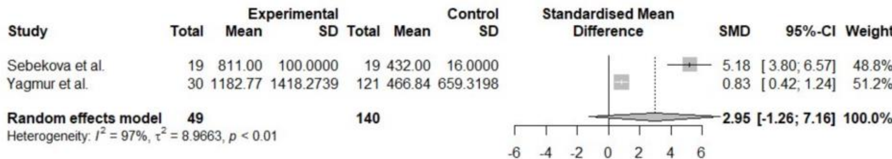

Figure 4. Pairwise comparisons of serum AGE (CML) levels between patients with ALD and healthy controls [38,39].

3.3. Diagnostic Accuracy

Only three studies reported data on the diagnostic accuracy of AGEs in NAFLD (Table 2). The AUC of 0.7 to 0.8 was considered acceptable, 0.8 to 0.9 excellent and >0.9 outstanding [40]. The performance of AGE fluorescence in distinguishing between low and moderate steatosis was acceptable (AUC 0.76). CML, CEL and pentosidine performed poorly in distinguishing healthy controls from patients with NAFLD; however, when coupled with sRAGE (a soluble variant of the AGE receptor), CEL/sRAGE, AGE (defined as the sum of CML, CEL, and pentosidine levels)/sRAGE and AGE fluorescence/sRAGE presented excellent discriminatory ability (AUC 0.83–0.85), exceeding the performance of both AGEs and sRAGE when used as separate markers. GA-AGE separated NAFL from NASH with acceptable performance (AUC 0.78). None of the studies with ALD patients reported diagnostic accuracy of AGEs.

Table 2. Diagnostic accuracy of AGEs.

Study	Comparison	Reference Test	Index Test	AUC	Sensitivity	Specificity
Swiderska et al. [30]	Low steatosis (BARD score 0–1) vs. moderate steatosis (BARD score 2–4)	BARD score	AGE fluorescence (>2.77 AFU/mg)	0.76	70%	84%
Palma-Duran et al. [32]	Healthy controls vs. NAFLD	Elevated liver enzymes, ultrasound hepatic steatosis evidence, and exclusion of other liver injuries	CML, CEL, pentosidine, CML/sRAGE	<0.78 **	NA	NA
			CEL/sRAGE (>6.9 mmol/pmol)	0.85	81%	77%
			AGE */sRAGE (>7.8 mmol/pmol)	0.85	81%	77%
			AGE fluo/sRAGE (>87.4 AU/ng)	0.83	80%	79%
Hyogo et al. [35]	NAFL vs. NASH	Biopsy	GA-AGE (>8.53/mL)	0.78	66.7%	88.9%

*—AGE represents the sum of CML, CEL, and pentosidine serum levels; **—no exact value was reported. AFU—arbitrary fluorescence unit, AUC—area under curve, NAFLD—non-alcoholic fatty liver, CML—N-(carboxymethyl)lysine, CEL—N-(carboxyethyl)lysine, GA-AGE—glyceraldehyde-derived AGE, sRAGE—soluble variant of the AGE receptor, BARD score—risk of advanced fibrosis in NAFLD.

4. Discussion

The goal of our systematic review and meta-analysis was to compare serum levels of various AGEs between patients with NAFLD or ALD and healthy controls. In addition, we have presented data regarding diagnostic accuracy in differentiating NAFLD from healthy controls and early NAFLD from more advanced stages. Types of the obtained levels of AGE compounds include CML, CEL, imidazolone, GA-AGE, glucose-AGE, AGE-1, pentosidine and AGE fluorescence.

The results of our study show that chronic liver diseases are associated with elevated concentrations of some fractions of AGEs. Interestingly, NAFLD was not associated with significant differences in serum concentrations of CML, which was frequently used as a proxy for all the subtypes of AGEs. However, the levels of CML were significantly higher in patients with AC compared with healthy controls. As we did not identify any study

examining NAFLD-associated cirrhosis, we are unable to provide a definitive answer whether the difference stems from the stage (i.e., NAFL versus AC) or etiology (alcoholic versus non-alcoholic) of the liver disease. However, Yagmur et al. [39] provide some indirect evidence that cirrhosis itself—and not etiology—is responsible for higher CML levels in AC. Apart from patients with AUD, they have included other etiologies of liver injury—virus hepatitis, biliary, autoimmune and unspecified, other etiologies. They have shown that, irrespectively of etiology, cirrhotic patients had higher levels of CML than groups with chronic liver diseases but without cirrhosis. In addition, they have established a positive correlation between how advanced cirrhosis is and the concentration of CML. Unfortunately, no NAFLD group was included in that study [39]. Further evidence that CML is associated with the pathophysiology of NAFLD is provided by Gaens et al. [41]. They have shown that the accumulation of CML in the liver is significantly associated with the grade of hepatic steatosis. CEL, AGE with biochemical properties similar to CML [42] had significantly higher concentrations in patients with NAFLD than in healthy controls. Both NAFLD and ALD were associated with increased levels of AGE fluorescence.

Only one of the included studies reported the accuracy of AGEs in the diagnosis of NAFLD. None of the AGEs performed well as a sole diagnostic marker. However, when using AGEs together with sRAGE as a ratio (e.g., CEL/sRAGE), the performance significantly increased reaching an AUC of at least 0.83. This increase in the diagnostic accuracy can be explained by the physiological role of sRAGE, which serves as a receptor decoy for AGE ligand [43]. Through this mechanism, increased serum concentrations of sRAGE might lead to reduced activation of the AGE/RAGE axis, which is pivotal in the AGE-related hepatocyte injury [17].

Unfortunately, the data regarding differences in serum concentrations of AGEs in early and advanced chronic liver diseases were scarce. For ALD, the only AGE studied in this context was the fluorescent fraction, which did not differ in patients with SAH and AC. However, for NAFLD, there was a significant increase in AGE fluorescence between early (without fibrosis) and advanced disease. Additionally, AGE fluorescence distinguished low steatosis from moderate with acceptable performance. Serum concentrations of imidazolone and CML did not differ significantly between patients with NAFL and NASH. The only AGE which was significantly elevated in the patients with NASH compared with NAFL was GA-AGE. As a diagnostic marker, it had acceptable AUC (0.78), relatively high specificity (88.9%), and low sensitivity (66.7%). Therefore, our results hint that further research into different fractions of AGEs (perhaps combined with sRAGE as ratio) in various stages of NAFLD might result in clinically useful biomarkers. Out of currently available biomarkers for distinguishing NASH from simple steatosis, cytokeratin-18 is the most widely studied, with the largest trial on the topic reporting an AUC of 0.65 [44]. Distinguishing between NAFL and NASH without biopsy is currently challenging—none of the noninvasive tests have been validated for the diagnosis of NASH [4].

Although the exact mechanism of the development and progression of NAFLD and ALD has not yet been fully elucidated, a growing body of evidence shows that AGE might play a causative role. AGE, after binding to its specific receptor activates numerous pathways, including nuclear factor-κB (NF-κB), which is among the most widely studied compounds associated with liver injury [45]. Activation of NF-κB increases the production of proinflammatory cytokines (such as tumor necrosis factor α, interleukins 1 and 6), which has been linked with liver injury in the animal models of both NAFLD and ALD [46,47]. In addition, the binding of AGE to RAGE activates mitogen-activated protein kinases (MAPK) and c-Jun N-terminal protein kinase (JNK). The JNK/MAPK pathway is strongly associated with insulin resistance. The critical involvement of JNK1 isoform in the development of NAFLD and progression to NASH has been established using animal models. JNK1-knockout mice fed a high-fat diet had lower steatosis compared with wild type mice [48]. In the murine methionine- and choline-deficient diet model of steatohepatitis, knockout of JNK1 (but not JNK2) was associated with reduced susceptibility to NASH [49].

Apart from endogenous formation, AGEs can be provided with diet. The average daily consumption of AGE is approximately 75 mg. The main exogenous sources of AGEs include highly processed food (especially fried) and soft drinks containing high fructose corn syrup or sugar. Two main factors associated with the increased formation of dietary AGEs are high cooking temperatures and long cooking times [50]. It is not clear what fraction of dietary AGEs is absorbed into the circulation, with estimates varying between 10 and 80% [51,52]. This issue could have important practical implications—if dietary AGEs were found to significantly contribute to serum AGE levels, a low AGE diet would be a potentially viable option in reducing the severity of liver injury in NAFLD and ALD. The dietary modifications leading to the reduction of AGE content in food are relatively simple and include shorter cooking times, using lower cooking temperatures and poaching instead of frying [50]. In addition, as high pH increases the formation of dietary AGEs [53], marinating food in vinegar prior to processing might be another viable strategy. Currently, no trial examining low AGE diet in NAFLD and ALD has been performed on human subjects. However, a recent meta-analysis reported that a low AGE diet was associated with a reduction of weight and a positive impact on the markers of insulin resistance [54]. In addition, Leung et al. has shown, that diet rich in dietary AGEs was associated with increased liver injury and inflammation in an animal model of NAFLD [55].

Our study has several limitations. First, a low number of the obtained studies precluded us from performing sensitivity and more nuanced analyses such as meta-regression. In addition, for NAFLD, half of the included studies did not use a biopsy to establish the diagnosis, reducing the reliability of obtained results. Furthermore, the included papers had a cross-sectional design, which precludes making strong whether AGEs play a causative role in liver injuries. However, as we have discussed above, several studies support this notion.

Recently, another subtype has been described in human tissues, of which in vitro analogue could be synthesized in anhydrous conditions [56]. This subtype is different from glucose-related AGEs, and it is formed in physiological conditions most likely in other than known biosynthetic pathways. Our further studies will consider the relationship between its level and various clinical parameters.

In conclusion, NAFLD and ALD are associated with increased concentrations of several AGEs, and the size of the effect depends on the studied fraction. The diagnostic accuracy of AGEs is poorly studied, however the GA-AGE appears to be promising in distinguishing the patients with NAFL and NASH. Our study strengthens the link between AGEs and chronic liver diseases and shows that further research on the topic is needed and might result in novel diagnostic approaches.

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PUBMED:

("advanced glycation end product*" OR "advanced glycation endproduct*" OR "advanced glycation end-product*" OR "Glycation End Products, Advanced"[MeSH] OR imidazolone OR (CML NOT "chronic myeloid leukemia") OR RAGE OR AGER OR "Receptor for Advanced Glycation End Products"[MeSH])

AND

(("fatty liver"[MeSH] OR hepatitis[MeSH] OR "liver cirrhosis"[MeSH] OR steatosis OR steatohepatitis OR "nonalcoholic steatohepatitis" OR "non-alcoholic steatohepatitis" OR NAFLD OR NASH OR "fatty liver disease" OR FLD)

OR

("alcoholic steatohepatitis" OR "alcoholism"[MeSH] OR "Liver Diseases, Alcoholic"[MeSH] OR ALD OR ASH OR "alcoholic steatohepatitis" OR "alcoholic hepatitis"))

EMBASE:

("advanced glycation end product*" OR "advanced glycation endproduct*" OR "advanced glycation end-product*" OR 'Glycation End Products, Advanced'/exp OR imidazolone OR (CML NOT "chronic myeloid leukemia") OR RAGE OR AGER OR 'Receptor for Advanced Glycation End Products '/exp)

AND

(('fatty liver'/exp OR 'hepatitis'/exp OR 'liver cirrhosis'/exp OR steatosis OR steatohepatitis OR "nonalcoholic steatohepatitis" OR "non-alcoholic steatohepatitis" OR NAFLD OR NASH OR "fatty liver disease" OR FLD)

OR

("alcoholic steatohepatitis" OR 'alcoholism'/exp OR 'Liver Diseases, Alcoholic'/exp OR ALD OR ASH OR "alcoholic steatohepatitis" OR "alcoholic hepatitis"))

CENTRAL:

#1 ("advanced glycation end product*" OR "advanced glycation endproduct*" OR "advanced glycation end-product*" OR imidazolone OR (CML NOT "chronic myeloid leukemia")) (Word variations have been searched)

#2 MeSH descriptor: [Glycation End Products, Advanced] explode all trees

#3 RAGE OR AGER

#4 MeSH descriptor: [Receptor for Advanced Glycation End Products] explode all trees

#5 #1 OR #2 OR #3 OR #4

#6 MeSH descriptor: [Fatty Liver] explode all trees

#7 MeSH descriptor: [Hepatitis] explode all trees

#8 MeSH descriptor: [Liver Cirrhosis] explode all trees

#9 steatosis OR steatohepatitis OR "nonalcoholic steatohepatitis" OR "non-alcoholic steatohepatitis" OR NAFLD OR NASH OR "fatty liver disease" OR FLD

#10 #6 OR #7 OR #8 OR #9

#11 MeSH descriptor: [Alcoholism] explode all trees

#12 MeSH descriptor: [Liver Diseases, Alcoholic] explode all trees

#13 "alcoholic steatohepatitis" OR ALD OR ASH OR "alcoholic steatohepatitis" OR "alcoholic hepatitis"

#14 #11 OR #12 OR #13

#15 #5 AND (#10 OR #14)

Article

Alcoholic Liver Disease Is Associated with Elevated Plasma Levels of Novel Advanced Glycation End-Products: A Preliminary Study

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Abstract: Elucidating the biochemical mechanisms associated with the progression of alcoholic liver disease (ALD) to more advanced stages such as alcoholic hepatitis (AH) remains an important clinical and scientific challenge. Several hypotheses point to the involvement of advanced glycation end-products (AGEs) in alcohol-associated liver injuries. Recently, we determined the structure of a synthetic, melibiose-derived AGE (MAGE), which was an analog of the novel AGE subgroup AGE10. The primary objective of our study was to determine whether AGE10 was associated with alcoholic hepatitis. The secondary objective was to provide a diagnostic accuracy of AGE10 in AH. To achieve this objective, we examined the plasma levels of AGE10 in 65 healthy individuals and 65 patients with AH. The AGE10 level was measured using a competitive ELISA. Our study confirmed that patients with AH had significantly higher plasma concentrations of AGE10 compared with healthy controls (184.5 ± 71.1 µg/mL and 123.5 ± 44.9 µg/mL, respectively; $p < 0.001$). In addition, AGE10 showed an acceptable performance as a diagnostic marker of AH, with an AUC of 0.78. In conclusion, AH was associated with elevated levels of novel advanced glycation end-product AGE10.

Keywords: advanced glycation end-products; liver disease; alcohol

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1. Introduction

The excessive use of alcohol is one of the most important contributors to global mortality. With 3 million deaths attributable to alcohol abuse, it accounted for 5.3% of all deaths in 2016. The effect of alcohol overconsumption on mortality is higher than HIV/AIDS, diabetes, or hypertension [1]. According to the World Health Organization, in 2016, approximately 132.6 million disability-adjusted life years were lost to the consequences of alcohol abuse [1]. The social and economic consequences of alcohol use disorder (AUD) are further exacerbated by the age structure of the deaths attributed to alcohol; deaths and disability from alcohol occur in relatively young people, with alcohol abuse being responsible for 13.5% of all deaths in people aged 20–39 years [1]. One of the leading causes of mortality in AUD is alcoholic liver disease (ALD) and its consequences. The most advanced form of ALD—alcoholic cirrhosis—accounts for 47.9% of all liver cirrhosis deaths [2]. Of all the chronic heavy drinkers, almost 100% will develop an alcoholic fatty liver. However, in this group, only 10 to 35% will progress to alcoholic steatohepatitis (ASH), and 8 to 20% will end up with cirrhosis [3]. The set of clinical features associated with ASH is called alcoholic hepatitis (AH). Elucidating the pathophysiological events responsible for the development of ASH and cirrhosis is of great clinical significance. The early detection of patients at a greater risk of progression to ASH would allow the development of more focused, individualized treatment approaches.

The diagnosis and accurate staging of ALD remain a challenge. A liver biopsy combined with a documented excessive consumption of alcohol remains the diagnostic gold standard for ALD. However, it is associated with a 2% risk of severe complications [4] and, as a consequence, the European Association for the Study of the Liver (EASL) guidelines for the management of ALD do not recommend its routine use [5]. For this reason, non-invasive tests of the liver function,—i.e., gamma-glutamyl transpeptidase (GGTP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)—remain the mainstay of an ALD diagnosis.

