Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Radosław Konieczny

Wpływ uszkodzenia bariery jelitowej, mierzonego poziomem cytruliny i poziomu N-tlenku trimetyloaminy w surowicy na rokowanie u pacjentów z chorobami sercowo-naczyniowymi.

Rozprawa doktorska

Promotor: dr hab. n. med., Wiktor Kuliczkowski

Wrocław, 2022

Podziękowania:

Pragnę złożyć podziękowania promotorowi mojej pracy,

Panu dr.hab. n.med., Wiktorowi Kuliczkowskiemu

za wskazówki udzielone podczas przygotowywania publikacji,

Pani Prof. dr hab. n.med. Elżbiecie Poniewierce

za życzliwość i motywację do rozwijania aktywności naukowej,

Paniom Dr Ewie Żurawskiej- Płaksej oraz Dr Hannie Czapor- Irzabek

za przeprowadzone analizy laboratoryjne,

Krystianowi Papierowi za pomoc w edycji rycin.

Pracę dedykuję Żonie,

Rodzicom, Siostrze

i Przyjacielowi

Wykaz skrótów użytych w pracy

ACEI- angiotensin-converting-enzyme inhibitors- Inhibitory konwertazy angiotensyny

ARB- Angiotensin receptor blockers- antagoniści receptora angiotensyny

ASA- Acetylsalicylic acid- kwas acetylosalicalowy

ASCVD-Atherosclerotic cardiovascular disease- choroba sercowo-naczyniowa na podłożu miażdzycy

CABG-coronary artery by-pass graft- pomostowanie aortalno wieńcowe

CCS- Canadian Cardiovascular Society (grading for angina pectoris)- skala zaawansowania dławicy piersiowej Kanadyjskiego Towarzystwa Kardiologicznego

CVD- cardiovascular disease- choroba sercowo-naczyniowa

eGFR- estimated glomerular filtration rate- szacowane przesączanie kłebuszkowe

FMO-flavin-containing monooxygenase-flawinomonooksygenaza 3

HBA1c- glycated hemoglobin A1C- hemoglobina glikowana A1c

HDL-C- high density lipoprotein cholesterol- lipoproteina wysokiej gęstości

HR-hazard Ratio- Hazard względny

hsCRP- high sensitivity C-reactive protein-wysoko czułe oznaczenie białka c-reaktywnego

IQR-interquartile range- rozstęp międzykwartylowy

LDL-C- low density lipoprotein cholesterol- lipoproteina niskiej gęstości

LVEF- left ventricular ejection fraction- frakcja wyrzutowa lewej komory serca

MACE- Major adverse cardiovascular events- poważne zdarzenia sercowo- naczyniowe

NYHA- New York Heart Association (functional classification)- skala Nowojorskiego Towarzystwa Kardiologicznego nasilenia objawów niewydolności serca.

PCI- percutaneous coronary intervention- przezskórna śródnaczyniowa angioplastyka wieńcowa

Q- quartile- kwartyl

TC- total cholesterol- całkowite stężenie cholesterolu

TG- triglycerides- trójglicerydy

TF-tissue Factor- czynnik tkankowy

TMAO-trimethylamine-N-oxide- N-tlenek trimetyloaminy

TMA-trimethylamine- trimetyloamina

TM-thrombomodulin- trombomodulina

Spis treści

Wykaz publikacji stanowiących składowe rozprawy doktorskiej:

Cykl publikacji naukowych stanowiących treść rozprawy tworzą:

1. Konieczny, R. A., Kuliczkowski, W. (2022). Trimethylamine N-oxide in cardiovascular disease. *Advances in clinical and experimental medicine: official organ Wroclaw Medical University*, *31*(8), 913–925. (IF 1.736, MEiN 70)

2. Konieczny, R. A, Żurawska-Płaksej, E., Kaaz, K., Czapor- Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*,*147*(4), 443–452. (If 2.342, MEiN 40)

3. Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor- Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine: official organ Wroclaw Medical University*, *31*(10), 1121–1128. (IF 1.736, MEiN 70)

1.Wstęp

1.1.Wprowadzenie

Według Światowej Organizacji Zdrowia (ang. WHO-World Health Organization), choroby sercowo-naczyniowe (CVD) pozostają główną przyczyną śmierci i niepełnosprawności na całym świecie [1]. Dzięki rozwojowi metabolomiki i metagenomiki, mikrobiota jelitowa coraz częściej jest wskazywana jako modulator przebiegu CVD [2]. Aktywność mikrobioty jelitowej prowadzi do powstania cząsteczek, które po wchłonięciu z jelita uczestniczą bezpośrednio lub pośrednio w patogenezie CVD. Przykładem takiej cząsteczki jest n-tlenek trimetyloaminy (TMAO).

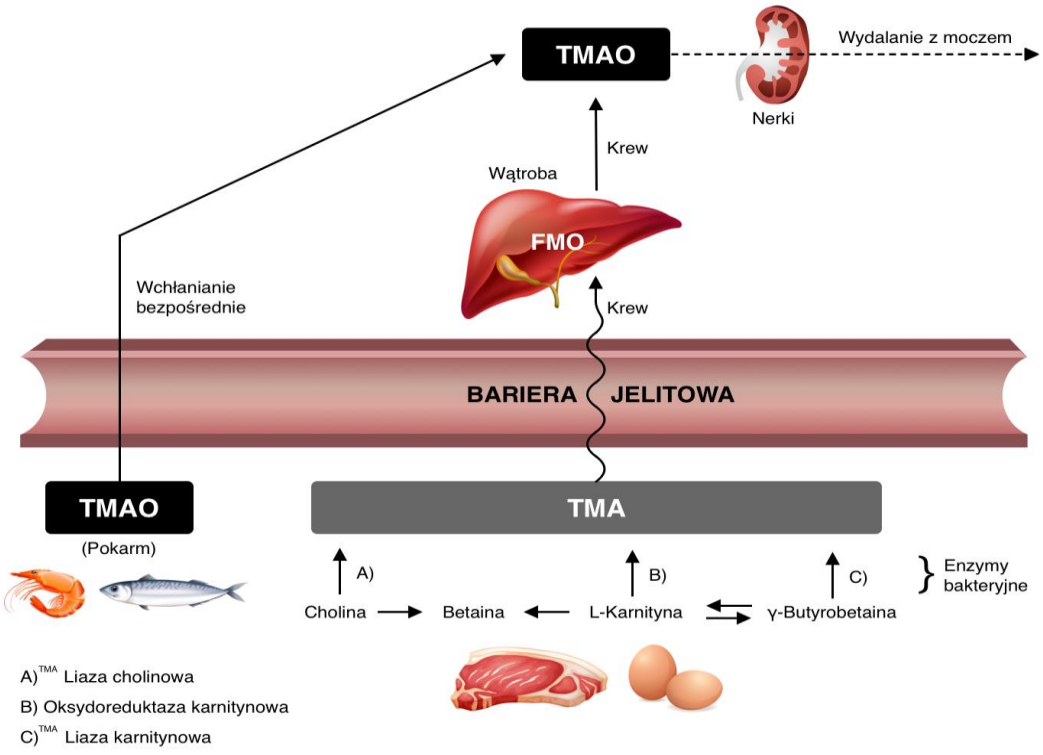
1.2.TMAO

TMAO powstaje w ludzkim organizmie na drodze enzymatycznego utlenienia metabolitu mikrobioty jelitowej – trimetyloaminy (TMA). Jest także wchłaniany bezpośrednio z pokarmu [3]. Uproszczony schemat metabolizmu i wchłaniania TMAO przedstawiono na Rycinie 1.