The invasive nature of a liver biopsy has prompted the development of novel, non-invasive tests for the accurate staging of ALD. Several biochemical parameters of liver fibrosis associated with ALD have been proposed. Currently, the individual biochemical marker with the highest diagnostic accuracy is hyaluronic acid [6]. However, biochemical panels combining multiple individual markers (such as an enhanced liver fibrosis test [7] or PGAA [8]) provide an even better diagnostic performance. In the case of alcoholic steatohepatitis, the spectrum of currently established biomarkers is much more limited, with the M30 and M65 epitopes of cytokeratin-18 (CK18) having the widest support in the literature [9]. Despite these early advances, it is worth highlighting that current guidelines do not recommend their use in clinical practice.

The pathophysiology of ALD is complex and involves multiple partially overlapping molecular pathways. The major pathophysiological foundations of ALD include the generation of reactive oxygen species (ROS) [3], microbiome alterations [10], and the accumulation of advanced glycation end-products (AGEs) [11]. AGEs, also called glycotoxins, are a group of long-lived, structurally diverse compounds. They are formed through several complex networks of biochemical reactions. The most important pathway begins with a reaction, which occurs between the reducing sugars and free amino groups of a protein, resulting in the generation of an unstable Schiff base. Subsequent rearrangements of the Schiff base lead to the formation of a stable Amadori product, which undergoes further oxidation, glycation, and cross-linking—generally called Maillard reactions—resulting in AGEs [12]. Both the Schiff base and Amadori product can further increase the rate of AGE generation by entering the Namiki and Wolff pathways, respectively, which produce dicarbonyls (such as methylglyoxal) [13]. Dicarbonyls are a group of highly reactive molecules, with up to 20,000 times higher glycating activity compared with glucose [14]. Apart from the Maillard reaction, dicarbonyls can also be generated through glycolysis, ketone body metabolism, lipid peroxidation, and the polyol pathway [15]. Although dicarbonyls are generally found in low concentrations in physiological conditions, due to their extreme glycating activity they significantly contribute to the formation of AGEs [14]. Their influence on the AGE pool can be further exacerbated in the pathological state; for example, hyperglycemia shifts glucose towards the polyol pathway, which leads to a significant increase in the generation of dicarbonyls [16].

The accumulation of AGEs has been hypothesized to play a role in a diverse range of diseases, including non-infectious liver disease [17], thyroid gland pathologies [18], diabetes [15], or even the development of psychotic symptoms [19]. Although the exact mechanism is still not fully elucidated, the involvement of glycotoxins in ALD is well-documented in the literature [17]. The relationship between AGEs and the development of liver disease appears to be bidirectional. On one side, the current leading hypothesis of AGE involvement in ALD (the so-called toxic AGE theory) postulates that interactions between acetaldehyde- or glyceraldehyde-derived AGEs and their receptor (RAGE) lead to the activation of molecular pathways, resulting in the release of proinflammatory cytokines and an increased generation of reactive oxygen species, which causes liver injuries [11]. On the other side, the liver is responsible for the removal of AGEs [20]; hence, a liver injury might lead to a further accumulation of glycotoxins.

To date, the molecular structure has been characterized only for a relatively small fraction of all AGEs (notable examples include carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), imidazolone, and pentosidine). Recently, the structure of a synthetic, melibiose-

derived AGE (MAGE) was determined, which was an analog of a novel compound. As shown by the lack of cross-reactivity of anti-MAGE antibodies with other known subtypes of AGEs [21] as well as no significant correlation between MAGEs and fluorescent AGEs [18], this newly discovered subtype did not belong to any known group of AGEs; thus, it was called AGE10 [22]. Several interesting properties of MAGEs have already been confirmed. AGE10-modified proteins have been shown to be present in both diabetic patients and healthy controls, with different patterns of protein modification depending on the disease status; in diabetic patients, albumin and IgG were mainly involved whereas in healthy patients, it was mostly IgG and IgA [21]. The AGE10 concentration is significantly correlated with multiple risk factors for cardiovascular diseases such as hypertension and lower levels of HDL [23]. Serum from diabetic patients has been shown to contain MAGE-specific auto-antibodies [24]. In addition, synthetic MAGEs exhibited a significant genotoxicity against human peripheral blood lymphocytes and on multiple human cell lines [25]. In this study, we attempted to elucidate whether this novel subtype of AGE was associated with alcoholic hepatitis. As the early detection of AH is crucial for preventing irreversible damage to the liver, our secondary objective was to provide the diagnostic accuracy of AGE10 in ALD.

2. Methods

2.1. Participants

We included 65 patients with alcoholic hepatitis recruited at the Jan Mikulicz-Radecki University Teaching Hospital in Wroclaw, Poland. The inclusion criteria were an excessive intake of alcohol, a clinical diagnosis of alcoholic steatohepatitis (i.e., sudden onset or progression of jaundice, or increased levels of ALT or AST, with ALT and AST under 400 IU/l and an AST/ALT ratio over 1.5), and a lack of serious non-alcoholic-related comorbidities (such as HBV and HCV, renal dysfunction, hepatocellular carcinomas, sepsis, or multiorgan failure). An excessive intake of alcohol was defined as a daily consumption of more than 3 standard drinks for men and 2 for women (a standard drink corresponded with roughly 14 g of pure alcohol). A total of 65 healthy controls (HC) were recruited from the Tadeusz Dorobisz Regional Center for Blood Donation and Haemotherapy. The inclusion criteria for the controls were a lack of a significant medical history and the standard criteria for blood donation. All study participants gave their written consent; the study followed the guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Wroclaw Medical University (numbers KN-713/2020, KB-187/2019).

2.2. Sample Handling

Whole blood samples were drawn into a vacuum blood collection tube containing EDTA. The collected samples were centrifuged. The supernatant was then aliquoted, frozen, and stored at −80 °C until the analysis.

2.3. Determination of AGE10 Content in the EDTA Plasma

The AGE10 content was determined using a competitive enzyme-linked immunosorbent assay (ELISA). First, 96-well MaxiSorp plates (Nunc®, Sigma-Aldrich, Darmstadt, Germany) were coated with synthetic high molecular mass MAGEs (HMW-MAGEs) for 5 h at room temperature. The plates were washed three times using phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBST). The plates were then blocked overnight at 4 °C with 10% skimmed milk. The plasma samples were thawed at room temperature and 50 µL of each sample was taken into an Eppendorf safe-lock test tube, diluted twice with PBS, and incubated for 45 min with 150 µL of non-commercial monoclonal IgE anti-MAGE antibodies. Concurrently, the standard of serially diluted low-molecular mass MAGEs (LMW-MAGEs) was prepared with an antibody incubation step analogous to the plasma samples. A total of 100 µL of the antibody sample solution was then transferred to coated plates and incubated for two hours. The plates were washed three times with PBST. A solution of horseradish peroxidase-conjugated rabbit IgE (Acris Antibodies GmbH, Herford, Germany) diluted

1:7000 in PBS was added and the plates were incubated at room temperature for 2 h. After three washes with PBST, an o-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich, Darmstadt, Germany) substrate was added and the plates were incubated at room temperature for 5 min. The absorbance was read at 450 nm with an Enspire plate reader (Perkin Elmer, Waltham, MA, USA). The AGE10 content of each sample was calculated using the standard curves. The LMW-MAGE, HMW-MAGE, and non-commercial anti-MAGE antibodies were prepared as described previously [24].

2.4. Statistical Analyses

The statistical analyses were performed using R v4.0.3 [26]. All results were represented as means with corresponding standard deviations (SD). The statistical significance was determined at a p-value under 0.05. As the Shapiro–Wilk test revealed a significant deviation of AGE10 concentrations from the normal distribution, we used non-parametric statistical tests. Group-wise comparisons were performed using the Mann–Whitney U-test. Correlations between the age, ALT, AST, GGTP, and bilirubin and the AGE10 concentration were assessed using Kendall′s rank correlation. To examine the diagnostic accuracy of AGE10, the sensitivity and specificity were calculated and a receiver operating characteristic (ROC) plot [27] was constructed. The optimal test cut-off value was determined using Youden′s J statistic [28], which was calculated with the following equation:

Higher J values corresponded with a higher test accuracy for a given cut-off value.

3. Results

3.1. Impact of the Demographic Characteristics of the Study Population on AGE10

Detailed characteristics of the study population are provided in Table 1. The plasma concentration of AGE10 did not differ between sexes (p = 0.54) or across ages (p = 0.29).

Table 1. Characteristics of the study population.

Parameters	HC	AH
Age	52	49.62
Sex (M/F)	44/21	45/20
AST	-	144.67
ALT	-	71.45
Bilirubin	-	8.46
GGTP	-	638.65

3.2. Impact of Alcohol Consumption and Liver Function on AGE10

The mean concentration of AGE10 was significantly higher in the AH group than in the healthy controls (184.5 ± 71.1 µg/mL and 123.5 ± 44.9 µg/mL, respectively; $p < 0.001$, Figure 1). There was no significant correlation between the AGE10 concentration and the markers of the liver function (AST, ALT, bilirubin, and GGTP with respective p-values of 0.53, 0.28, 0.86, and 0.30). The association between AGE10 and the study population characteristics is depicted in Figure 2.

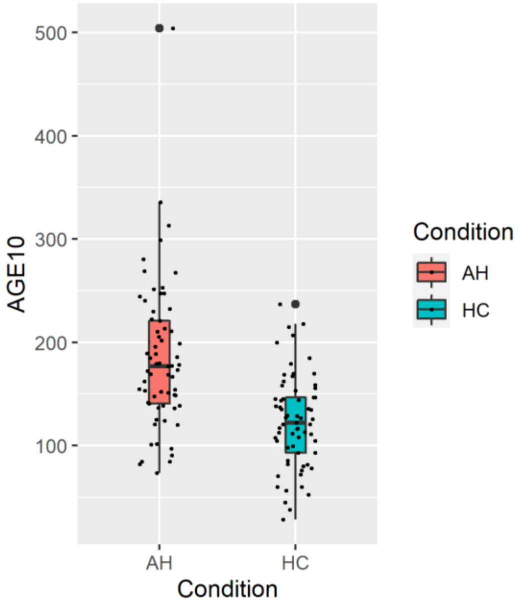

Figure 1. AGE10 concentration (µg/mL) in groups. AH: alcoholic hepatitis; HC: healthy control.

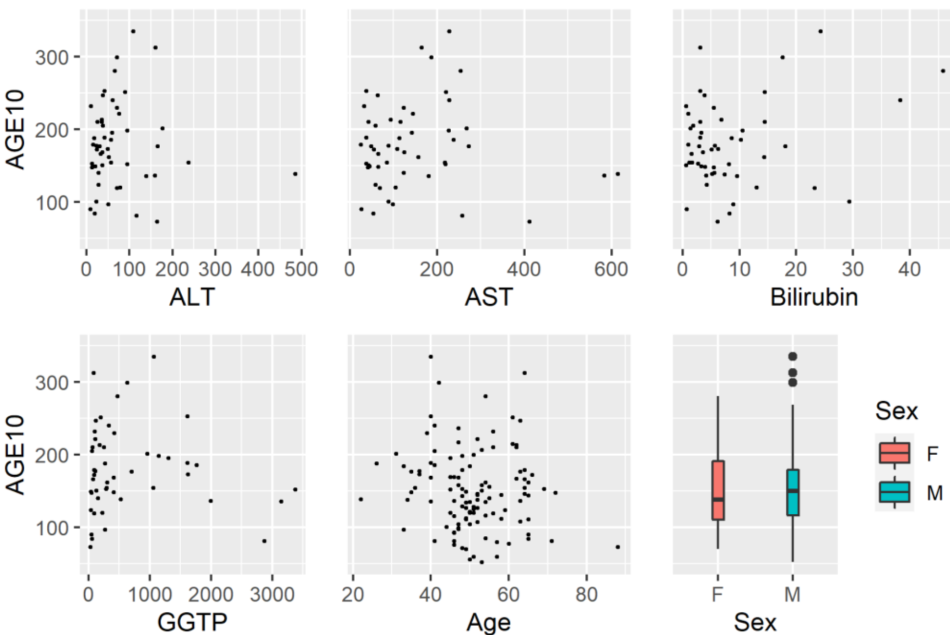

Figure 2. Association between AGE10 and patient characteristics.

3.3. Diagnostic Accuracy of AGE10 in Distinguishing AH from HC

The diagnostic accuracy of AGE10 is presented as a ROC plot in Figure 3. The AGE10 concentration was able to distinguish AH from the healthy controls, with an AUC of 0.78; this is considered to be an acceptable performance for a diagnostic test [29]. The highest Youden's *J* value was obtained at a cut-off value of 147.25 µg/mL, with a corresponding 75% sensitivity and 72% specificity.

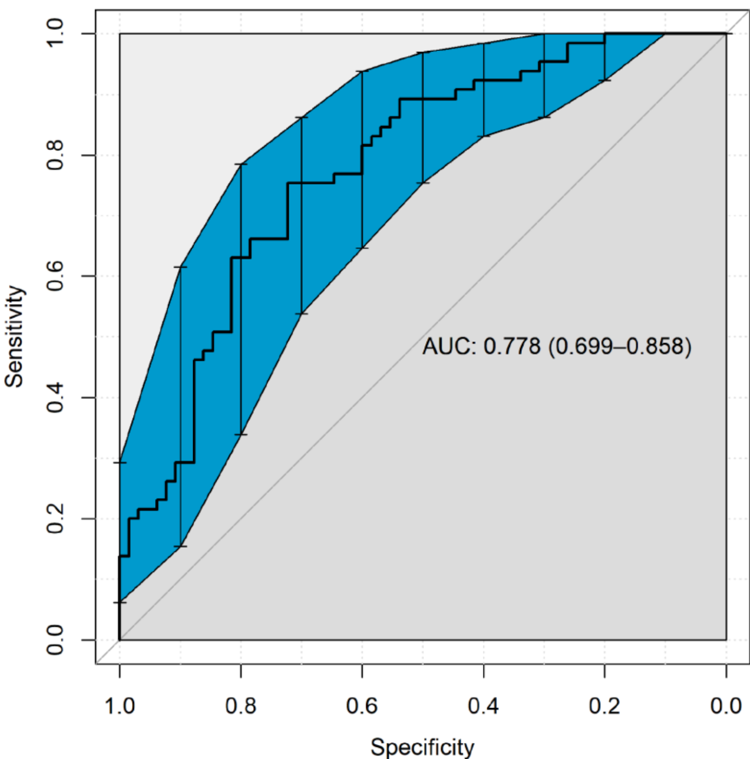

Figure 3. Receiver operating characteristic plot. The area in blue corresponds with 90% confidence intervals.

4. Discussion

The objective of our study was to establish whether a novel subtype of AGEs, a melibiose-derived AGE analog, was associated with AH. We revealed that patients with AH possessed a higher plasma concentration of AGE10 than the healthy controls. In addition, we verified the diagnostic potential of AGE10 and confirmed that it had an acceptable performance in discriminating between AH and HC. Although the presence of AH was associated with significantly increased AGE10 levels, interestingly, there was no significant correlation between the AGE10 concentration and the ALT, AST, bilirubin, and GGTP levels.

Whether AGEs are a cause or a result of a liver injury remains a topic of ongoing discussion. The strongest support for AGEs as a result rather than a cause of a liver injury comes from Butscheid et al. They showed that the early stages of liver disease were not associated with significantly higher levels of CML or imidazolone compared with healthy controls [30]. Świderska et al. reported conflicting results; in their study, both advanced and early liver disease presented with significantly higher serum levels

of AGEs than healthy controls (however, patients with advanced NAFLD had increased AGE levels compared with the early disease) [31]. Considering the existence of established mechanisms by which AGEs exert their harmful effect and the liver is involved in AGE clearance, it is most plausible that liver disease both causes and is caused, at least partially, by AGEs. The mechanism of a potential liver injury caused by AGEs is strongly intertwined with other pathophysiological events associated with ALD (i.e., microbiome alterations and ROS generation). There are two main mechanisms by which endogenous AGEs exert their harmful effects. Their direct action causes the impairment of proteins through cross-linking [32,33]. The other mechanism is an interaction between AGEs and their receptor (RAGE). The binding of AGEs to RAGE leads to the activation of multiple pathways associated with a liver injury, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK)/c-Jun N-terminal protein kinase (JNK) pathways [32,33]. The involvement of NF-κB in liver injuries stems most likely from its proinflammatory effect [34]. The pathological activation of JNK leads to an increased production of ROS (which, through several downstream pathways, causes the subsequent and further activation of JNK, leading to a self-sustaining amplification loop) [35].

AGEs are provided to the body from both endogenous and exogenous sources. The major exogenous source is the consumption of a highly processed, Western diet and soft drinks high in fructose corn syrup [36]. The most important endogenous source of AGEs in patients abusing alcohol is alcoholysis in the liver, which results in the accumulation of acetaldehyde and the subsequent generation of acetaldehyde-derived AGEs [11]. However, the exact source of AGE10 remains a mystery. It remains to be elucidated whether MAGEs/AGE10 are present in the food and if they can be absorbed from the intestines. As for endogenous generation, the source of melibiose for MAGE/AGE10 generation is also not clear. There are two possible sources for supplying melibiose for an endogenous MAGE/AGE10 synthesis. Melibiose could be provided with food (such as honey [37] or a plant-based diet [38]) and then readily absorbed through paracellular junctions [39]. Another source for melibiose is fermentation by several bacterial genera present in the gut microbiome such as *Bifidobacterium*, *Lactobacillus*, or *Lactococcus* [40–42]. As AH is associated with increased relative abundances of *Lactobacillus* and *Bifidobacterium* [10], we considered microbial fermentation, and not supplementation by diet, to be the most plausible source of melibiose and, subsequently, a potential explanation for the increased AGE10 concentration in AH.

The role of exogenous (I.e., dietary) AGEs remains an important area of study. The absorption rate of AGEs depends on their chemical characteristics and varies between 10 and 30% [13]. The AGE content in food depends both on the characteristics of the raw product and on the way in which it was prepared. Generally, the AGE content is highest in animal-derived, fatty, and protein-rich foods. The amount of AGEs in a single, standard serving varies from values as low as 20 kU (in certain soy-derived products) to exceeding 10,000 kU (in highly processed meats) [43]. As up to 30% of dietary AGEs are absorbed, and as the AGE content in several foods is extremely high, the exogenous supply of AGEs is typically higher than the endogenous production. This led us to the conclusion that dietetic intervention is a valid approach for reducing plasma AGE levels. Although the impact of a low AGE diet on liver disease has not yet been evaluated in human subjects, there is indirect evidence supporting its protective effect. A recent meta-analysis confirmed that a low AGE diet resulted in a significant reduction of insulin resistance, fasting glucose, total cholesterol, and LDL [44]. In addition, a high AGE diet aggravated liver injuries in a murine model [45]. Several practical guidelines have already been proposed to reduce the intake of dietary AGEs. Uribarri et al. provided a convenient database describing the AGE (CML) content of 549 different types of food [43]. Apart from avoiding food with an inherently high AGE content, simple changes in food preparation might produce a staggering reduction in AGEs. Examples include marinating food in vinegar (which reduces the formation of AGEs through an increase in the pH), avoiding frying, and a reduction of the cooking time and temperature [46,47].

The pathophysiological impact of dietary AGEs is not limited to their contribution to the blood AGE pool. Dongen et al. showed that dietary AGEs induced changes in the mice microbiome [48]. Multiple studies have confirmed that ALD is associated with alterations in the gut microbiome [10]. The benefits of a fecal microbiota transplant (FMT) in ALD were confirmed in a recent placebo-controlled, double-blinded, randomized clinical trial. The FMT group had a significantly lower rate of AUD-related serious adverse effects compared with the placebo [49]. Interestingly, microbiome alterations associated with a high AGE diet and with AH showed an overlap. In both AH and a high AGE diet, melibiose-producing *Bifidobacterium* was enriched [48]. This result suggests the potential involvement of dietary AGEs in microbiome alterations associated with a liver injury. Due to fermentation-dependent melibiose production in the gut microbiome, we hypothesized that the relationship between AGEs and the microbiome might be bidirectional, with AGEs influencing the microbiome and the microbiome providing substrates for further AGE synthesis.

The biomarkers associated with a liver injury can be broadly divided into two groups: the direct markers of a hepatocyte injury and the markers of the immune response. Currently, the most promising non-invasive biomarkers of a liver injury are the M65 and M30 serum cytokeratin-18 epitopes. CK18 is an intermediate filament protein abundantly expressed in multiple cell types, including hepatocytes [50]. The M30 epitope is produced through caspase-mediated cleavage and the M65 epitope includes both caspase-cleaved and full-length CK18 [51]. Consequently, specifically targeting the M30 epitope is a specific marker of early apoptosis, and the M65 epitope is a more general marker of cell death [52]. Both M30 and M65 CK18 have been proposed as potential biomarkers of a liver injury. As hepatocyte death occurs in multiple types of liver disease, CK18 epitopes are not specific for an alcohol-induced injury. Mueller et al. [9] confirmed that although both M30 and M65 were associated with multiple histological parameters related to liver injuries, neither could differentiate patients with ALD from the NAFLD group. A recent, large meta-analysis incorporating 41 studies examining the diagnostic accuracy of M30 and M65 epitopes for the detection of non-alcoholic steatohepatitis and fibrosis revealed that the AUC significantly differed across the studies (from 0.69 to 0.82 for M30 and 0.69 to 0.91 for M65 [53]). Although the majority of the studies on CK18 were performed on patients with non-alcoholic liver disease, there are reports that the diagnostic performance for ALD is similar. With an AUC of 0.78, AGE10 is a promising biomarker for AH. This AUC puts the accuracy of AGE10 on a par with M30 and M65 (with respective AUCs of 0.776 and 0.784 for ALD [9]), the epitopes of cytokeratin-18. Apart from biomarkers that have been focused directly on detecting liver damage, there are multiple studies that have examined the accuracy of detecting factors related to the immune response associated with a liver injury, including soluble CD163 (sCD163), ST2 receptor, and several proinflammatory microRNAs (such as miRNA-192 [54]) [55]. CD163 is a macrophage- and monocyte-specific hemoglobin–haptoglobin scavenger receptor [56]. During inflammation, metalloproteinase cleaves CD163 near the cell membrane, resulting in the release of sCD163 into the circulation [57]. Increased levels of sCD163 have been detected in multiple types of liver injury, including HCV-induced [58], Wilson's disease, and alcoholic hepatitis [59]. The diagnostic accuracy of sCD163 has been seldom reported and the studies mostly focused on examining its potential as a prognostic factor. In a study on acute liver disease with multiple etiologies, sCD163 was shown to predict a fatal outcome, with an AUC ranging from 0.64 to 0.8 [60]. ST2 is a receptor for interleukin-33 (IL-33) and exists in two forms, full-length and soluble (sST2) [61]. Similar to sCD162, sST2 has been studied mainly as a prognostic factor. Higher levels of sST2 are correlated with more severe stages of ALD [61]; however, to our knowledge, none of the studies on the topic have examined the diagnostic accuracy in discriminating the stages of ALD or predicting fatal outcomes. microRNA-192 (miRNA-192) is the second (after miRNA-122) most abundant microRNA expressed in the liver [62]. The expression of miRNA-192 was significantly larger in patients with alcoholic hepatitis compared with healthy controls [54]. In a recent study by Kim et al. [63], the

authors were able to discriminate patients with a fatty liver from a group with steatohepatitis, with and AUC of 0.771. The performance was even better when using panels combining 4 or 8 microRNAs, with respective AUCs of 0.875 and 0.924. Although the preliminary results regarding the use of immunological markers for the diagnosis and prediction of ALD severity are promising, this approach has several shortcomings. The inflammatory response is present in multiple forms of liver injury (including NASH, ALD, or HBV-induced liver injury); hence, detecting higher levels of inflammation-associated biomarkers does not help to determine the underlying etiology. In addition, most of the inflammation-associated biomarkers are not liver-specific; for example, higher levels of sST2 have been detected in multiple highly inflammatory states other than liver disease such as aortic dissections, heart failure, and sepsis [64–66].

Similar to the M30 and M65 epitopes of CK18, the diagnostic use of AGEs in the context of liver disease has been mainly focused on NAFLD [17]. Panels that incorporated a soluble variant of the AGE receptor (sRAGE) along with different types of AGEs performed significantly better than only measuring AGEs (with an AUC ranging from below 0.78 up to 0.85) [67]. The performance of AGEs in differentiating a non-alcoholic fatty liver from non-alcoholic steatohepatitis was acceptable, with an AUC of 0.78 (which was associated with a relatively high specificity of 88.9% and a weak sensitivity of 66.7%) [68]. A similar pattern of a relatively high specificity (84%) and a low sensitivity (70%) was observed in the diagnostic accuracy of discriminating low-grade hepatic steatosis from moderate [31]. Although several authors have confirmed that ALD is associated with an increased concentration of classic subtypes of AGEs (such as CML and GA-AGE), none have provided the diagnostic accuracy for discriminating between ALD and healthy controls or between the different stages of ALD [69–71].

Our study had several limitations. First, the diagnosis was established by clinical criteria and not a biopsy. Although this approach is widely used in both scientific publications and in clinical practice, it is worth noting that a liver biopsy is the method with the highest sensitivity and specificity in the diagnosis and grading of ALD. The use of a biopsy would have allowed us to detect whether the AGE10 concentration significantly correlated with any individual histological features of ALD such as ballooning, necrosis, or fibrosis. However, due to the preliminary nature of our study, the use of an invasive diagnostic method associated with a relatively high risk of serious complications could not be justified. The majority of the AH group included in our study had a high Maddrey score, which further contributed to the increased risk of a biopsy. Although the alcoholic hepatitis group and healthy controls included in our study were age- and sex-matched, there were other potentially important confounding factors that could have influenced our results. As dietary AGEs are generally important contributors to the AGE pool (however, whether that is the case for AGE10 remains to be elucidated), we could not exclude the possibility that potential differences in the dietary habits between the groups also contributed to the different AGE10 concentrations. Another limitation was the lack of a comparison group with other liver disorders. Based on the data from our study, it was impossible to tell whether the AGE10 increase was specific to alcoholic liver disease or if it would be elevated in other types of liver injury such as NAFLD or viral infections. Determining these associations is an important direction for our subsequent studies. In conclusion, our study showed that AH was associated with significantly increased plasma AGE10 levels and that the AGE10 concentration exhibited an acceptable diagnostic accuracy in distinguishing between patients with AH and healthy controls.

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Data Availability Statement: Data supporting the results will be made available by the authors upon reasonable request.

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Article

Microbiome Alterations in Alcohol Use Disorder and Alcoholic Liver Disease

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Abstract: Microbiome alterations are emerging as one of the most important factors that influence the course of alcohol use disorder (AUD). Recent advances in bioinformatics enable more robust and accurate characterization of changes in the composition of the microbiome. In this study, our objective was to provide the most comprehensive and up-to-date evaluation of microbiome alterations associated with AUD and alcoholic liver disease (ALD). To achieve it, we have applied consistent, state of art bioinformatic workflow to raw reads from multiple 16S rRNA sequencing datasets. The study population consisted of 122 patients with AUD, 75 with ALD, 54 with non-alcoholic liver diseases, and 260 healthy controls. We have found several microbiome alterations that were consistent across multiple datasets. The most consistent changes included a significantly lower abundance of multiple butyrate-producing families, including *Ruminococcaceae*, *Lachnospiraceae*, and *Oscillospiraceae* in AUD compared to HC and further reduction of these families in ALD compared with AUD. Other important results include an increase in endotoxin-producing *Proteobacteria* in AUD, with the ALD group having the largest increase. All of these alterations can potentially contribute to increased intestinal permeability and inflammation associated with AUD and ALD.

Keywords: alcohol; gut microbiota; microbiome; liver disease

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1. Introduction

Alcohol use disorder (AUD) is one of the most significant contributors to the global burden of mortality and disease [1]. It is a risk factor for more than 200 diseases, of which more than 40 are fully attributable to alcohol [2]. Of these, alcoholic liver disease (ALD) is the largest contributor to the health harm caused by AUD [1]. ALD is responsible for almost 50% of all deaths attributed to liver diseases in adults [1]. Considering the significant increase in the prevalence of AUD [3] and, in the best case, the moderate effectiveness of currently approved therapies [4], new therapeutic approaches are urgently needed.

ALD is divided into several stages, ranging from relatively benign and reversible hepatic steatosis to severe and irreversible cirrhosis. Hepatic steatosis will affect 90% of heavy drinkers, however, only 10–35% will progress to alcoholic steatohepatitis (ASH) and 8–20% to cirrhosis [5]. Identification of individuals at increased risk of progression to more advanced stages of ALD could potentially help the development of novel and more personalized approaches for the treatment and prevention of ALD by, for example, finding patients who could potentially benefit from early fecal microbiota transplantation from a donor with desired microbiome composition. Although significant advances have been made in recent years, the pathophysiology of ALD development and progression remains largely unknown. Multiple, partially overlapping mechanisms have been proposed as potential causes of liver injury, including advanced glycation end-products [6], oxidative stress [7], and genetic factors [8]. A growing body of evidence shows that

microbiome alterations are another factor that influences the development and course of ALD [9].

Significant technological advances have reduced the cost of sequencing faster than predicted by Moore's law [10]. This has enabled the development of cultivation-independent methods for studying the human microbiome. Of these methods, due to their low cost and high accuracy, 16S rRNA sequencing remains the most widely used. Rapid progress in sequencing technologies is accompanied by significant advances in bioinformatic methods associated with the study of the microbiome. Advances include novel denoising methods, enabling better removal of spurious sequences caused by PCR errors [11], more accurate taxonomic classification methods [12], and more robust methods for differential abundance (DA) testing [13]. Although often overlooked, the impact of the choice of correct bioinformatic methods for microbiome analysis is tremendous; in some cases, frequently used tools for differential abundance testing have been shown to have a false discovery rate (FDR) as high as 70%, meaning that up to 70% of bacteria discovered as significant could be wrong [13]. Analysis of 14 differential abundance testing methods on 38 datasets has shown that the proportion of bacteria discovered as significant strongly depends on the used method; for example, the use of the Wilcoxon test on data normalized as centered log ratio (CLR) resulted in the detection of up to 90% of bacteria as significantly different between studied groups; when using ANCOM-II on the same datasets, on average only 0.8% of bacteria were detected as significant. The authors concluded that ANCOM-II produced the most consistent and conservative results of all the methods studied [14]. The recently developed novel method for differential abundance testing called ANCOM-BC provides further improvement over ANCOM-II, with an additional reduction in FDR while maintaining adequate power [13].

Studies examining the microbiome in AUD used different, and in some cases, suboptimal bioinformatic tools for reads preparation and differential abundance testing, including statistical tests which do not account for the compositional nature of the microbial abundance data, lack of adjustment for multiple testing, and tools with high FDR such as LEfSe. As discussed above, these inconsistencies could potentially influence the obtained results. For this reason, to provide a more accurate and consistent assessment of the microbiome, we have applied a standard workflow consisting of state-of-the-art bioinformatics methods characterized by the best performance in benchmarking studies to datasets that examine the microbiome composition in AUD and ALD. In addition, the inclusion of multiple datasets has enabled us to use dataset identifiers as confounding variable, which reduced variability due to non-bioinformatic technical differences, such as choice of primers and DNA extraction methods.

2. Results

2.1. Diversity Analysis and Variance Contribution

The variance contribution of the disease status differed significantly between the datasets and ranged from less than 1% to 20% (Figure 1). Most studies reported variance contribution of disease status smaller than 10%. In the combined dataset approach, the variance contribution of the batch effect was approximately two times larger than that of disease status.