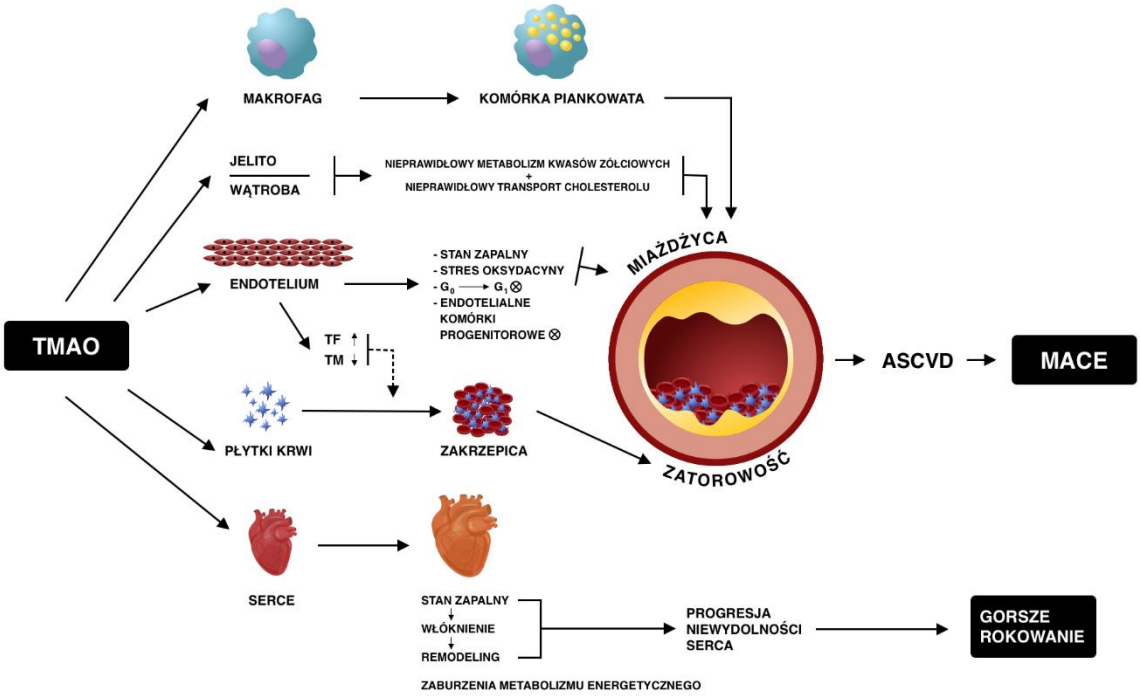
Udział TMAO w procesie aterogenezy, indukcji stresu oksydacyjnego, nadreaktywności płytek krwi czy włóknieniu i remodelingu mięśnia sercowego znajduje odzwierciedlenie w badaniach populacji pacjentów obciążonych chorobami układu krążenia [4-7]. Wykazano w nich, że wyższe stężenie TMAO w surowicy zwiększa ryzyko zgonu i innych niekorzystnych zdarzeń sercowo- naczyniowych w przebiegu choroby wieńcowej, niewydolności serca oraz częstości powikłań migotania przedsionków [8-10]. Uproszczony schemat procesów patofizjologicznych w których pośrednio lub bezpośrednio uczestniczy TMAO przedstawiono na Rycinie 2.

Postuluje się, że parametry bariery jelitowej mogą być czynnikiem istotnie wpływającym na wchłanianie tej cząsteczki i jej prekursorów [11]. Z tego powodu przepuszczalność bariery jelitowej powinna być uwzględniana w całościowej ocenie ryzyka związanego z podwyższonym stężeniem TMAO w surowicy[11]. Dane dotyczące procesu wchłaniania TMAO oraz TMA, z przewodu pokarmowego są ograniczone[12].Analizy tego typu, w kontekście CVD przeprowadzono dotychczas głównie na modelu zwierzęcym[13-15]. Dopiero w marcu 2022 roku opublikowano badanie przeprowadzone na małej (n=29) grupie pacjentów ze zdekompensowaną niewydolnością serca, w którym analizowano korelację stężenia TMAO z parametrami bariery

jelitowej [16]. W badaniu tym wykazano brak istotnej korelacji stężenia TMAO z przepuszczalnością bariery jelitowej.



Rycina 1. Metabolizm i wchłanianie TMAO z przewodu pokarmowego.



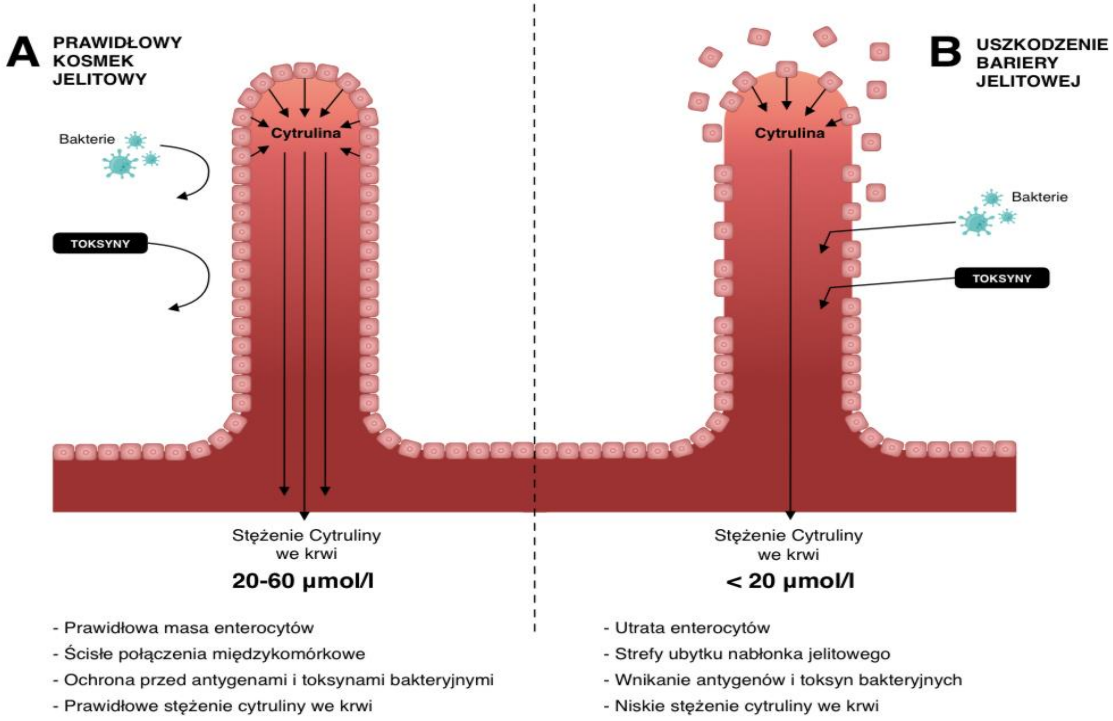
Rycina 2. Procesy patofizjologiczne układu krążenia wiązane pośrednio lub bezpośrednio z TMAO.

1.3.Uszkodzenie bariery jelitowej w chorobach układu krążenia

Chorobom układu krążenia towarzyszy uszkodzenie bariery jelitowej i utrata jej funkcji ochronnej [17]. Pierwsze badania na ten temat dotyczyły uszkodzenia bariery jelitowej w przewlekłej niewydolności serca [18,19]. Wykazano także związek uszkodzenia bariery jelitowej z chorobą wieńcową, zawałem mięśnia sercowego oraz nadciśnieniem tętniczym [20-24]. Powstaje tzw. błędne koło, w którym choroba układu krążenia powoduje uszkodzenie bariery jelitowej i szybsze przenikanie czynników powodujących progresję zmian w sercu oraz naczyniach krwionośnych [17].