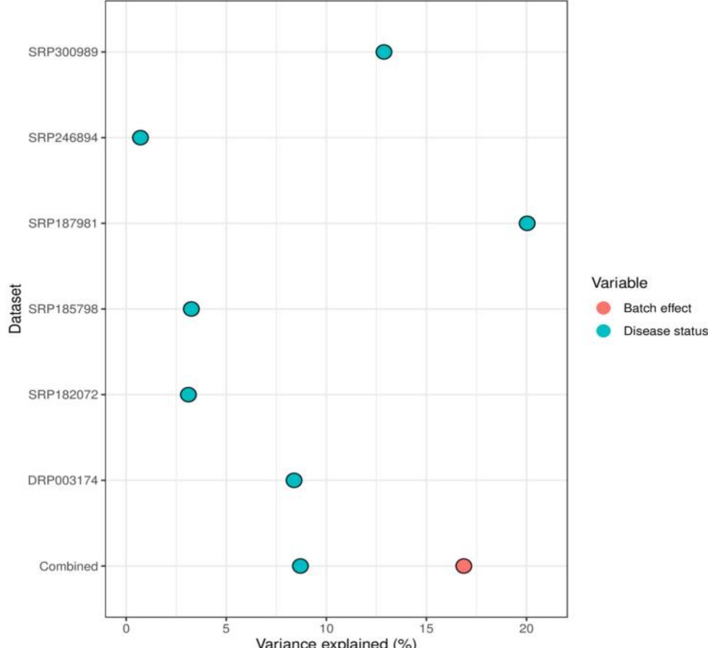

Figure 1. Microbiome variance explained by batch effect (i.e., dataset ID) and disease status (alcohol use disorder vs healthy control).

Alpha diversity did not differ significantly between HC and AUD, both for estimated and observed measures (Figure 2). The rarefication–extrapolation curves for these comparisons are provided in Supplementary Figure S2. Patients with ALD had a lower alpha diversity for all three measures compared with AUD for both estimated and observed measures (Figure 3). The non-alcoholic liver diseases group had higher alpha diversity than the ALD group; however, the species richness differed significantly only for estimated values (Figure 4).

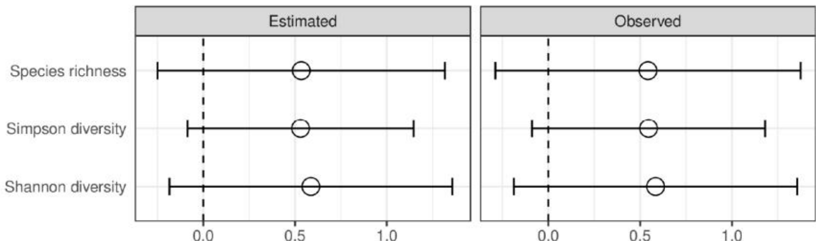

Figure 2. Observed and estimated alpha diversity for comparison between patients with alcohol use disorder and healthy controls denoted as Agresti's generalized odds ratio and summarized with random effects model; values greater than 0 indicate greater richness in healthy controls. An open dot denotes statistically insignificant results. Error bars indicate 95% confidence intervals.

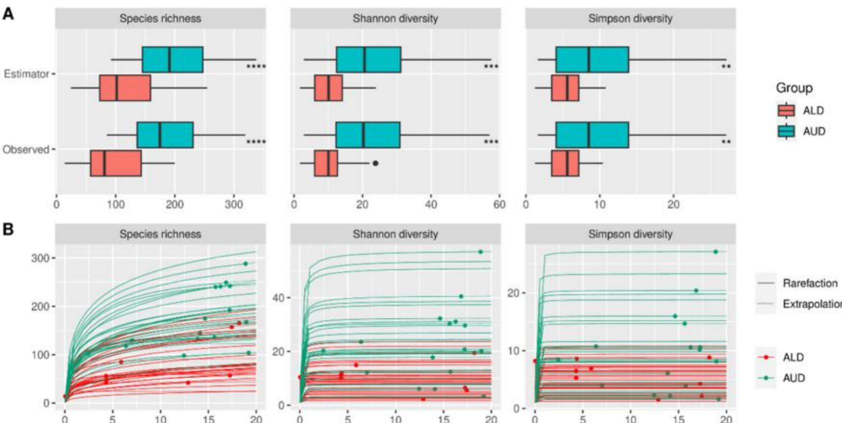

Figure 3. (**A**) Observed and estimated alpha diversity for comparison between patients with alcoholic liver disease and alcohol use disorder (without liver disease). (**B**) Rarefication–extrapolation curves for alpha-diversity measures. Each curve represents the alpha diversity of a single sample. Dots represent observed read depths, which were extrapolated to a common read depth of 20,000. Curves without dots indicate samples that had the read depth already above 20,000 and thus were rarefied to that level; ALD—alcoholic liver disease, AUD—alcohol use disorder, **—$p <=0.01$, ***—$p <=0.001$, ****—$p <= 0.0001$.

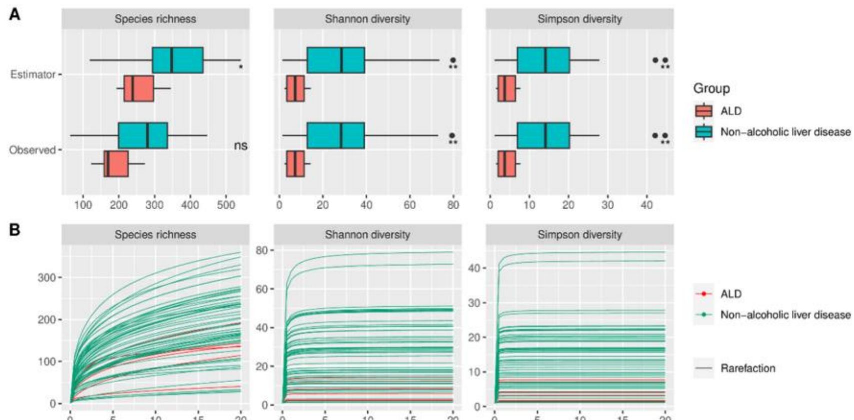

Figure 4. (**A**) Observed and estimated alpha diversity for comparison between patients with alcoholic liver disease and patients with other, non-alcoholic etiologies of liver disease. Black dots denote outliers. (**B**) Rarefication–extrapolation curves for alpha-diversity measures. Each curve represents the alpha diversity of a single sample rarefied to a common read depth of 20,000; ALD—alcoholic liver disease, *—$p <=0.05$, **—$p <=0.01$, ns—non-significant.

In three out of five data sets (DRP003174, SRP187981, and SRP300989), beta diversity was significantly different for the comparison of HC versus AUD (Figure 5). Beta-diversity differed significantly also for the ALD vs. nonalcoholic liver disease and AUD vs ALD comparison (both examined by one dataset each, SRP185798 and SRP187981, respectively).

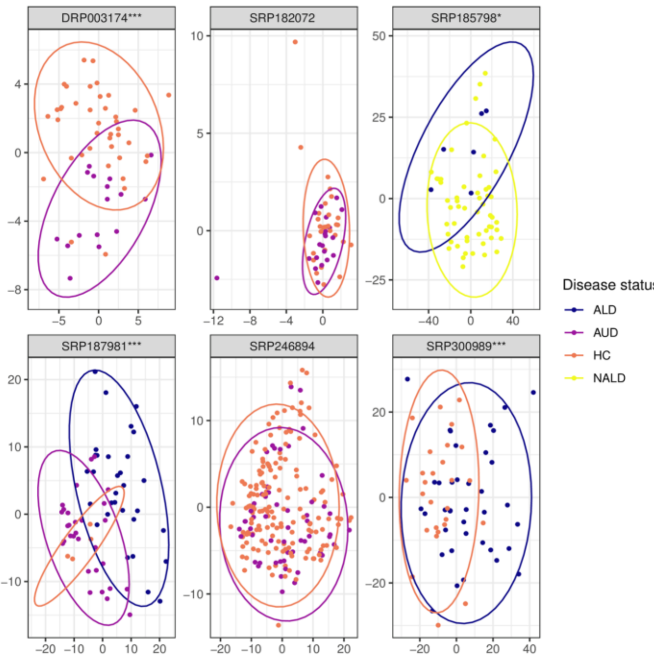

Figure 5. Principal coordinate analysis (PCoA) plots of beta-diversity measured with Euclidean distance after normalization using PhilR. The label above each graph indicates the SRA identifier for the corresponding dataset; ALD—alcoholic liver disease, AUD—alcohol use disorder, HC—healthy controls, NALD—other, non-alcoholic liver disease, *—$p <= 0.05$, ***—$p < = 0.001$.

2.2. Alcohol Consumption Causes Unfavorable Changes in the Fecal Microbiome

The complete results for all comparisons at each of the studied taxonomic levels are presented in Figure 6 and Supplementary Figures S3–S8.

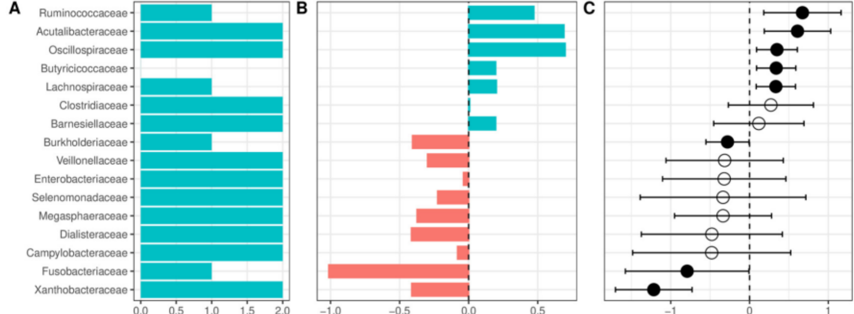

Figure 6. Changes in the relative abundance between patients with alcohol use disorder and healthy controls at the family level. (**A**) Number of datasets in which the given family was detected as significant. (**B**) Change in the relative abundance of a given family in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. (**C**) Change in the relative abundance of a given family after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error bars indicate 95% confidence intervals. Only bacteria

detected as significant in at least two datasets or significant in the random-effects analysis are presented.

2.3. Alcohol Use Disorder versus Healthy Controls

At the phylum level, patients with AUD had a lower relative abundance of *Firmicutes*, *Cyanobacteria*, and a *Verrucomicrobiota*, and a higher abundance of *Proteobacteria* and *Fusobacteria* (Supplementary Figure S3). The decrease in the relative abundance of *Firmicutes* was accompanied by a nonsignificant decrease of the *Firmicutes/Bacteroidetes* ratio (F/B ratio, a frequently used marker of gut health) in most of the datasets. This result remained nonsignificant after pooling with a random-effects model (Supplementary Figure S9). At the class level, patients with AUD were enriched in *Fusobateria*, *Gammaproteobacteria*, and *Negativicutes*, whereas HC had a higher abundance of *Clostridia*, *Verrucomicrobiae*, and *Alphaproteobacteria* (however, several classes had very wide confidence interval crossing 0, Supplementary Figure S4). At the order level, the highest increase of relative abundance in AUD was observed for *Fusobacteriales* and the largest reduction for *Oscillospirales* (Supplementary Figure S5). Patients with AUD compared with HC presented with a reduced relative abundance of *Acutalibacteraceae* and several families containing short-chain fatty acid (SCFA) producing bacteria (*Ruminococcaceae*, *Oscillospiraceae*, *Butyricicoccaceae*, *Lachnospiraceae*, *Clostridiaceae*, and *Barnesiellaceae*, Figure 6). Patients with AUD were enriched in *Enterobacteriaceae*; however, confidence intervals were wide and crossed 0. Among the more abundant families in AUD patients, only *Burkholderiaceae*, *Fusobacteriaceae*, and *Xanthobacteraceae* had confidence intervals that did not cross 0 and were detected as significant in the random effects model. Changes at the family level were partially repeated at the genus level (Supplementary Figure S6), and several genera from *Lachnospiraceae* (*Scatomonas*, *Fusicatenibacter*, *Roseburia*, *Choladousia*, *Dorea_A*, *Lachnoclostridium*, *Roseburia*, *Eubacterium*), *Ruminococcaceae* (*Bittarella*), and *Butyricicoccaceae* (*Agathobaculum*) were decreased in AUD, and two genera from *Burkholderiaceae* (*Duodenibacillus*, *Parasutterella*) were increased.

2.4. Alcoholic Liver Disease versus Alcohol Use Disorder without Liver Disease

Patients with ALD showed a further reduction in *Firmicutes* and *Cyanobacteria*, and an increase of *Proteobacteria* compared to AUD without liver disease; other changes included a reduction of *Methanobacteriota* and a very slight increase in *Campylobacterota* (Supplementary Figure S7a). The F/B ratio did not differ significantly between ALD and AUD without liver disease (Supplementary Figure S10). Patients with ALD compared with AUD showed further enrichment of *Campylobacteriota*, an increase of *Gammaproteobacteria*, and a reduction of *Clostridia* and *Methanobacteria* (Supplementary Figure S7b). The most notable changes at the order level were a reduction of *Oscillospirales* and an increase of *Enterobacterales* in patients with alcoholic liver disease (Supplementary Figure S7c). ALD was associated with further reduction in *Acutalibacteraceae* and SCFA-producing families compared with AUD. Nine families were detected as more abundant in ALD, with the largest increase in *Enterobacteriaceae*, *Veillonellaceae*, *Neisseriaceae*, and *Campylobacteraceae* (Supplementary Figure S7d). Changes at the genus level included further reduction of genera from *Lachnospiraceae* (*Mediterraneibacter*, *Dorea*, *Scatomonas*, *Blautia_A*), *Ruminococcaceae* (*Ruminococcus_C*), and *Butyricicoccaceae* (*Agathobaculum*) families and an increase in several, potentially pathogenic genera such as *Streptococcus* in the ALD group (Supplementary Figure S7e).

2.5. Alcohol liver Disease versus Other, Non-Alcoholic Causes of Liver Diseases

Compared with other causes of liver disease, ALD was characterized by lower relative abundances of *Verrucomicrobiota*, and a higher abundance of *Campylobacterota* and *Patescibacteria* (Supplementary Figure S8a). Interestingly, compared with other causes of liver disease, ALD was characterized by a lower abundance of *Clostridia*, an increase of *Bacilli*, and slight alterations of *Verrucomicrobiae*, *Campylobacteria*, and *Saccharimonadia*

(Supplementary Figure S8b). At the order level, ALD was associated with the highest reduction of *Lachnospirales* and the highest increase in *Lactobacillales* (Supplementary Figure S8c). Compared to other causes of liver disease, ALD was characterized by a lower abundance of *Oscillospiraceae*, *Lachnospiraceae*, and *Butyricicoccaceae* (and multiple other families, Supplementary Figure S8d) and a higher abundance in 10 families, with *Lactobacillaceae* having the largest increase. Consistently, patients with ALD were enriched in the *Lactobacillus* genus compared with other causes of liver disease (Supplementary Figure S8e).

2.6. AUD and Liver Disease Cause Alterations in Predicted Functional Metagenomic Profiles

Multiple pathways enriched in AUD patients were related to heme synthesis. In addition, several aerobic pathways and pathways related to citrate (TCA) and glyoxylate cycle (Figure 7) were enriched in AUD. Patients with ALD showed further enrichment of pathways related to TCA and glyoxylate cycles and biosynthesis of heme. Additionally, among pathways with the largest increase in ALD was the LPS-related super pathway of (Kdo)2–lipid A biosynthesis; among other changes, the most consistent was the enrichment of multiple pathways related to menaquinol biosynthesis (Supplementary Figure S11). Recently, we have shown that alcoholic liver disease is associated with an increase in plasma concentration of a novel advanced glycation end product (AGE10). Synthetically obtained melibiose-derived AGE mimics this AGE10 epitope. We hypothesized that microbiome alterations could be associated with increased availability of melibiose in the gut. Melibiose can be delivered to the human organism from a plant diet or provided by gut microbiota. In dysbiosis, melibiose could be translocated to circulation thus contributing to AGE10 increase, whereas in healthy conditions, melibiose is hydrolyzed with microbial α-galactosidase. For this reason, we performed additional analysis of PICRUST2 data with the Kyoto Encyclopedia of Genes and Genomes as a reference database, targeting α-galactosidase (KEGG identifier K07407), which catalyzes the reaction of melibiose hydrolysis. The abundance of α-galactosidase did not differ significantly between healthy control, patients with AUD, and patients with ALD.

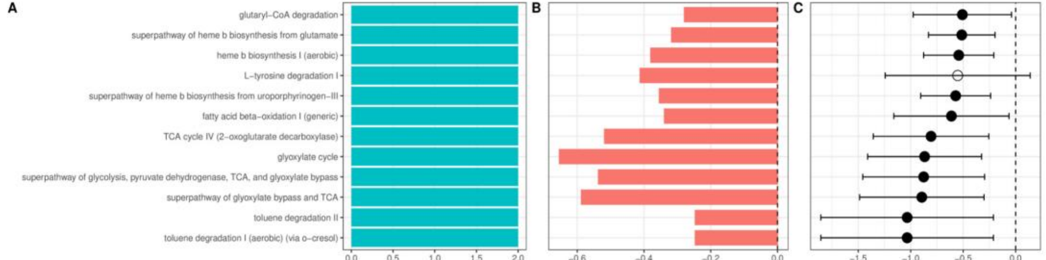

Figure 7. Changes in the relative abundance of the inferred metagenomic pathways between patients with alcohol use disorder and healthy controls a. Number of datasets in which given pathway was detected as significant. b. Change in the relative abundance of a given pathway in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. c. Change in the relative abundance of a given pathway after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error bars indicate 95% confidence intervals. Only pathways detected as significant in at least two datasets or significant in random-effects analysis are presented.

2.7. Diagnostic Accuracy of Deep Learning in Predicting the Disease Status

The metrics related to the diagnostic accuracy of deep learning are provided in Figure 8. Although the diagnostic accuracy for the combined dataset had an acceptable

performance with 0.71 AUC, when applied to individual datasets we observed significant variance, with values of AUC ranging from 0.92 to only 0.02. The F1 score ranged from 0.37 to 0.81. The lowest AUC and F1-score were obtained for dataset SRP187981. To reduce overfitting, we have increased the lambda value of L2 regularization; however, it did not reduce variance across datasets and led to a further decrease of all metrics for the SRP187981 dataset. We hypothesized that weak performance on this dataset might be associated with significant class imbalance (i.e., a much larger number of AUD samples compared with HC). To combat this effect we performed random oversampling, which resulted in a significant increase of AUC to 0.25 (Supplementary Figure S12).

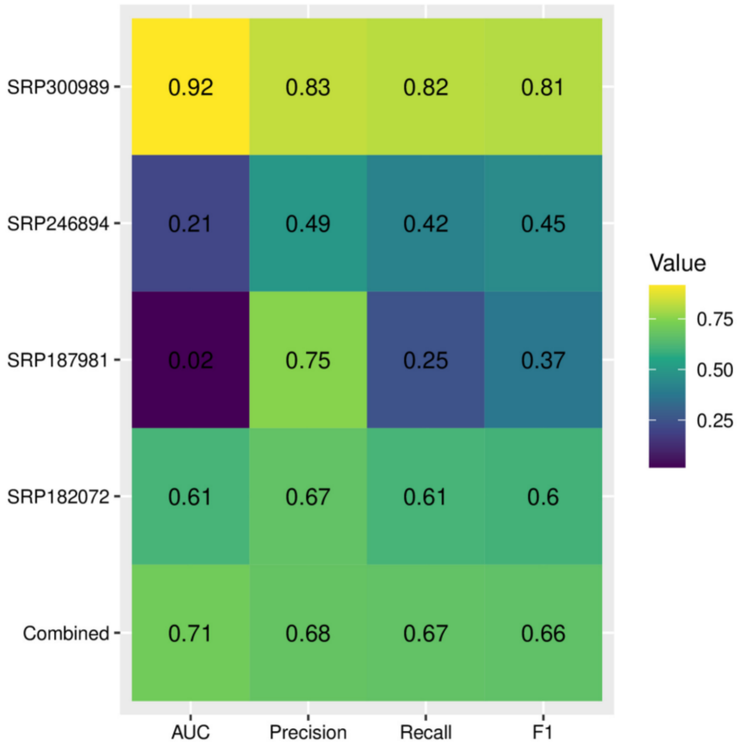

Figure 8. Accuracy of the deep learning model for microbiome-based differentiation between alcohol use disorder and healthy controls; AUC—area under the curve, F1—F1-score, harmonic mean of precision and recall. Larger values indicate better performance.

3. Discussion

The last decade has witnessed an exponential increase in studies that examine the microbiome in a wide range of diseases. During that time, several reports on microbiome alterations in AUD and ALD have been published [15–20]. The incorporation of multiple datasets in an analysis enables a more robust estimation of the dysbiosis patterns associated with AUD. However, differences in the methodologies used and the significant batch effect between studies make a direct comparison of the results unreliable. The use of raw reads from different datasets has allowed us to apply a consistent bioinformatical pipeline that reduced the variance associated with the choice of statistical tools. In addition, it allowed the estimation of the batch effect and then correcting for it. The batch effect in our study was a greater contributor to variance than the effect of disease status, a result

consistent with previous reports that examined multiple microbial datasets [21]. The use of statistical methods that account for it resulted in finding consistent patterns of microbiome alterations associated with AUD and ALD.

Alpha-diversity analysis showed significant differences between patients with AUD, ALD, and other (nonalcoholic) liver diseases. No significant changes were detected between the AUD and HC groups. However, rarefication–extrapolation curves revealed that in most of the included studies, sequencing depth was not sufficient to capture the true diversity. Although the use of Hill's numbers with asymptotic extrapolation provides more robust results than frequently used rarefication to common sequencing depth [22], it is still an estimation that could potentially differ from the true underlying diversity. For most of the datasets, beta diversity was significant, with the principal coordinate analysis (PCoA) plots showing some level of separation between the HC, AUD, and ALD groups.

Deep learning analysis has shown a strong variance in accuracy across datasets, even with increased L2-regularization (which is one of the most commonly used methods for the reduction of overfitting in neural networks). This shows that the good performance of deep learning models for microbiome-based predictions on individual datasets does not necessarily translate across datasets, confirming the common knowledge that training deep learning models requires diverse datasets. In the case of microbiome-based predictions, where the batch effect is often larger than the effect of disease status, this is especially crucial.

Our results show alterations in the microbiome that are consistent across multiple datasets. Patients with AUD were characterized by a reduction in *Firmicutes*, which was mostly attributed to a reduction in the *Clostridia* class. At the family level, this reduction can be explained by the lower abundance of *Ruminococcaceae*, *Lachnospiraceae*, *Oscillospiraceae*, and *Butyricicoccaceae* which contain multiple butyrate-producing bacteria. Patients with ALD showed a further reduction in most of these families compared to those with AUD without liver disease. The role of butyrate in maintaining gut health is multidirectional and is maintained through multiple mechanisms. Butyrate is the main source of energy for colonocytes [23]. Butyrate beta-oxidation induces physiological hypoxia in the colon [24]. Reduction of butyrate-producing bacteria, resulting in smaller availability of substrate for beta-oxidation leads to greater availability of oxygen, making the colonic environment more favorable to facultative anaerobes. This is reflected in our results: we have shown a significant increase in facultative anaerobes (such as the Enterobacteriaceae family) and a significant increase in multiple aerobic pathways in inferred metagenomic analysis. Due to the preferential use of butyrate as an energy source, a relatively small portion (about 5%) is absorbed into circulation [25]. However, even in small quantities, butyrate appears to have a strong anti-inflammatory effect on the host. This effect is achieved through multiple pathways, including interactions with G-protein coupled receptors (most notably GPR41 and GPR43 [26]), activation of nuclear factor kappaB (NF-kB), activation of PPAR-gamma, and inhibition of IFN-gamma signaling [27]. An additional mechanism through which the reduction of *Ruminococcaceae*, *Lachnospiraceae*, and other butyrate-producing bacteria could influence the course of AUD and its complications is through their effect on the intestinal barrier. Butyrate has been shown to have a protective effect on alcohol-induced intestinal barrier impairment, leading to a reduction in intestinal permeability [28]. The increase in intestinal permeability associated with the lower availability of butyrate allows increased endotoxin translocation, leading to sustained systemic inflammation [29]. Patients with AUD with and without liver disease have increased intestinal permeability; however, the increase is larger in patients with ALD [30]. This is consistent with our results, where patients with AUD had a smaller relative abundance, compared to HC, of the *Ruminococcaceae* family (which includes multiple intestinal barrier-protective bacteria), and patients with ALD showed further reduction of this family compared to AUD without liver disease. Patients with ALD are characterized by increased circulating levels of LPS, and the level of endotoxin correlates with the severity of liver injury [31]. Our results indicate that AUD is associated with a significantly

higher abundance of *Proteobacteria* and that this increase is larger for patients with ALD. The increase in *Proteobacteria* is mainly attributed to the higher abundance of *Gammaproteobacteria*. The increase in this class is significantly correlated with higher serum levels of LPS [32]. Furthermore, the immunogenicity of LPS derived from *Proteobacteria* is significantly stronger compared to bacteria from other phyla [33]. Increased intestinal permeability combined with a greater abundance of highly immunogenic *Proteobacteria*-derived LPS are important contributors to systemic inflammation associated with AUD and ALD. At the family level, the most notable increase in bacteria from the *Proteobacteria* phylum was observed in *Enterobacteriaceae* and *Burkholderiaceae. Enterobacteriaceae* play an important role in the course of liver diseases. In patients with hepatic encephalopathy, and they have been shown to be associated with systemic inflammation and worsening of cognition [34]. Furthermore, *Enterobacteriaceae* are responsible for most cases of spontaneous bacterial peritonitis, which is the most common infection in patients with liver cirrhosis [35]. *Burkholderiaceae* further increases ethanol-associated inflammation. It is significantly correlated with IFN-gamma-inducible protein 10 (IP-10, sometimes called CXCL10), which exacerbated the inflammatory response in the murine model of ALD [36]. *Fusobacteria*, the phylum with the largest increase in AUD compared to HC in our study, further contributes to inflammation. It has been shown to be significantly correlated with higher levels of pro-inflammatory cytokines IL-2 and IL-13 [34]. In light of a report showing that supplementation with *Lactobacillus rhamnosus GG* ameliorates liver injury in a murine model [37], the increase in *Lactobacilli* in ALD compared to other causes of liver disease might appear paradoxical. However, a previous shotgun metagenomic study of ALD has shown that the increase in *Lactobacillus* was mostly attributed to oral species (such as *Lactobacillus salivarius*) and did not include *Lactobacillus rhamnosus*. We provide two potential mechanisms for the higher abundance of *Lactobacillus* in ALD compared to other causes of liver injury. First, it could be attributed to alcohol-induced disturbances in bile acids metabolism. Feces of patients with ALD had a lower concentration of deoxycholic acid (DCA) [38], which exhibits strong antimicrobial properties. Reduction in a DCA could result in a colonic environment more favorable for bacteria typically present in the oral microbiome (including *Lactobacillus*) [39]. Second, the higher abundance of *Lactobacillus* could be attributed to its metabolic abilities, namely, the ability to metabolize ethanol [40]. Inferred metagenomic analysis revealed some interesting alterations in AUD. The glyoxylate cycle enables the use of ethanol as a source of acetyl coenzyme A [41]. Enrichment of this pathway could be one of the adaptive strategies employed by bacteria more abundant in the AUD. Another interesting insight is provided by the enrichment of multiple pathways associated with the synthesis of heme. Dietary heme has been shown to alter the composition of the microbiome and increase intestinal inflammation [42]. Whether microbiome-derived heme has similar biological effects remains to be elucidated.

Our study has an important limitation. Although we have provided a comprehensive examination of the microbiome changes associated with AUD and ALD, the exploratory nature of our study means that we cannot establish causality based on our findings. To fully understand the associations identified in our study, it is crucial to conduct further, mechanistic studies that aim to establish causality.

4. Materials and Methods

4.1. Characteristics of the Included Datasets and Overview of the Pipeline

We have included publicly available datasets from NCBI's Sequence Read Archive (SRA) or European Nucleotide Archive (ENA), which provided raw 16S rRNA sequencing data and corresponding metadata for the following groups: (1) patients with AUD and healthy controls (HC), (2) patients with AUD without liver disease and patients with ALD, (3) patients with ALD and liver disease of other etiology. The characteristics of the included studies are presented in Table 1. The overview of the pipeline is illustrated in Supplementary Figure S1. Datasets providing samples for patients with AUD and HC were

analyzed using two approaches: the combined dataset approach (where data from all qualifying studies was pooled for downstream quality control and statistical analysis) and the individual dataset approach (where each study was analyzed separately). Since only one dataset was available for both comparison of ALD with other causes of liver disease and with AUD (with respective accession IDs SRP185798 and SRP187981), they were analyzed using an individual study approach only. Statistical analysis was performed using R, version 4.1 [43].

Table 1. Characteristics of the included datasets.

Dataset	Region	Platform	Primers	Country	Samples
DRP003174 [15]	V1–V2	454 GS FLX Titatnium and Junior (Roche Applied Science, Penzberg, Upper Bavaria, Germany)	27F/338R	Japan	AUD *n* = 16, HC *n* = 40
SRP182072 [16]	V3–V4	Illumina HiSeq (Illumina, USA)	342F/806R	Norway	AUD *n* = 21, HC *n* = 30
SRP185798 [17]	V3–V4	Illumina MiSeq and HiSeq (Illumina, USA)	341F/805R	USA	ALD *n* = 6, NALD *n* = 54
SRP187981 [18]	V4	Illumina MiSeq (Illumina, USA)	515F/806R	USA, Mexico, Europe	ALD *n* = 31, AUD *n* = 30, HC *n* = 4
SRP246894 [19]	V4	Illumina MiSeq (Illumina, USA)	515F/806R	USA	AUD *n* = 55, HC *n* = 159
SRP300989 [20]	V3–V4	Illumina MiSeq (Illumina, USA)	338F/806R	China	ALD *n* = 38, HC *n* = 27

4.2. Data Preparation

The entire workflow was run using Snakemake version 7.14.0 [44]. Primers were removed using Cutadapt [45] with a minimum read length set to 30 and other parameters set to default (i.e., a maximum error rate of 0.1). The reads were then merged (with a maximum number of mismatches set to 1 and minimum % identity of alignment set to 80), truncated to 250 bases, and quality filtered (with a maximum expected error of 1.0). Obtained sequences were denoised using the UNOISE3 algorithm implemented in USEARCH v11.0.667 [11], [46], resulting in the generation of zero-radius operational taxonomic units (zOTUs). zOTUs are generally equivalent to OTUs (however, with some notable differences, e.g., in contrast to normal OTUs, zOTUs undergo denoising) with a 100% identity threshold, that is, each zOTU represents one correct biological sequence. The use of a 100% identity threshold is the optimal approach for the data from the V4 hypervariable region sequencing [47]. Taxonomy was determined using DECIPHER IDTAXA [12], with GTDB version 07-RS207 as a reference database [48]. The obtained zOTU table, corresponding metadata, and taxonomy have been imported into the Phyloseq object for downstream analysis [49]. Functional abundance prediction was performed using PICRUSt 2 [50] with the Metacyc database as a reference. The plots were generated using ggplot2 version 3.3.6 [51].

4.3. Variance Contribution and Beta-Diversity

Before calculating variance contribution and beta diversity, the zOTU tables were normalized using PhilR [52]. The beta diversity was then assessed using Euclidean distance. The use of PhilR with Euclidean distance has several advantages over commonly used methods for asserting beta diversity. It incorporates phylogenetic information, but contrary to other phylogenetically-aware methods (such as UniFrac), it accounts for the compositional nature of microbiome datasets, which is crucial for the reduction of spurious results [53]. The statistical significance of beta diversity was determined using

PERMANOVA with 999 permutations. The variance contribution was calculated using redundancy analysis (RDA, implemented in the Vegan package version 2.6–2 [54]). The disease status (both for the individual and combined dataset approach) and the SRA identifier (only for the combined approach) were used as constraining variables.

4.4. Alpha Diversity

To account for differences in the read depth between samples (which influences alpha-diversity estimates [55]), samples were normalized using asymptotic extrapolation. In contrast to ordinary rarefication to a given read length, the asymptotic extrapolation does not require throwing away valid reads and, as a result, enables a more accurate estimation of alpha diversity [22]. Alpha diversity was measured using Hill numbers with q values equal to 0 (species richness), 1 (Hill–Shannon diversity), and 2 (Hill–Simpson diversity). The use of Hill numbers solves several problems associated with traditional alpha diversity measures such as Shannon or Simpson diversity; it offers conceptually simpler interpretation (e.g., reduction of 1/3 of the species in a community results in a 1/3 reduction of Hill diversity; in contrast for both ordinary Shannon and Simpson indices, the reduction is much smaller than anticipated). For a more comprehensive description of asymptotic estimation and Hill numbers, we refer the reader to the seminal paper by Chao et al. [22]. Both asymptotic estimation and Hill number calculation were performed using the iNEXT package [56].