1.3.1 Cytrulina

Cytrulina jest aminokwasem syntetyzowanym prawie wyłącznie przez enterocyty środkowej i szczytowej części kosmków proksymalnego odcinka jelita cienkiego [25]. Oznaczenie stężenia cytruliny w surowicy pozwala w sposób pośredni ocenić masę czynnych metabolicznie enterocytów. Jego spadek świadczy o uszkodzeniu bariery jelitowej [25-27]. Potwierdzono przydatność cytruliny jako biomarkera niedokrwienia jelita u pacjentów z niedrożnością tętnicy krezkowej oraz markera utraty nabłonka jelitowego po śródzabiegowej okluzji naczyń jelita cienkiego [28,29]. U pacjentów w stanie krytycznym, obniżony poziom cytruliny może świadczyć o uszkodzeniu bariery jelitowej i jest w tej grupie niezależnym czynnikiem rokowniczym zgonu[30-33]. Rolę cytruliny jako biomarkera w ocenie bariery jelitowej przedstawiono na Rycinie 3



Rycina 3. Cytrulina jako biomarker bariery jelitowej

2. Przesłanki do badań

Dotychczas nie przeprowadzono analizy wpływu stężenia TMAO na śmiertelność długoterminową w polskiej populacji pacjentów obciążonych chorobami układu krążenia.

Ocena wpływu uszkodzenia bariery jelitowej na stężenie TMAO była przeprowadzona jedynie na niewielkich populacjach pacjentów obciążonych chorobami układu krążenia [16]. Do oceny bariery jelitowej wykorzystano biomarkery inne niż cytrulina.

Brakuje badań oceniających stężenie cytruliny jako czynnika rokowniczego w obserwacji długoterminowej.

3. Cele rozprawy doktorskiej:

1. Ocena przydatności klinicznej stężenia TMAO jako niezależnego czynnika rokowniczego u polskich pacjentów obciążonych chorobami układu krążenia w obserwacji długoterminowej.

2. Ocena przydatności klinicznej stężenia cytruliny jako niezależnego czynnika rokowniczego u pacjentów obciążonych chorobami układu krążenia w obserwacji długoterminowej.

3. Ocena wzajemnego powiązania stężenia TMAO i uszkodzenia bariery jelitowej ocenionego stężeniem cytruliny. Zbadanie takiej korelacji pozwoli lepiej zrozumieć wzajemny wpływ tych czynników na rokowanie pacjentów z chorobami układu krążenia.

4.Materiał i Metody

Pracę poglądową zawartą w publikacji 1 przygotowano zgodnie z protokołem Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P). Przegląd literatury wykonano 3 listopada 2021 roku w bazie PubMed używając słów kluczowych TMAO, atherosclerosis (ang. miażdżyca), coronary artery disease (ang. choroba naczyń wieńcowych), atrial fibrillation (ang, migotanie przedsionków) heart failure (ang. niewydolność serca), gut microbiota (mikrobiota jelitowa). Wyniki wybranych badań oraz metaanaliz omówiono w tekście oraz zebrano w tabelach.

Populację badań opisanych w pracach oryginalnych 2 i 3 stanowili kolejni pacjenci Kliniki Kardiologii Uniwersyteckiego Szpitala Klinicznego we Wrocławiu, hospitalizowani od marca 2013 do listopada 2015 roku, którzy wyrazili świadomą, pisemną zgodę na pobranie materiału biologicznego- próbki surowicy. Pacjenci, którzy nie wyrazili zgody na oddanie próbki na potrzeby przyszłych badań lub ich stan zdrowia nie pozwalał na wyrażenie świadomej zgody zostali wykluczeni z badania. Próbki surowicy pobierano w 1 lub 2 dniu hospitalizacji, na czczo, aby zapewnić możliwie podobne warunki pobrania materiału. Próbki były niezwłocznie po pobraniu schładzane do temperatury -80 stopni Celsjusza i przechowywane w BioBanku Polskiego Ośrodka Rozwoju Technologii we Wrocławiu (PORT). Szczegółową charakterystykę badanej populacji przedstawiono w Tabeli1.

Używając spektrofotometrii mas sprzężonej z kolumnową chromatografią cieczową retrospektywnie oznaczono stężenie TMAO oraz cytruliny w próbkach surowicy 1036 pacjentów. Szczegółowy opis metodyki oznaczeń laboratoryjnych przedstawiono w publikacjach 2 i 3.

Informację o ewentualnym wystąpieniu zgonu i jego dacie pozyskano z rejestru NFZ- stan na dzień 19.02.2020.

Stężenie TMAO i cytruliny w surowicy skorelowano ze śmiertelnością 5-letnią oraz dostępnymi danymi klinicznymi z rejestrowanej hospitalizacji.

Analizę statystyczną przeprowadzono dla całej populacji n=1036 oraz subpopulacji wyodrębnionych na podstawie dominującego rozpoznania klinicznego (ostry zespół wieńcowy n=177, przewlekły zespół wieńcowy n=441, przewlekła niewydolność serca=292, migotanie przedsionków n=277). Wszystkie obliczenia wykonano w programie TIBCO Software Inc. (2017).

Statistica (data analysis software system), version 13. https://www.tibco.com/ Wszystkie hipotezy statystyczne weryfikowano na poziomie istotności p=0.05. Szczegółowy opis metodyki analizy statystycznej przedstawiono w publikacjach 2 i 3.

5. Wyniki

5.1. Przegląd literatury-publikacja 1.

Otwarcie cyklu publikacji stanowi praca 1: **Trimethylamine N-oxide in cardiovascular disease.** W pierwszej części przeglądu omówiono złożony metabolizm TMAO oraz powiązane procesy patofizjologiczne. Podsumowanie wyników dotychczasowych badań na temat szkodliwości TMAO przedstawiono w tekście oraz zbiorczej tabeli. Uwzględnia ona rodzaj badania, charakterystykę populacji, długość obserwacji, punkty końcowe, uzyskane wyniki oraz stężenie TMAO w porównanych subpopulacjach. W podobny sposób przedstawiono wyniki metaanaliz dotyczących związku TMAO z ryzykiem sercowo-naczyniowym oraz badań ukierunkowanych na redukcję szkodliwości TMAO u ludzi. Publikację zakończono wskazaniem kierunków kolejnych badań dotyczących wpływu mikrobioty jelitowej na choroby układu krążenia.

Na podstawie przeglądu literatury można stwierdzić, że TMAO jest użytecznym markerem ryzyka sercowo naczyniowego. Użyteczność kliniczna oznaczeń stężenia TMAO wymaga jednak potwierdzenia w prospektywnych, interwencyjnych badaniach klinicznych. Praca stanowi wstęp do cyklu publikacji i w sposób usystematyzowany podsumowuje aktualny stan wiedzy na temat TMAO w kontekście chorób układu krążenia.

5.2 Prace oryginalne

Druga praca z cyklu, '**All-cause mortality and trimethylamine-N-oxide levels in patients with cardiovascular disease'** przedstawia ocenę wpływu stężenia TMAO w surowicy na śmiertelność 5-letnią w populacji 1036 polskich pacjentów hospitalizowanych na oddziale kardiologicznym.

5.2.1.Charakterystyka populacji.

Mediana wieku pacjentów wynosiła 62 lata, 61% populacji stanowili mężczyźni. W czasie 5- letniej obserwacji zanotowano 171 zgonów co stanowiło 16,5% pacjentów uczestniczących w badaniu. Pacjentów podzielono na grupę, w której wystąpił zgon oraz grupę która przeżyła okres 5-letniej obserwacji.

Większość analizowanych zmiennych klinicznych i biochemicznych była różna w grupie, gdzie wystąpił zgon w porównaniu z grupą pacjentów przeżywających. Osoby, które zmarły były starsze i byli to częściej mężczyźni. Wykryto u nich wyższe stężenie TMAO i hsCRP oraz wyższy poziom HbA1c. Obserwowano niższy poziom cholesterolu całkowitego, frakcji HDL i trójglicerydów. Niższa była u nich wartość przesączania kłębuszkowego i frakcji wyrzutowej lewej komory serca. Częściej stosowali kwas acetylosalicylowy, doustną antykoagulację, beta blokery, statyny, diuretyki pętlowe i blokery receptora angiotensyny. Częściej obserwowano u nich również ostry i przewlekły zespół wieńcowy, przewlekłą niewydolność serca, migotanie przedsionków, cukrzycę, nadciśnienie tętnicze i nikotynizm. W grupie pacjentów zmarłych częściej występowały przebyte zabiegi angioplastyki wieńcowej i pomostowania aortalno-wieńcowego.