4.5. Differential Abundance Testing

Differential abundance testing was performed using ANCOM-BC [13]. ANCOM-BC is one of the compositionally aware methods for DA testing which accounts for uneven sampling using a novel method of bias correction. It has been shown to significantly reduce FDR compared to other approaches while maintaining adequate statistical power [13,57]. A comprehensive discussion of the statistical properties and assumptions underlying ANCOM-BC can be found in the manuscript by Lin et al. [13]. Differential abundance testing was performed at phylum, class, order, family, and genus levels. Since sequencing of individual subregions of 16S rRNA (e.g., V4) does not achieve the taxonomic resolution required for accurate classification of species [58], differential abundance testing at this level was not performed.

4.6. Combined Dataset Analysis

In the combined approach, all datasets that provided samples for patients with AUD and HC were analyzed as one dataset, with a pipeline analogous to the individual study approach. To obtain globally aligned reads (i.e., starting and ending at the same position of 16S rRNA), we have used 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) primers. The dataset DRP003174 was excluded from the combined approach due to sequencing of the V1–V2 region, which could not be globally aligned with V3–V4. The significance testing was performed analogously to the individual study approach (with one notable difference of using SRA ID as a concomitant variable in ANCOM-BC). Alpha-diversity and CLR-transformed microbial abundances from the individual study approach were transformed to Agresti's generalized odds ratios using the genodds package [59] and summarized with the random-effects model using the meta package [60].

4.7. Deep Learning Analysis

We used the PopPhy-CNN convolutional neural network for taxonomy-based prediction of disease status (AUD versus HC) [61]. The model was trained using default settings (L2Lambda = 0.001). Due to the high variance across datasets, we re-trained the model with increased lambda values (0.003 and 0.01) to strengthen the L2-regularization. L2-regularization reduces the weights features by adding the sum of squares of feature

weights to the loss function. Higher values of lambda result in bigger punishment of large feature weights. Due to a significant class imbalance in one of the datasets, we performed random oversampling (ROS). ROS is a technique that combats class imbalance by multiplying randomly chosen samples from a minority class in the training set. The metrics used for the evaluation of the model were precision, recall, an area under the curve (AUC), and F1-score (harmonic mean of precision and recall).

5. Conclusions

In conclusion, we have shown that the batch effect is one of the largest contributors to variance across different datasets that examine AUD. The most consistent changes in the microbiome in AUD were related to a reduction in SCFA-producing bacteria and an increase in bacteria associated with inflammation. Inferred metagenomic analysis showed that ALD is associated with an increase in multiple pathways related to bacterial heme synthesis, which so far has not been studied in the context of alcoholic liver disease. Deep learning analysis has shown significant variance in the microbiome-based prediction of AUD. Our findings confirm that AUD is associated with negative microbiome alterations, which could be mechanistically linked to liver injury.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24032461/s1.

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

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Supplementary file

Title: Microbiome alterations in alcohol use disorder and alcoholic liver disease

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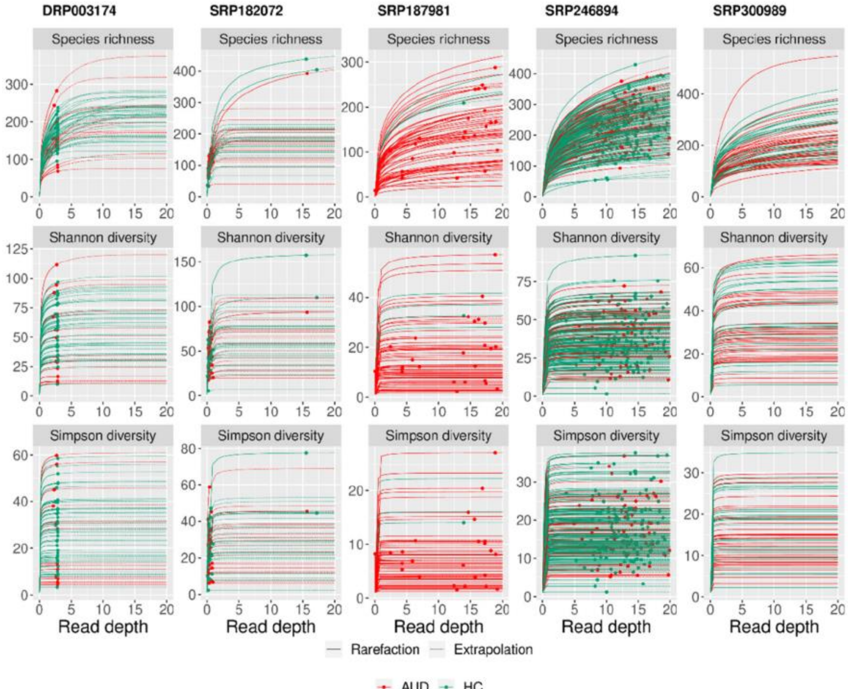
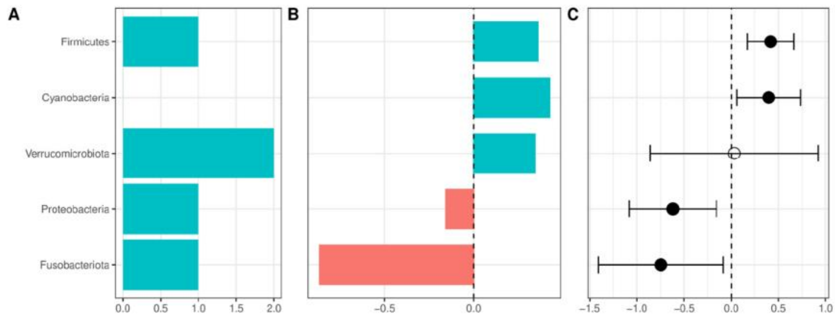
Supplementary Figure S1. Simplified overview of the analytical pipeline

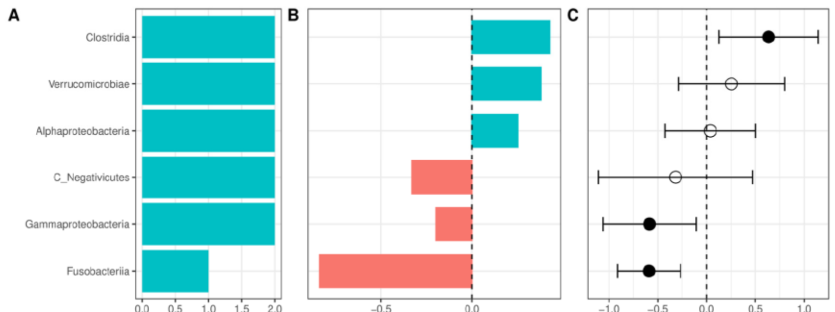


Supplementary Figure S2. Rarefication-extrapolation curves for alpha-diversity analyses

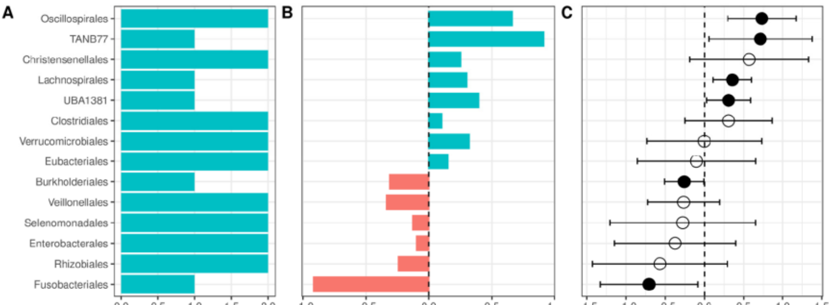


Supplementary Figure S3. Changes in the relative abundance between patients with alcohol use disorder and healthy controls at the phylum level. a. Number of datasets in which the given phylum was detected as significant. b) Change in the relative abundance of a given phylum in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. c. Change in the relative abundance of a given phylum after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote

greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error bars indicate 95% confidence intervals. Only bacteria detected as significant in at least two datasets or significant in random-effects analysis are presented.

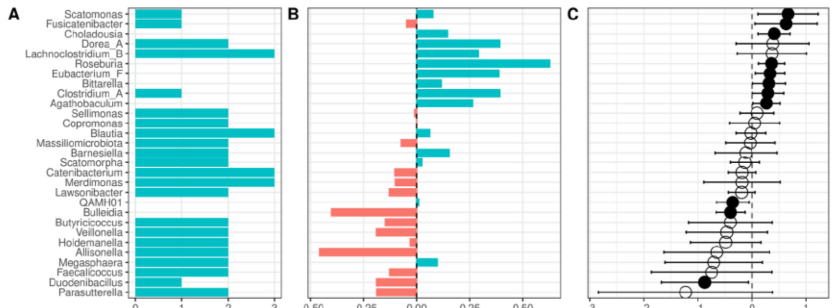


Supplementary Figure S4. Changes in the relative abundance between patients with alcohol use disorder and healthy controls at the class level. a. Number of datasets in which the given class was detected as significant. b) Change in the relative abundance of a given class in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. c. Change in the relative abundance of a given class after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error bars indicate 95% confidence intervals. Only bacteria detected as significant in at least two datasets or significant in random-effects analysis are presented.

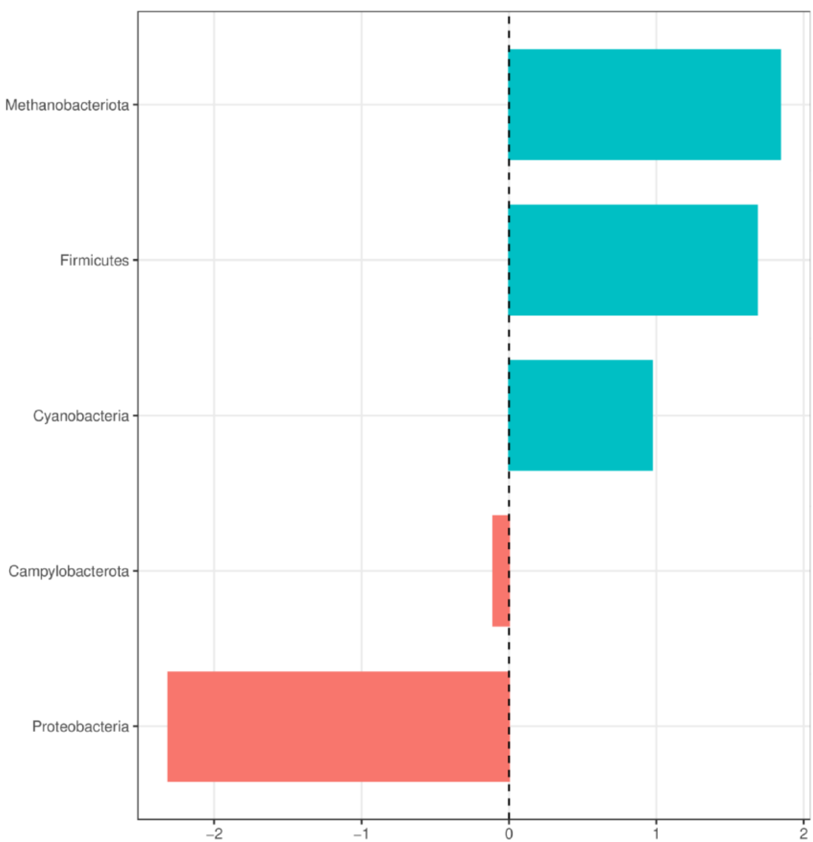


Supplementary Figure S5. Changes in the relative abundance between patients with alcohol use disorder and healthy controls at the order level. a. Number of datasets in which the given order was detected as significant. b) Change in the relative abundance of a given order in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. c. Change in the relative abundance of a given order after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error

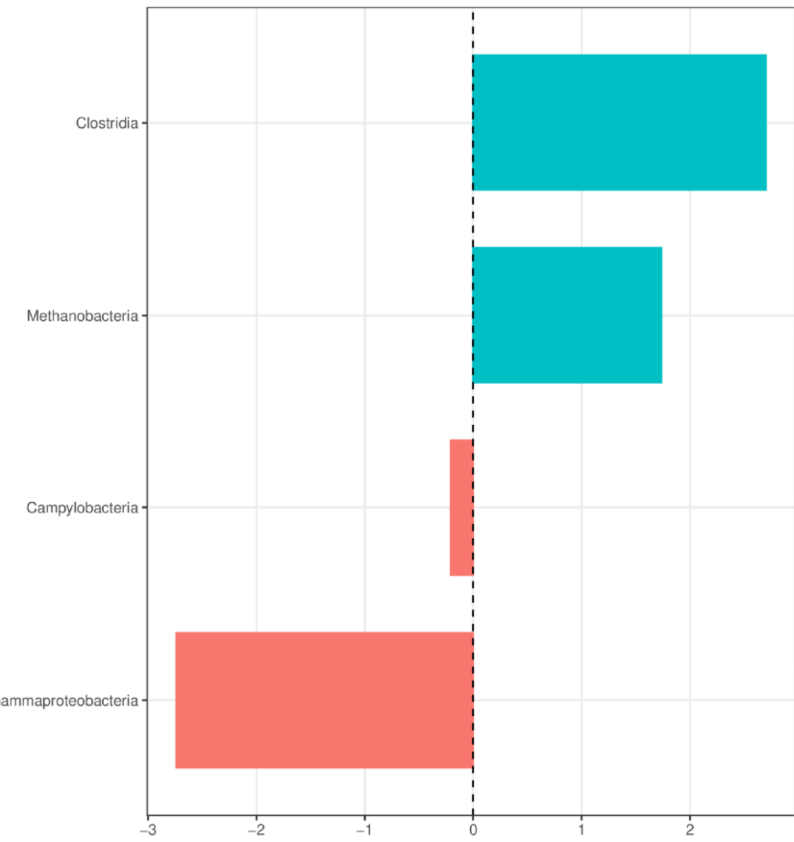
bars indicate 95% confidence intervals. Only bacteria detected as significant in at least two datasets or significant in random-effects analysis are presented.



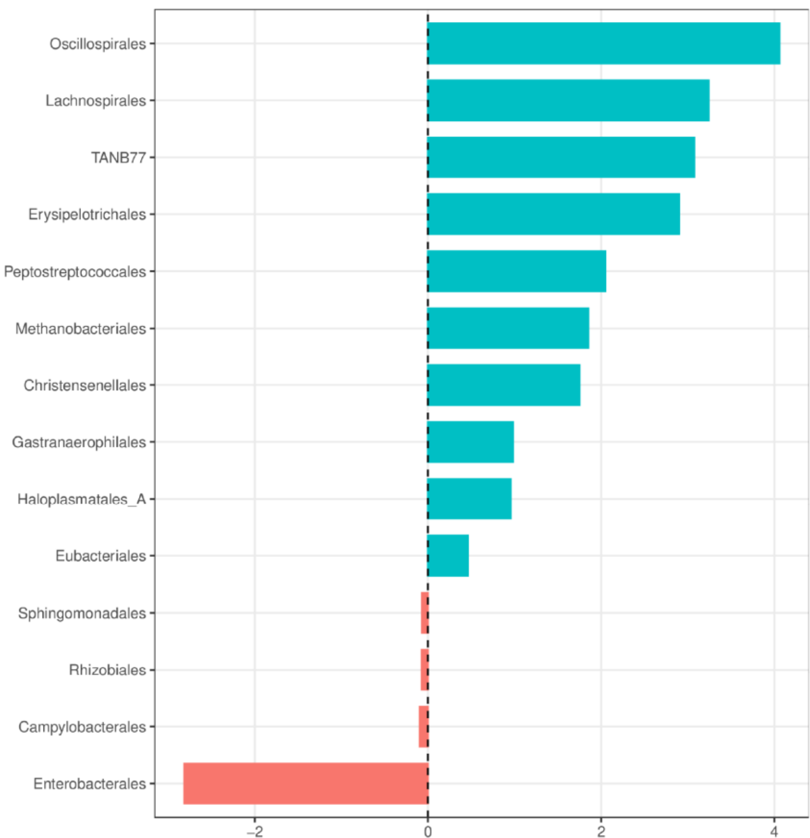
Supplementary Figure S6. Changes in the relative abundance between patients with alcohol use disorder and healthy controls at the genus level. a. Number of datasets in which the given genus was detected as significant. b) Change in the relative abundance of a given genus in the combined dataset reported as log-ratio. Blue denotes bacteria more abundant in healthy controls, and red more abundant in alcohol use disorder. c. Change in the relative abundance of a given genus after summarizing results from individual datasets using the random-effects model, expressed as a mean difference in centered log ratios. Values larger than zero denote greater abundance in healthy controls. A full dot denotes a statistically significant effect. Error *bars indicate 95% confidence intervals. Only bacteria detected as significant in at least two datasets or significant in random-effects analysis are presented.*



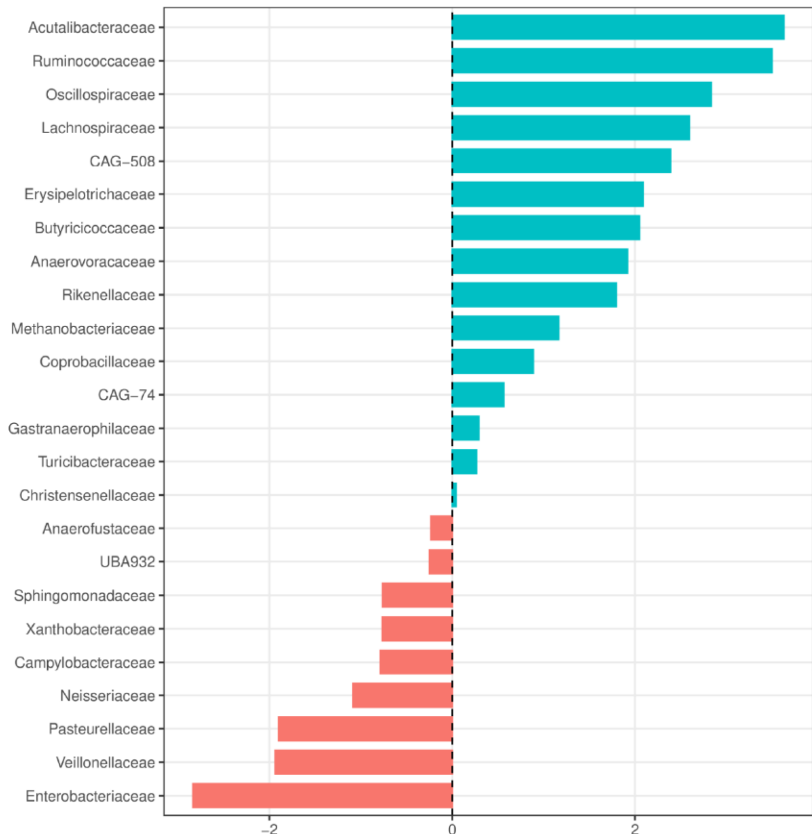
Supplementary Figure S7. Changes in the relative abundance of bacteria at different taxonomy levels between patients with alcoholic liver disease and alcohol use disorder, expressed as log-ratios. Only bacteria detected as significant in ANCOM-BC are presented. Values greater than 0 indicate larger abundance in alcohol use disorder without liver disease. a) Phylum



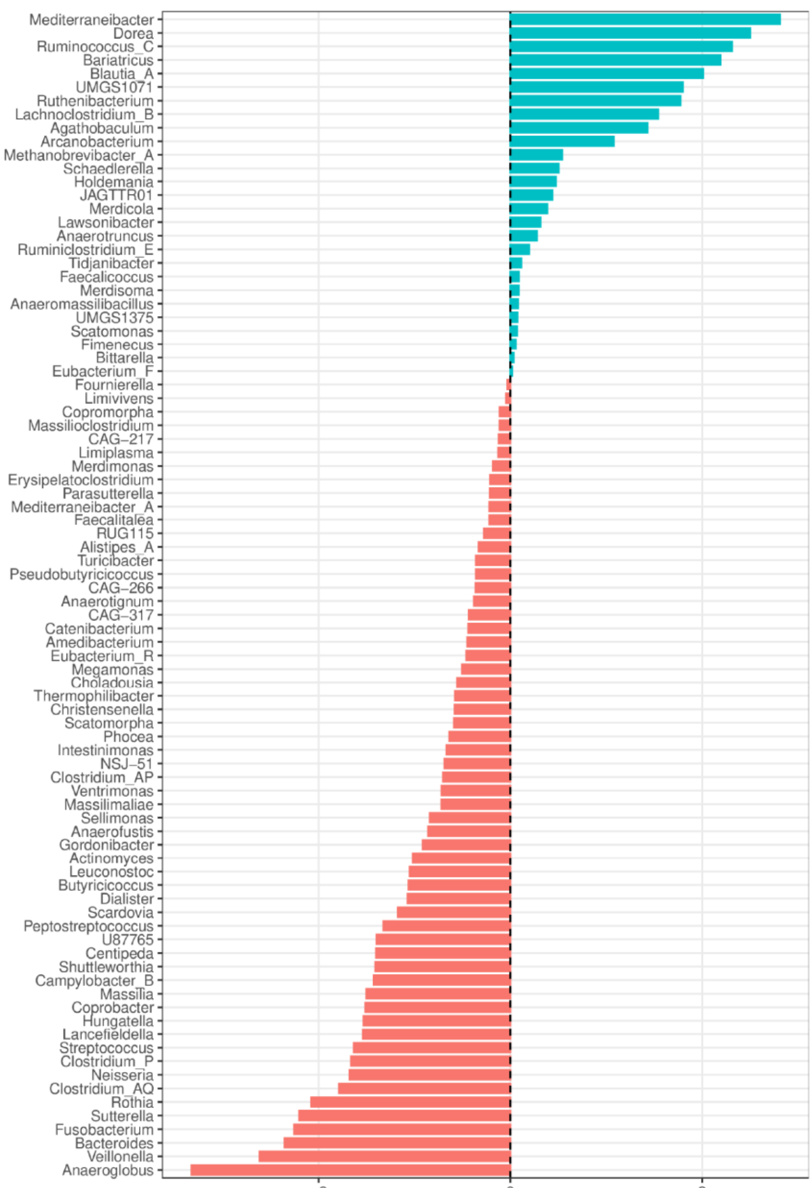
Supplementary Figure S7. b) Class



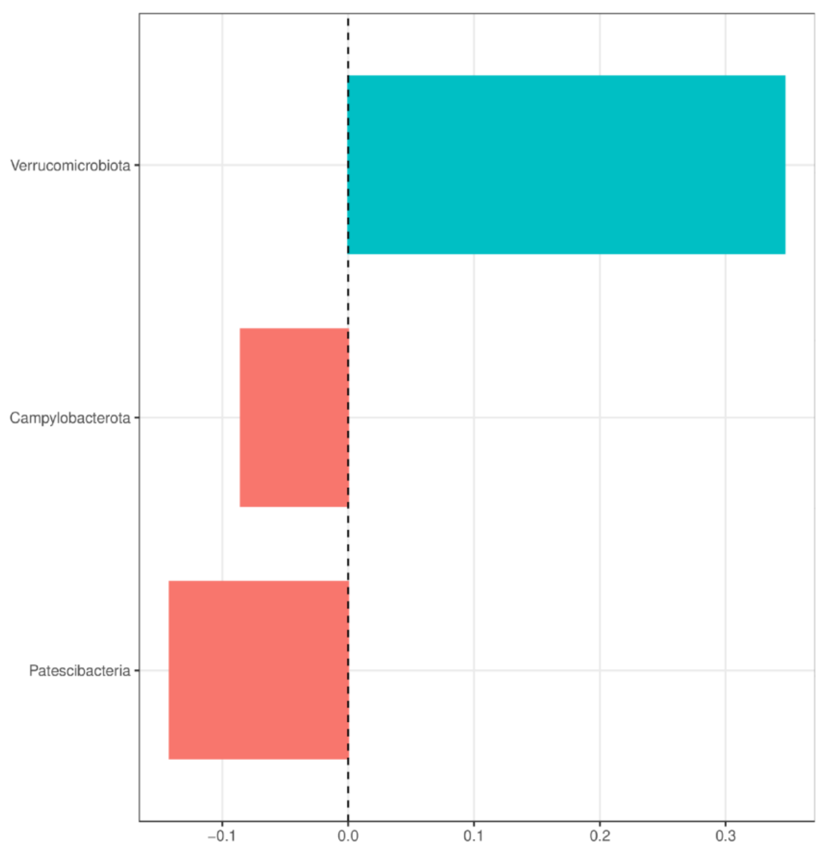
Supplementary Figure S7. c) Order



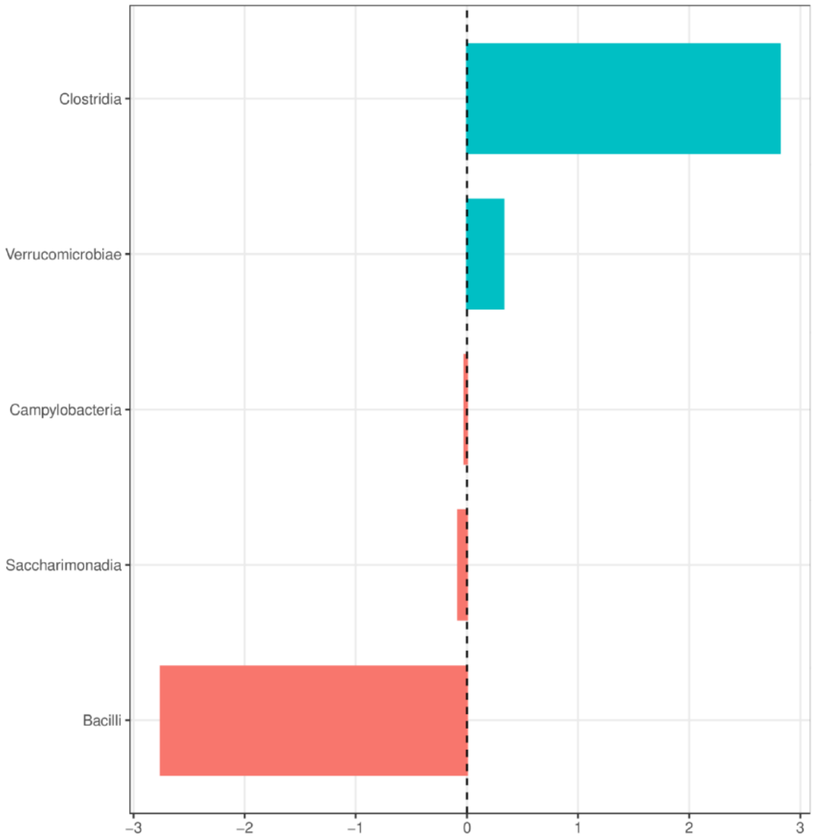
Supplementary Figure S7. d) Family



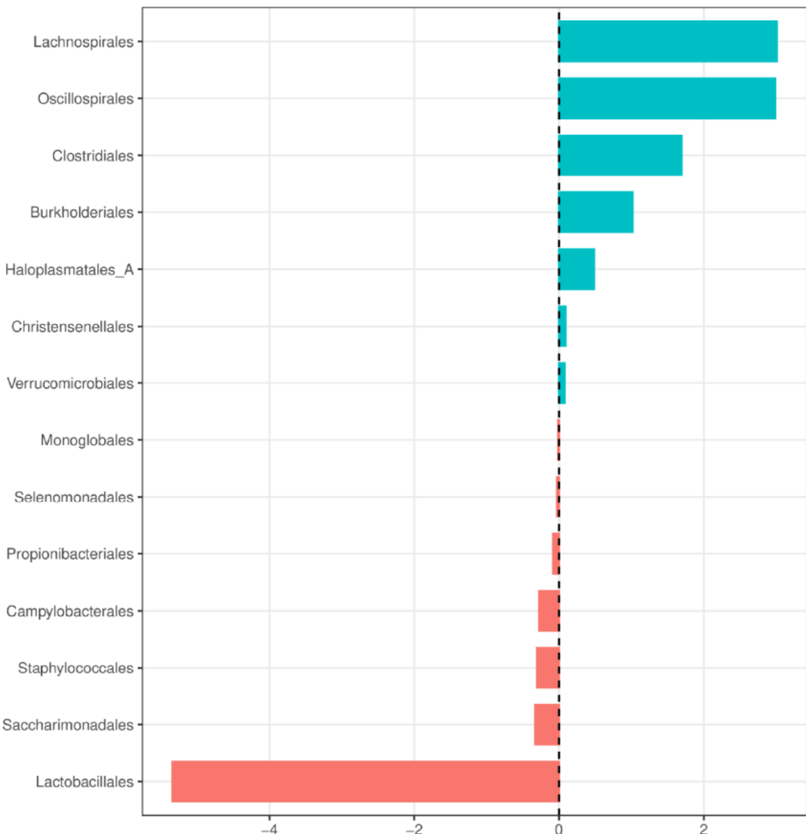
Supplementary Figure S7. e) Genus



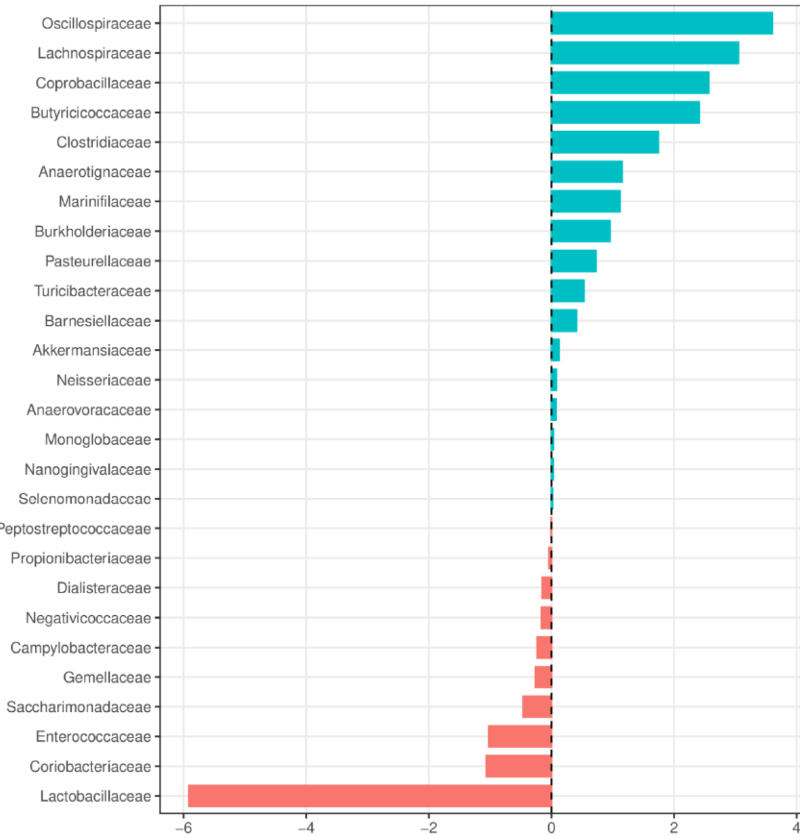
Supplementary Figure S8. Changes in the relative abundance of bacteria at different taxonomy levels between patients with alcoholic liver disease and other causes of liver disease, expressed as log-ratios. Only bacteria detected as significant in ANCOM-BC are presented. Values greater than 0 indicate larger abundance in alcohol use disorder without liver disease. a) Phylum