Pełną charakterystykę populacji dostępne dane biochemiczne i kliniczne z hospitalizacji przedstawiono w Tabeli 1.

Tabela 1. Charakterystyka populacji

Zmienna	Cała grupa (n=1036)	Zgon w trakcie 5-letniej obserwacji		wartość p
		Tak (n=171)	Nie (n=865)	
Wiek (lata)	62,0±14,1	68,9±11,1	60,7±14,3	p<0,0001
Płeć (% mężczyzn)	61,1	73,0	58,8	p<0,001
TMAO (μM)	4,06 (2,79-6,01)	5,65 (3,48-8,94)	3,86 (2,70-5,62)	p<0,0001
Cytrulina (μM)	22,5 (17,8-27,9)	21,5 (16,5-27,6)	22,8 (18,0-28,0)	p=0,18
Ostry zespół wieńcowy (%)	17,0	32,7	19,4	p<0,001
Przewlekły zespół wieńcowy (%)	42,5	57,1	40,0	p<0,0001
Przewlekła niewydolność serca (%)	28,1	54,7	23,4	p<0,0001
Migotanie przedsionków (%)	26,7	43,2	23,4	p<0,0001

Inne zaburzenia rytmu (%)	16,4	14,7	85,2	p=0,48
Zaburzenia przewodnictwa (%)	44,0	13,1	86,8	p=0,010
PCI w trakcie hospitalizacji (%)	21,0	31,1	23,3	p=0,051
PCI w wywiadzie (%)	14,4	25,7	16,9	p=0,017
CABG w wywiadzie (%)	7,6	12,2	6,7	p=0,012
Cukrzyca (%)	24,7	35,2	22,6	p<0,001
Nadciśnienie tętnicze (%)	71,7	81,8	69,7	p=0,0012
Nikotynizm (%)	23,2	30,0	21,9	p=0,026
NYHA (% w grupie przewlekłej niewydolności serca)	I - 7,0 II- 55,5 III - 34,0 IV - 3,3	I - 8,7 II - 48,3 III - 37,7 IV - 5,3	I - 6,3 II - 58,8 III - 32,3 IV - 2,6	p=0,16
CCS (% w grupie przewlekłego zespołu wieńcowego)	I - 10,2 II - 54,3 III - 32,2 IV - 3,1	I - 6,0 II - 57,5 III - 33,3 IV - 3,0	I - 11,3 II - 53,5 III - 32,0 IV - 3,1	p=0,65
LVEF (%)	60 (50-65)	50 (37-65)	65 (55-66)	p<0,0001
eGFR (ml/min)	68 (55-80)	58 (44-72)	69 (58-81)	p<0,0001
hsCRP (mg/l)	3,47 (1,33-13,3)	7,79 (2,64-30,8)	3,11 (1,24-10,1)	p<0,0001
HbA1c (%)	5,70 (5,40-6,20)	5,90 (5,50-6,65)	5,70 (5,40-6,10)	p=0,018
TC (mg/dl)	182 (149-216)	171 (135-207)	184 (151-218)	p=0,0028

LDL (mg/dl)	104 (80-136)	97 (33-249)	106 (83-136)	p=0,094
HDL (mg/dl)	45 (37-55)	42 (34-51)	46 (38-55)	p<0,001
TG (mg/dl)	124 (95-169)	115 (89-155)	127 (96-172)	p=0,018
ASA (%)	54,8	64,0	52,9	p=0,0085
Klopidogrel (%)	29,4	28,1	35,1	p=0,075
NOAC (%)	28,9	43,4	26,0	p<0,0001
ACE-I (%)	70,2	76,1	69,0	p=0,066
ARB (%)	7,19	3,01	8,02	p=0,022
Beta bloker (%)	81,2	92,2	79,0	p<0,0001
Statyna (%)	79,1	89,2	77,0	p<0,001
Diuretyk pętlowy (%)	19,1	43,4	14,3	p<0,0001

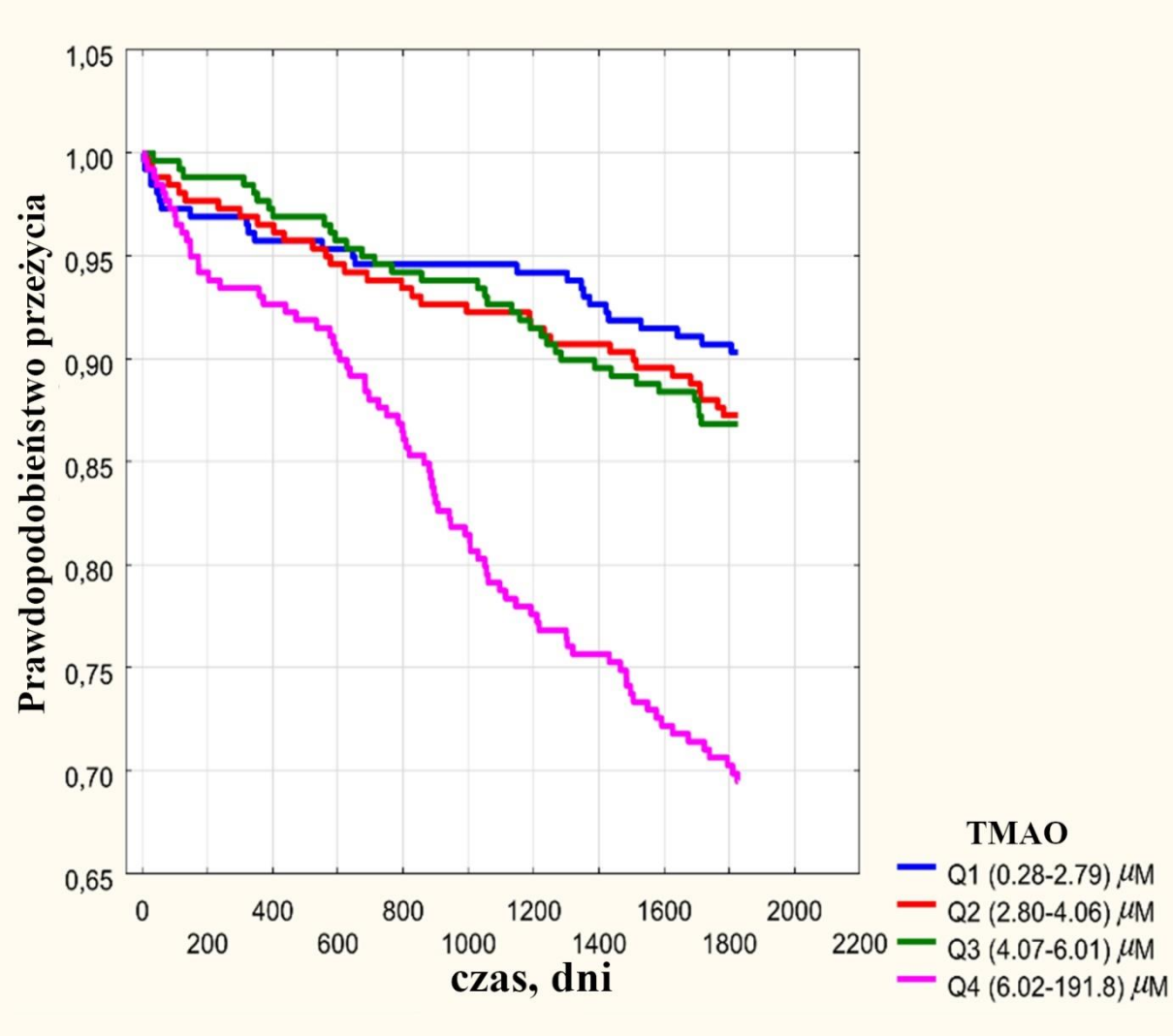
5.2.2. Wpływ stężenia TMAO na śmiertelność w całej grupie badanej

W analizie jednoczynnikowej proporcjonalnego hazardu Coxa wyższe stężenie TMAO w całej grupie badanej było istotnym statystycznie predykatorem śmiertelności 5-letniej (HR 1,01 przy 95% CI 1,006-1,018 i p<0,0001). Opisana zależność straciła swoją istotność statystyczną w analizie wieloczynnikowej, uwzględniającej wymienione powyżej czynniki związane ze śmiertelnością

tj. wiek, płeć, choroby współistniejące oraz nieprawidłowości badań biochemicznych.