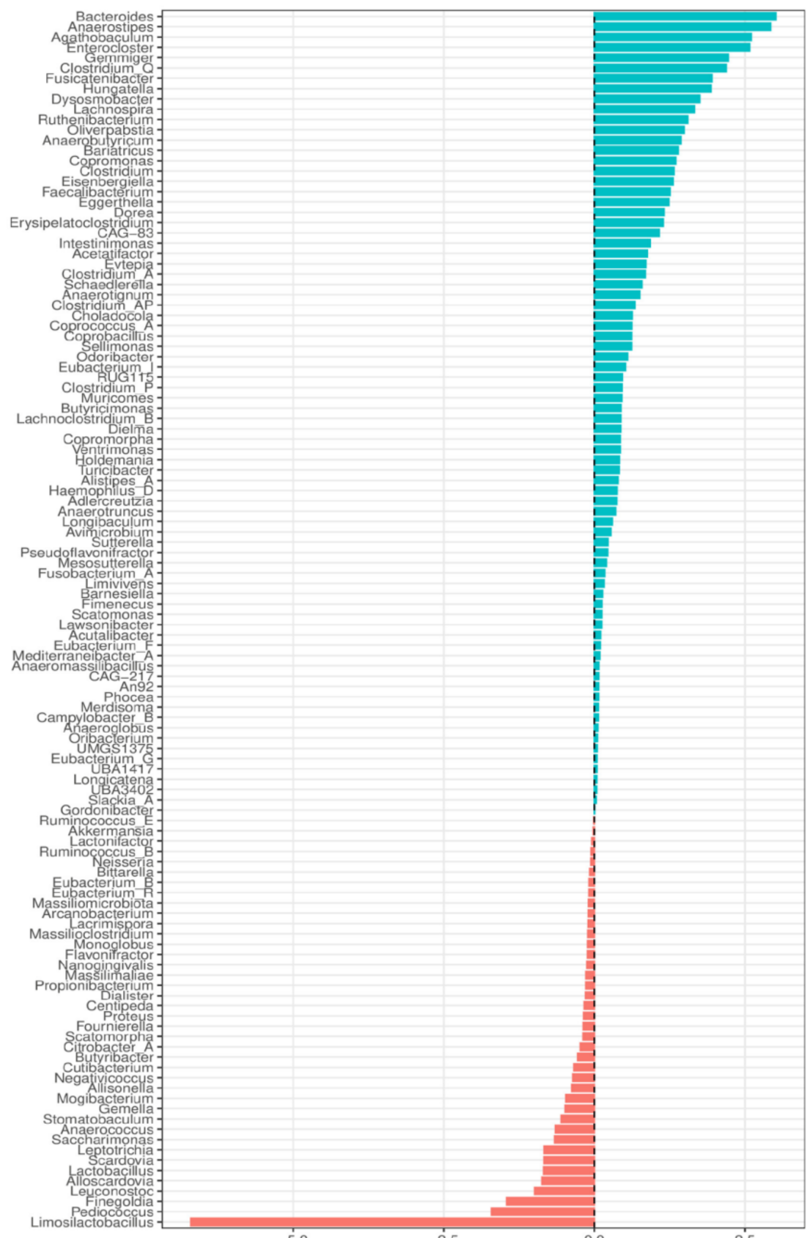
Supplementary Figure S8. b) Class



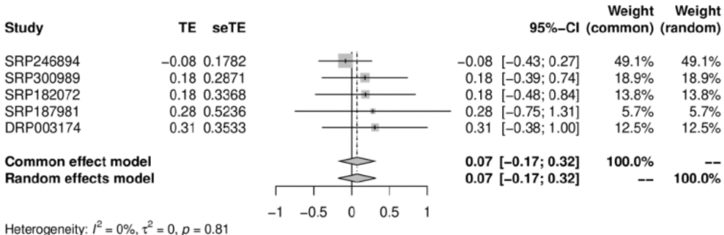
Supplementary Figure S8. c) Order



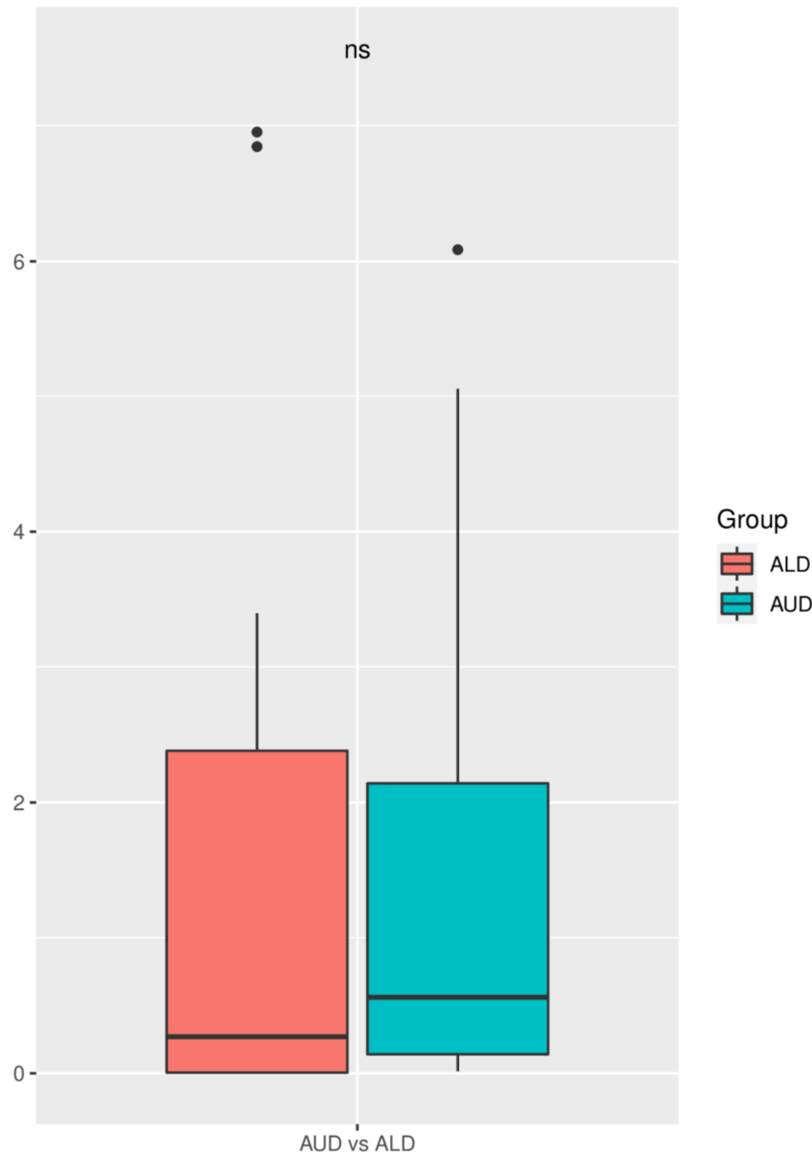
Supplementary Figure S8. d) Family



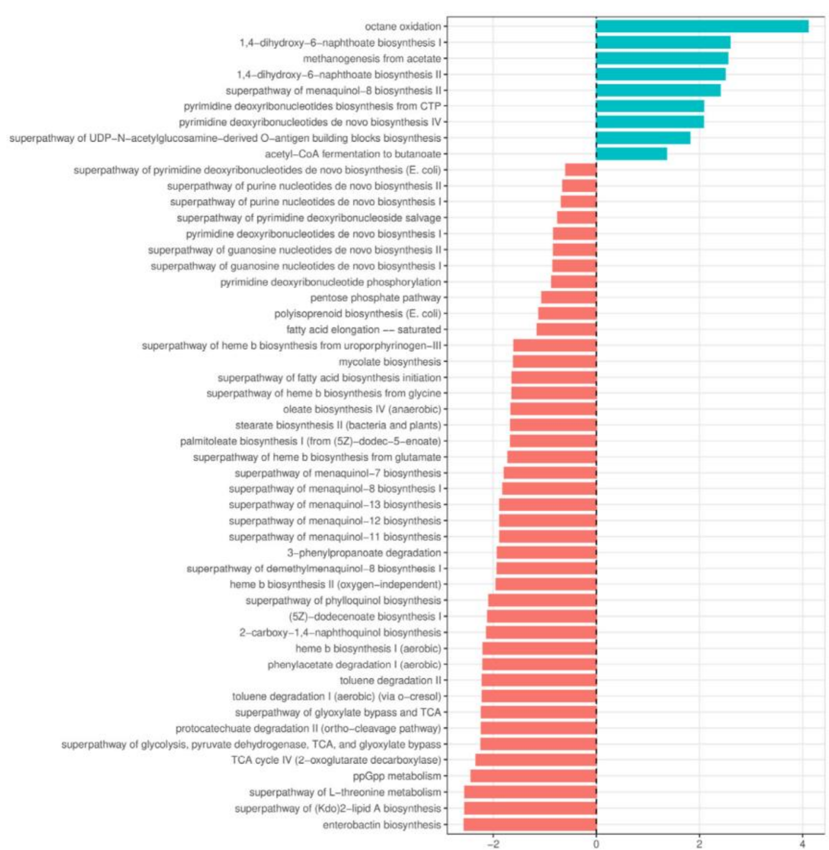
Supplementary Figure S8. e) Genus



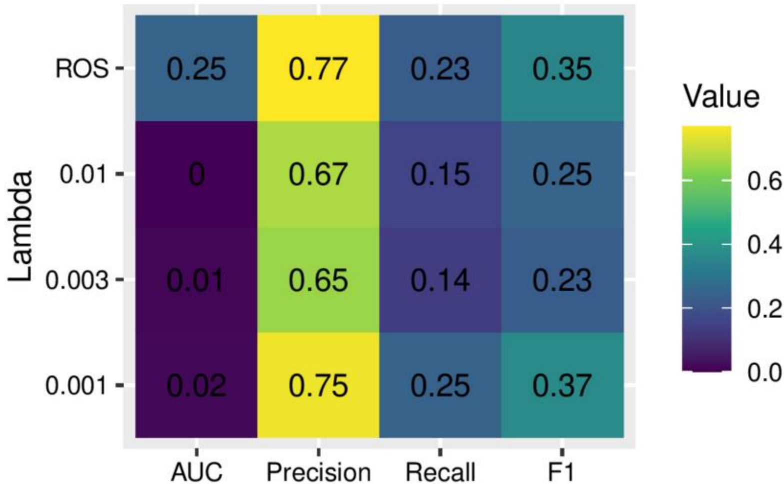
Supplementary Figure S9. Comparison of Firmicutes/Bacteroidetes (F/B) ratio between patients with alcohol use disorder and healthy control. Values larger than 0 denote greater F/B ratio in healthy controls.



Supplementary Figure S10. Comparison of Firmicutes/Bacteroidetes (F/B) ratio between AUD and ALD; AUD - alcohol use disorder, ALD - alcoholic liver disease, ns - non-significant result



Supplementary Figure S11. Changes in the relative abundance of the inferred metagenomic pathways between patients with alcoholic liver disease and alcohol use disorder, expressed as log-ratios. Only pathways detected as significant in ANCOM-BC are presented. Values greater than 0 indicate larger abundance in alcohol use disorder without liver disease.



Supplementary Figure S12. Accuracy of deep learning for prediction of disease status for the SRP187981 dataset with different values of lambda for L2 regularization and with inclusion of random oversampling (ROS)

11. Wnioski

1. Alkoholowe zapalenie wątroby związane jest z istotnie wyższym stężeniem AGE10 w osoczu.
2. W badanej grupie wiek i płeć nie wpływały istotnie na poziom AGE10 w osoczu.
3. Wśród pacjentów z alkoholowym zapaleniem wątroby, poziom wykładników uszkodzenia wątroby (AST, ALT, bilirubina i GGTP) nie wpływał istotnie na stężenie AGE10 w osoczu.
4. Dokładność diagnostyczna AGE10 wynosi 0.778, dla optymalnego punktu odcięcia czułość wynosi 75% a swoistość 72%.
5. ALD jest powiązane z istotnie wyższymi stężeniami klasycznych typów AGE. Żadna z prac zakwalifikowanych do przeglądu systematycznego i metaanalizy nie raportowała ich dokładności diagnostycznej.
6. AUD i ALD powiązane są z licznymi zaburzeniami mikrobiomu, z których najważniejszy jest spadek w rodzinach bakterii wytwarzających krótkołańcuchowe kwasy tłuszczowe oraz wzrost w typie produkującym silnie immunogenny lipopolisacharyd.
7. Poziom mikrobowej α-galaktozydazy nie różni się istotnie pomiędzy AUD, ALD i grupą kontrolną.

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Alcoholic Liver Disease Is Associated with Elevated Plasma Levels of Novel Advanced Glycation End-Products: A Preliminary Study, Nutrients 2022 Vol.14 no.24 art.5266

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Advanced glycation end-products in common non-infectious liver diseases: systematic review and meta-analysis. Nutrients 2021 Vol.13 no.10 art.3370

Kamil Litwinowicz, Ewa Waszczuk, Aleksandra Kuzan, Agnieszka Bronowicka-Szydełko, Kinga Gostomska-Pampuch, Piotr Naporowski, Andrzej Gamian
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Alcoholic Liver Disease Is Associated with Elevated Plasma Levels of Novel Advanced Glycation End-Products: A Preliminary Study, Nutrients 2022 Vol.14 no.24 art.5266

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Kamil Litwinowicz, Ewa Waszczuk, Andrzej Gamian
Advanced glycation end-products in common non-infectious liver diseases: systematic review and meta-analysis. Nutrients 2021 Vol.13 no.10 art.3370

Kamil Litwinowicz, Ewa Waszczuk, Aleksandra Kuzan, Agnieszka Bronowicka-Szydełko, Kinga Gostomska-Pampuch, Piotr Naporowski, Andrzej Gamian
Alcoholic Liver Disease Is Associated with Elevated Plasma Levels of Novel Advanced Glycation End-Products: A Preliminary Study, Nutrients 2022 Vol.14 no.24 art.5266

Kamil Litwinowicz, Andrzej Gamian
Microbiome Alterations in Alcohol Use Disorder and Alcoholic Liver Disease, International Journal of Molecular Sciences 2023 Vol.24 no.3 art.2461

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2012 – 2018	Studia na Uniwersytecie Medycznym im. Piastów Śląskich we Wrocławiu. Kierunek Lekarski, ukończone z wyróżnieniem.

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2021 – obecnie	European Society of Radiology

3. Dorobek naukowy

Liczba publikacji**:** 9 (6 jako pierwszy autor)

Sumaryczna liczba punktów MNiSW**:** 1075

Sumaryczny Impact Factor**:** 52,660

Publikacje:

Lp.	Opis bibliograficzny	IF	PK
1	**Litwinowicz Kamil**, Choroszy Marcin, Waszczuk Ewa: Changes in the composition of the human intestinal microbiome in alcohol use disorder: a systematic review, American Journal of Drug and Alcohol Abuse, 2020, vol. 46, nr 1, s.4-12. DOI:10.1080/00952990.2019.1669629	3,829	70
2	**Litwinowicz Kamil**, Choroszy Marcin, Wróbel Anna: Strategies for reducing the impact of cycling on the perineum in healthy males:	11,928	200

	systematic review and meta-analysis, Sports Medicine, 2021, vol. 51, nr 2, s.275-287. DOI:10.1007/s40279-020-01363-z		
3	**Litwinowicz Kamil**, Waszczuk Ewa, Gamian Andrzej: Advanced glycation end-products in common non-infectious liver diseases: systematic review and meta-analysis, Nutrients, 2021, vol. 13, nr 10, art.3370 [12 s.]. DOI:10.3390/nu13103370	6,706	140
4	Choroszy Marcin, **Litwinowicz Kamil**, Sobieszczańska Beata: Antybiotykoterapia w zapaleniu płuc a biofilm bakteryjny, Medycyna po Dyplomie, 2021, vol. 30, nr 2, s.89-90, 93-96	0	5
5	**Litwinowicz Kamil**, Choroszy Marcin, Ornat Maciej [i in.]: Bayesian network meta-analysis of face masks' impact on human physiology, Scientific Reports, 2022, vol. 12, art.5823 [11 s.]. DOI:10.1038/s41598-022-09747-z	4,996	140
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7	Choroszy Marcin, **Litwinowicz Kamil**, Bednarz Robert [i in.]: Human gut microbiota in coronary artery disease: a systematic review and meta-analysis, Metabolites, 2022, vol. 12, nr 12, art.1165 [23 s.]. DOI:10.3390/metabo12121165	5,581	100
8	Choroszy Marcin, Sobieszczańska Beata, **Litwinowicz Kamil** [i in.]: Co-toxicity of endotoxin and indoxyl sulfate, gut-derived bacterial metabolites, to vascular endothelial cells in coronary arterial disease accompanied by gut dysbiosis, Nutrients, 2022, vol. 14, nr 3, art.424 [17 s.]. DOI:10.3390/nu14030424	6,706	140
9	**Kamil Litwinowicz**, Andrzej Gamian: Microbiome Alterations in Alcohol Use Disorder and Alcoholic Liver Disease, International Journal of Molecular Sciences, 2023 vol.24 no.3 art.2461 [15s.]. DOI: 10.3390/ijms24032461	6,208	140