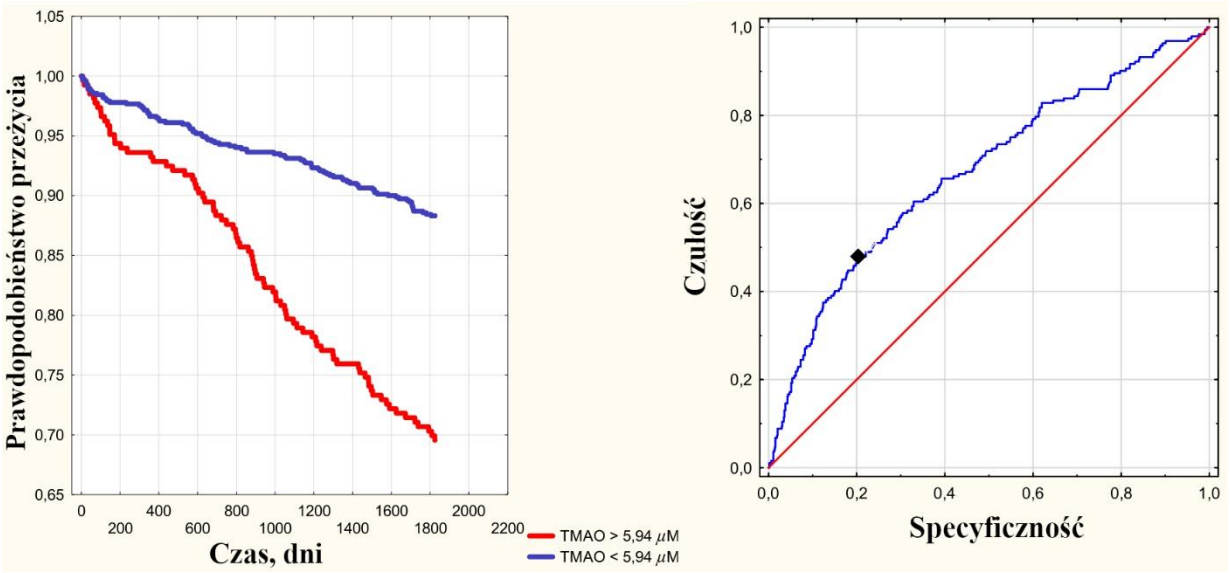
W celu dokładniejszej analizy wpływu poziomu TMAO na śmiertelność 5- letnią podzielono jego stężenie na kwartyle, które porównano za pomocą metody Kaplana-Meiera. *(Wykres 1)* Stwierdzono istotną różnicę w prawdopodobieństwie przeżycia pacjentów w najwyższym kwartylu poziomu TMAO w porównaniu z pozostałymi kwartylami (Chi kwadrat 49,61; p< 0,00001)

W analizie jednoczynnikowej dla kwartyli stężeń TMAO stwierdzono istotnie wyższą śmiertelność (HR 1,55 przy 95% CI 1,34-1,79 i p<0,0001) dla pacjentów w górnym kwartylu stężenia TMAO, jednak w analizie wieloczynnikowej zależność ta nie była istotna statystycznie.



Wykres 1 Krzywe Kaplana-Meiera prawdopodobieństwa przeżycia w czterech grupach pacjentów podzielonych na podstawie kwartyli stężenia TMAO. Q1, Q2, Q3,Q4 – kwartyle 1, 2, 3 i 4.

Przeprowadzona analiza ROC dla całej populacji badanej pozwoliła wyznaczyć punkt odcięcia stężenia TMAO wynoszący 5,94 µM, powyżej którego z 48% czułością i 80% specyficznością można wyznaczyć grupę pacjentów z podwyższonym ryzykiem zgonu w czasie 5-letniej obserwacji (pole powierzchni pod krzywą ROC 0,67; 95% CI 0,62-0,71; p<0,0001; Indeks Youdena=0.28)



Wykres 2 Po lewej: Prawdopodobieństwo przeżycia w całej grupie badanej w zależności od wyznaczonego w analizie ROC punku odcięcia stężenia TMAO = 5,94 µM (wartość statystyki log-rank = 7,07 przy p<0,00001). Po prawej Wykres analizy ROC z zaznaczonym czarnym rombem punktem odcięcia stężenia TMAO (5,94µM) powyżej którego z 48%czułością i 80% specyficznością można wyznaczyć grupę pacjentów z podwyższonym ryzykiem zgonu w obserwacji 5-letniej.

5.2.3 Wpływ TMAO na śmiertelność w podgrupach

Populację badaną podzielono na podgrupy pacjentów w zależności od rozpoznania dominującego. Następnie, podobnie jak dla populacji ogólnej, wprowadzono podział na pacjentów zmarłych oraz tych, którzy przeżyli obserwację 5-letnią. (Tabela 2)) Wykazano istotne różnice stężenia TMAO w podgrupach pacjentów z przewlekłym zespołem wieńcowym, przewlekłą niewydolnością serca oraz migotaniem przedsionków.

Analiza jednoczynnikowa (Tabela3.) przeprowadzona dla poszczególnych subpopulacji wykazała, że u pacjentów z przewlekłym zespołem wieńcowym, przewlekłą niewydolnością serca i migotaniem przedsionków podwyższone stężenie TMAO istotnie wpływało na śmiertelność.

Stwierdzony wzrost ryzyka w tych subpopulacjach nie jest jednak istotny klinicznie (HR1,01-1,02). W analizie wieloczynnikowej (Tabela 3.) stężenie TMAO w tych podgrupach nie miało istotnego wpływu na śmiertelność 5- letnią.

Tabela 2. Stężenie TMAO w podgrupach pacjentów (mediana, rozstęp kwartylowy)

Podgrupa badana	Cała podgrupa	Zgon w trakcie 5- letniej obserwacji		wartość p
Ostry zespół wieńcowy (n=177)	4,27 (2,87-5,92)	**Tak (n=37)**	**Nie (n=140)**	p=0,23
		4,42 (3,31-7,45)	4,15 (2,83-5,71)	
Przewlekły zespół wieńcowy (n=441)	4,29 (2,94-6,67)	**Tak (n=96)**	**Nie (n=345)**	p<0,00001
		6,26 (2,70-10,1)	4,00 (2,84-5,88)	
Przewlekła niewydolność serca (n=292)	4,91 (3,37-7,44)	**Tak (n=93)**	**Nie (n=199)**	p<0,001
		5,84 (4,12-8,94)	4,65 (3,27-6,74)	
Migotanie przedsionków (n=277)	4,86 (3,37-7,41)	**Tak (n=74)**	**Nie (n=203)**	p<0,0001
		6,61 (4,57-10,19)	4,46 (3,23-6,41)	

Tabela 3. Wyniki analizy jedno i wieloczynnikowej proporcjonalnego hazardu Coxa wpływu stężenia TMAO na śmiertelność 5- letnią w podgrupach pacjentów

Podgrupa badana	hazard względny (HR)	95% CI	wartość p
Analiza jednoczynnikowa proporcjonalnego hazardu Coxa			
Ostry zespół wieńcowy (n=177)	1,00	0,98-1,01	p=0,95
Przewlekły zespół wieńcowy (n=441)	1,01	1,00-1,02	p=0,001
Przewlekła niewydolność serca (n=292)	1,01	1,00-1,03	p=0,033
Migotanie przedsionków (n=277)	1,02	1,00-1,04	p=0,018
Analiza wieloczynnikowa proporcjonalnego hazardu Coxa			
Ostry zespół wieńcowy (n=180)	1,00	0,98-1,02	p=0,70
Przewlekły zespół wieńcowy (n=441)	1,02	0,99-1,04	p=0,089
Przewlekła niewydolność serca (n=292)	1,00	0,98-1,02	p=0,56
Migotanie przedsionków (n=277)	1,01	0,98-1,03	p=0,42

5.2.4. Korelacja stężenia TMAO z danymi klinicznymi

Dla danych ilościowych stężenie TMAO korelowało istotnie ($p<0,05$; korelacja R Spearmana) dodatnio z wiekiem pacjenta, poziomem hsCRP, HbA1c i trójglicerydów oraz ujemnie z wartością frakcji wyrzutowej lewej komory serca, wskaźnikiem eGFR i stężeniem cholesterolu frakcji HDL. Dla danych dychotomicznych stężenie TMAO korelowało istotnie ($p<0,05$; korelacja R Spearmana) dodatnio z obecnością cukrzycy, przewlekłego zespołu wieńcowego, przewlekłej niewydolności serca, nadciśnienia tętniczego i migotania przedsionków. Wszystkie wskaźniki wyżej wymienionej korelacji były słabe lub bardzo słabe, przy czym najsilniejsza z nich, ujemna, dotyczyła wartość przesączania kłębuszkowego.(Tabela 4.)

Tabela 4.Korelacje stężenia TMAO z danymi klinicznymi. Wszystkie przedstawione zmienne korelują istotnie ze stężeniem TMAO ($p<0,05$)

Zmienna	Wskaźnik korelacji R Spearmana
Wiek	0,28
hsCRP	0,12
HbA1c	0,27
TG	0,09
LVEF	-0,12
GFR	-0,42
HDL	-0,14
Cukrzyca	0,24
Przewlekły zespół wieńcowy	0,10
Przewlekła niewydolność serca	0,24
Nadciśnienie tętnicze	0,17
Migotanie przedsionków	0,19

5.2.5. Wielochorobowość a stężenie TMAO

Ze względu na częstą wielochorobowość pacjentów w grupie badanej prześledzono skalę tego zjawiska i jego związek ze stężeniem TMAO. Oceniano współwystępowanie ostrego zespołu wieńcowego, przewlekłego zespołu wieńcowego, przewlekłej niewydolności serca, migotania przedsionków, nadciśnienia tętniczego i rozpatrywanych łącznie zaburzeń rytmu serca lub zaburzeń przewodnictwa. Obecność tylko jednego z w/w schorzeń obserwowano u 130 pacjentów, dwóch u 260 pacjentów, trzech u 338 pacjentów, czterech u 191 pacjentów, pięciu u 85 pacjentów i wszystkich sześciu u 32 pacjentów. Wielochorobowość korelowała istotnie dodatnio ze stężeniem TMAO (wskaźnik korelacji tau Kendalla = 0,20 przy $p<0,05$). Obserwowano również istotne różnice w stężeniu TMAO pomiędzy wymienionymi podgrupami, w szczególności pomiędzy pacjentami z jednym lub dwoma zdiagnozowanym schorzeniami kardiologicznymi i pozostałymi.

Tabela 5. Stężenie TMAO w zależności od liczby zdiagnozowanych schorzeń kardiologicznych u danego pacjenta. W teście Kruskala-Wallisa istotne różnice obserwowano pomiędzy stężeniami TMAO w 1 i 2 grupie a pozostałymi (wartość statystyki $H=76,74$ i $p<0,0001$)

	Liczba zdiagnozowanych schorzeń kardiologicznych					
	1	**2**	**3**	**4**	**5**	**6**
TMAO(μM) (mediana, rozstęp kwartylowy)	**3,02** (2,19-4,38)	**3,47** (2,61-4,98)	**4,31** (2,93-6,41)	**4,55** (3,23-6,87)	**5,54** (3,61-8,56)	**5,93** (3,35-10,36)

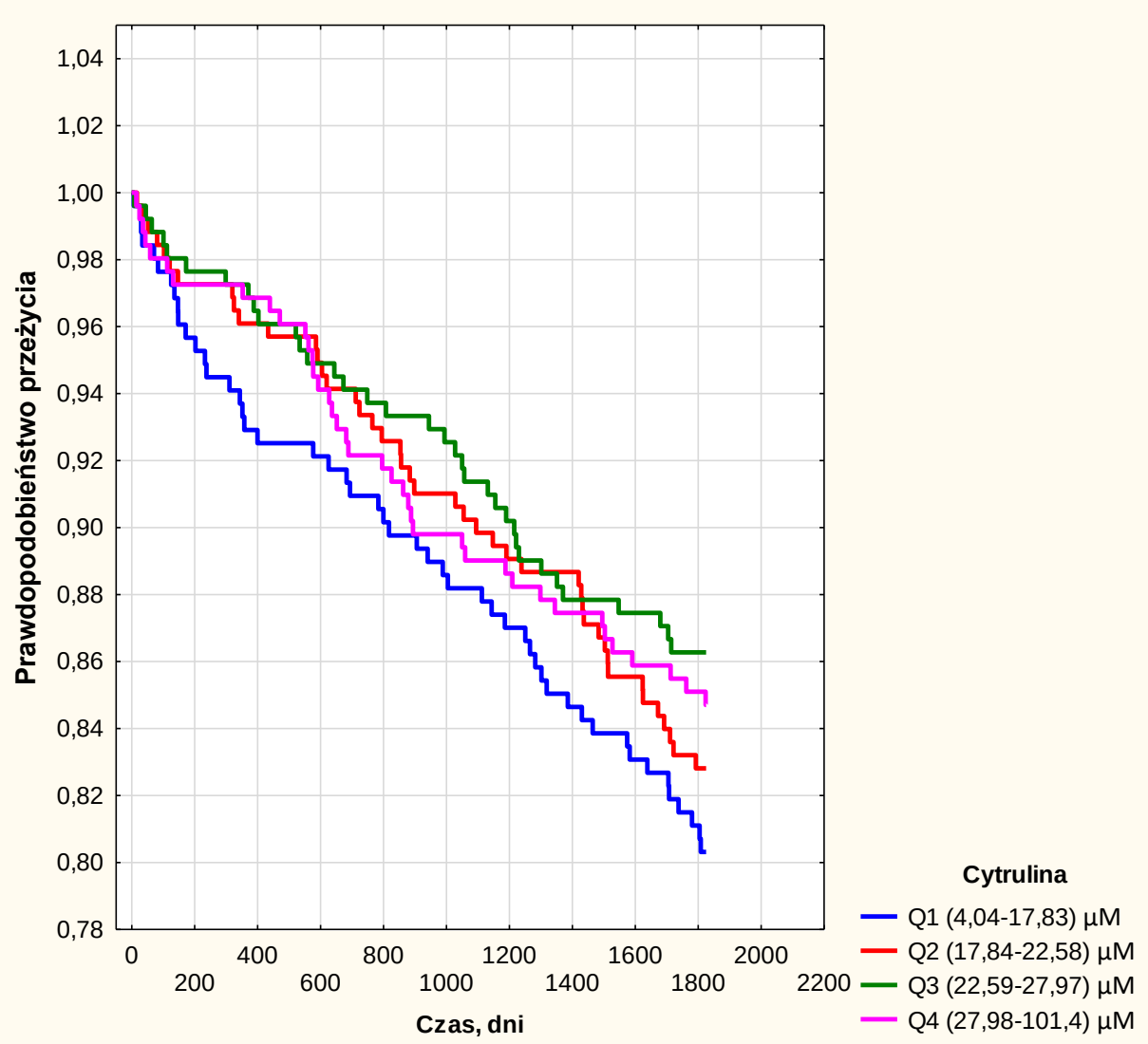
Omawiana praca wchodząca w skład rozprawy doktorskiej jest pierwszą, która ocenia zależność stężenia TMAO w surowicy ze śmiertelnością polskich pacjentów obciążonych chorobami układu krążenia. Badanie przeprowadzono na stosunkowo dużej populacji pacjentów przy długim okresie obserwacji. Dzięki temu, wyniki uzyskane zarówno dla populacji ogólnej oraz wyodrębnionych subpopulacji są statystycznie reprezentatywne. Ponieważ do badania rejestrowano kolejnych hospitalizowanych pacjentów, uzyskane wyniki można odnosić do ogólnej populacji pacjentów hospitalizowanych na polskich oddziałach kardiologicznych. Uwzględnione liczne parametry biochemiczne, antropomorficzne oraz dokładny wywiad chorobowy pozwoliły na przeprowadzenie szerokiej analizy wieloczynnikowej.

Przyjęta metodyka uniemożliwia uznanie stężenia TMAO jako istotnego klinicznie i niezależnego predykatora zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia. W omawianej pracy wykazano liczne korelacje stężenia TMAO z innymi parametrami klinicznymi. Procesy patofizjologiczne wiązane z TMAO, w praktyce klinicznej mogą współistnieć w różnym nasileniu i podlegają wpływom wielu zmiennych w czasie czynników zewnętrznych. W związku z tym, określenie TMAO jako czynnika sprawczego lub jedynie towarzyszącego chorobom układu krążenia jest bardzo trudne. Implikuje to także ostrożność w interpretacji dotychczasowych badań, w których TMAO wydawał się być obiecującym predykatorem zgonu.

5.2.6. Wpływ stężenia cytruliny na śmiertelność w całej grupie badanej

Trzecia praca z cyklu, **Citrulline and long-term mortality in patients with cardiovascular disease** stanowi rozwinięcie poprzedniej analizy o ocenę wpływu uszkodzenia bariery jelitowej- mierzonego stężeniem cytruliny na śmiertelność w badanej populacji.

W całej badanej populacji mediana stężenia cytruliny w surowicy wynosiła 22,5μM (IQR 17,8-27,9). Nie stwierdzono istotnej różnicy stężenia cytruliny pomiędzy grupą pacjentów zmarłych oraz przeżywających 5-letni okres obserwacji. Nie wykazano także statystycznie istotnego wpływu stężenia cytruliny na śmiertelność pacjentów z CVD w obserwacji 5-letniej HR 0.99 (95% CI 0.97-1.00 p=0,49). W analizie z użyciem podziału kwartylowego, stężenie cytruliny także nie wpływało na śmiertelność pacjentów (Wykres 3)



Wykres 3. Krzywe Kaplana-Meiera prawdopodobieństwa przeżycia w czterech podgrupach pacjentów podzielonych na podstawie kwartyli stężenia cytruliny. Q1, Q2, Q3, Q4 – kwartyle 1, 2, 3 i 4.

5.2.7. Wpływ stężenia cytruliny na śmiertelność w podgrupach

W celu pogłębionej analizy wpływu stężenia cytruliny na śmiertelność u pacjentów z chorobami układu krążenia przeanalizowano jego związek w podgrupach pacjentów z ostrym zespołem wieńcowym, przewlekłym zespołem wieńcowym, przewlekłą niewydolnością serca i migotaniem przedsionków. Stężenie cytruliny w podgrupach z dodatkowym podziałem na pacjentów zmarłych i przeżywających obserwację 5-letnią przedstawiono w Tabeli 6. Jedynie w podgrupie pacjentów z ostrym zespołem wieńcowym obserwowano istotnie mniejsze

stężenie cytruliny w grupie pacjentów, którzy zmarli w trakcie obserwacji 5-letniej w porównaniu z pacjentami, którzy przeżyli: 20,9 μM (15,2-25,8) vs 24,0 μM (18,5-30,1) p=0,025.

Tabela 6. Stężenie cytruliny w podgrupach pacjentów (mediana, rozstęp kwartylowy).

Podgrupa badana	Cała podgrupa	Zgon w trakcie 5-cio letniej obserwacji		wartość p
Ostry zespół wieńcowy (n=177)	23,3 (18,3-29,1)	**Tak (n=37)**	**Nie (n=140)**	p=0,025
		20,9 (15,2-25,8)	24,0 (18,5-30,1)	
Przewlekły zespół wieńcowy (n=441)	22,2 (17,5-28,1)	**Tak (n=96)**	**Nie (n=345)**	p=0,12
		21,0 (15,7-21,3)	22,4 (18,0-28,4)	
Przewlekła niewydolność serca (n=292)	22,2 (17,9-28,2)	**Tak (n=93)**	**Nie (n=199)**	p=0,50
		21,7 (16,6-28,2)	22,3 (18,3-28,3)	
Migotanie przedsionków (n=277)	22,2 (17,7-28,5)	**Tak (n=74)**	**Nie (n=203)**	p=0,89
		22,9 (16,5-30,1)	22,1 (18,1-28,4)	

5.2.8. Korelacje stężenia cytruliny z danymi klinicznymi

Nie wykazano istotnych statystycznie korelacji stężenia cytruliny z analizowanymi danymi klinicznymi.

5.2.9. Wielochorobowość a stężenie cytruliny

Nie obserwowano związku pomiędzy wielochorobowością, a stężeniem cytruliny.

5.2.10. Korelacja stężenia TMAO i cytruliny

Nie wykazano istotnej korelacji pomiędzy stężeniami TMAO i cytruliny (korelacja tau Kendala = 0,027).

Według wiedzy autorów jest to pierwsza praca oceniająca przydatność stężenia cytruliny jako niezależnego czynnika rokowniczego pacjentów z CVD w obserwacji długoterminowej. Uzyskane stężenia cytruliny są dość dalekie od normy. Pośrednio może to świadczyć o utracie nabłonka jelitowego w badanej populacji. Pomimo to, uzyskane wyniki uniemożliwiają uznanie stężenia cytruliny jako niezależnego predykatora zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia.

Oznaczenie cytruliny było wykorzystywane dotychczas głównie do oceny rokowania pacjentów w stanie krytycznym lub hospitalizowanych w oddziałach intensywnej terapii. W pracy będącej częścią cyklu publikacji, istotną statystycznie różnicę stężeń cytruliny w zależności od wystąpienia zgonu zaobserwowano jedynie w podgrupie pacjentów z OZW. Można uznać, że wśród całej badanej populacji, ta grupa pacjentów najlepiej odpowiada definicji stanu nagłego. Nie można więc wykluczyć, że wartość diagnostyczna i prognostyczna cytruliny jest wyższa w przypadku stanów ostrych niż chorób przewlekłych. Stoi to w sprzeczności z obniżeniem stężenia cytruliny w przebiegu przewlekłych chorób pierwotnie związanych z utratą nabłonka jelitowego jak choroba Crohna, celiakia czy zespół krótkiego jelita. Możliwe, że zmiany stężenia cytruliny w przebiegu przewlekłych chorób układu krążenia wykazują inną dynamikę i podlegają innym mechanizmom regulacyjnym. Wykazany pracy brak korelacji stężenia cytruliny z wielochorobowością pacjentów z CVD wydaje się przemawiać za takim wytłumaczeniem. Największe zaskoczenie budzi jednak brak statystycznie istotnej korelacji pomiędzy stężeniem cytruliny a danymi klinicznymi- w tym funkcją nerek (eGfr) czy parametrami stanu zapalanego (hscrp) w badanej populacji. Ścisłe powiązanie metabolizmu cytruliny z funkcją nerek czy nasileniem stanu zapalnego powinno ostatecznie wpłynąć na stężenie cytruliny, czego nie stwierdzono w przedstawionej pracy.

Przedstawione w pracy wyniki stanowią przede wszystkim ważne uzupełnienie badań wpływu uszkodzenia bariery jelitowej na wchłanianie TMAO u pacjentów obciążonych chorobami układu krążenia. Postulowana w literaturze potrzeba takiej analizy nie została dotychczas zrealizowana na tak dużej populacji jak w przedstawionej pracy. Dodatkowo, do oceny bariery jelitowej w kontekście wchłaniania TMAO wykorzystywano dotychczas biomarkery inne niż cytrulina. Brak istotnej korelacji stężenia cytruliny i TMAO wskazuje na inny, nieznany mechanizm transportu TMAO, który jest prawdopodobnie niezależny od ilości enterocytów.

6. Wnioski

1.W analizie jednoczynnikowej stężenie TMAO jest czynnikiem rokowniczym zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia. W analizie wieloczynnikowej zależność ta traci istotność statystyczną.

2.Uszkodzenie bariery jelitowej, mierzone stężeniem cytruliny w surowicy nie jest czynnikiem ryzyka zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia.

3.Nie występuje korelacja pomiędzy stężeniem TMAO oraz cytruliny w surowicy.

7. Ograniczenia metodologiczne prac stanowiących cykl publikacji

Cykl publikacji, będący przedmiotem rozprawy doktorskiej na każdym etapie tworzony był z intencją zachowania należytej staranności. Jako uzupełnienie wniosków należy wskazać ograniczenia metodologiczne, które potencjalnie mogły mieć wpływ na uzyskane wyniki oraz ich ostateczną interpretację.

a) Ramy objętościowe pracy poglądowej nr 1 uniemożliwiły omówienie wszystkich zagadnień związanych z metabolizmem TMAO. Potrzeba zamieszczenia w jednej publikacji zagadnień związanych z patofizjologią oraz wynikami dotychczasowych badań wymusiła także przedstawienie zgromadzonych danych w formie tabel oraz symbolicznych grafik.

Prace oryginalne 2 i 3 objęły tę samą populację i wykorzystywały podobne założenia metodologiczne, w związku z tym wykazują podobne ograniczenia.

b) Obydwa badania miały charakter retrospektywno- obserwacyjny i objęły pacjentów hospitalizowanych w jednym ośrodku. Rozwiązaniem tego problemu byłoby prospektywne badanie wieloośrodkowe, co często postuluje się w dotychczasowych badaniach dotyczących TMAO.

c) Retrospektywny charakter badań, pomimo analizy wieloczynnikowej nie wyklucza istnienia innych, niekontrolowanych czynników, które mogły mieć wpływ na wyniki badań laboratoryjnych oraz częstość zgonów. Wyniki prac 2 i 3 opierają się na pojedynczym pomiarze stężenia TMAO oraz cytruliny. Parametry te są zmienne w czasie i mogą zależeć od wielu czynników. Spośród najważniejszych, nie dysponujemy informacjami na temat wywiadu stosowania antybiotykoterapii w czasie poprzedzającym pobranie próbki surowicy, stężenia prekursorów TMAO, składu mikrobioty jelitowej oraz nawyków żywieniowych pacjentów. Czynniki te maksymalnie zredukowano pobierając próbki surowicy w możliwie podobnych warunkach dla wszystkich hospitalizowanych pacjentów oraz rejestrując liczne dostępne parametry kliniczne.

Optymalnym rozwiązaniem ograniczeń wynikających z użycia pojedynczego pomiaru stężenia cytruliny byłoby łączne oznaczenie kilku różnych biomarkerów bariery jelitowej, w seryjnych pomiarach, w odstępach czasowych dostosowanych do okresu obserwacji krótkoterminowej i długoterminowej. Dodatkowych danych dostarczyłaby także równoczesna ocena

histopatologiczna bioptatów śluzówki jelita lub testy czynnościowe wchłaniania z przewodu pokarmowego. Realizacja takiego planu, na tak dużej populacji pacjentów wydaje się jednak bardzo trudna ze względów organizacyjnych oraz finansowych. Należy także uwzględnić inwazyjność niektórych z wymienionych metod.

d) Dane dotyczące zgonów pacjentów w badanej populacji nie uwzględniają ich przyczyny. Długi czas obserwacji może sugerować, że nie wszystkie zarejestrowane zgony były konsekwencją chorób układu krążenia. Niestety rozwiązanie tego problemu w polskich warunkach może być trudne ze względu na duży udział tzw. kodów śmieciowych (ang. Garbage codes) powodujących nieścisłości w statystykach przyczyn zgonów.

e) W okresie obserwacji prac 2 i 3 nie rejestrowano kolejnych hospitalizacji, istotnych zdarzeń sercowo-naczyniowych, późniejszych chorób towarzyszących, zmian w farmakoterapii oraz podjętych przez pacjentów zmian stylu życia. Czynniki te mogłyby wpływać na stężenie TMAO, cytruliny a ostatecznie na śmiertelność pacjentów w okresie po rejestrowanej hospitalizacji.

f) Dysponujemy danymi z dostępnych publikacji o prawidłowych poziomach TMAO i cytruliny, jednak nie dysponujemy grupą kontrolną rekrutowaną równolegle z naszą grupą badaną w celu porównania wpływu poziomu omawianych cząsteczek na ryzyko zgonu.

9. Cykl prac

9.1. Publikacja nr 1

Reviews

Trimethylamine N-oxide in cardiovascular disease

Radosław Andrzej Konieczny[1,A–D], Wiktor Kuliczkowski[2,C,E,F]

[1] Clinical Department of Gastroentrology and Hepatology, Wroclaw Medical University, Poland
[2] Institute for Heart Diseases, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2022;31(8):913–925

Address for correspondence
Radosław Andrzej Konieczny
E-mail: rkonieczny.med@gmail.com

Funding sources
None declared

Conflict of interest
None declared

Received on December 20, 2021
Reviewed on March 17, 2022
Accepted on March 24, 2022

Published online on April 19, 2022

Abstract

Although traditional cardiovascular risk factors are well established and understood, mortality and morbidity in patients with cardiovascular disease (CVD) remains high. Exploring new pathophysiological pathways enables a better understanding of CVD at both the molecular and clinical levels. Gut microbiota as a potential modulator of CVD are the subject of extensive research. In recent years, trimethylamine N-oxide (TMAO), a biologically active molecule generated by the gut microbiota, has been widely tested in studies on various populations of patients. The ultimate TMAO levels depend on individual features and gut microbiota composition. Most of the research on TMAO has focused on atherosclerotic CVD and heart failure (HF). Studies conducted so far support the use of TMAO as a prognostic marker in CVD. Several studies describe diverse interventions aimed at reducing the concentration of TMAO and its harmful effects. This article summarizes the findings from research, discusses the major insights into TMAO metabolism and related pathophysiological processes, as well as indicates the directions for future research.

Key words: gut microbiota, coronary artery disease, heart failure, TMAO, trimethylamine oxide

Cite as
Konieczny RA, Kuliczkowski W. Trimethylamine N-oxide in cardiovascular disease. *Adv Clin Exp Med*. 2022;31(8):913–925. doi:10.17219/acem/147666

DOI
10.17219/acem/147666

Introduction

According to the World Health Organization (WHO), cardiovascular disease (CVD) remains the major cause of death and disability worldwide.[1] Exploring new pathophysiological pathways enables a better understanding of CVD at both the molecular and clinical levels. Owing to the development of metabolomics and metagenomics, the intestinal microbiota have been indicated as a potential modulator of the course of CVD. Trimethylamine N-oxide (TMAO) is a gut-derived metabolite whose usefulness has been evaluated in numerous studies. Despite promising results, TMAO is an example of a deeply researched gut microbiome biomarker which is still not used in everyday clinical practice. In order to determine the utility of a new biomarker, it is first necessary to assess its metabolism and related pathophysiological processes, followed by clinical trials.[2] This review summarizes extensive literature on TMAO and indicates gaps in knowledge existing after more than 10 years of research.

Objectives

The purpose of this article was to provide an overview of the metabolism of TMAO and associated pathophysiological processes, and the results of major studies. An attempt was also made to indicate the direction of further research.

Methodology

Literature search was carried out in the PubMed database on November 3, 2021 using the queries: "(TMAO) AND (atherosclerosis)", "(TMAO) AND (coronary artery disease)", "(TMAO) AND (atrial fibrillation)", "(TMAO) AND (heart failure)", and "(TMAO) AND (gut microbiota)". Selected key studies concern diverse groups of patients with CVD. Their results are discussed in the text and presented in tables below.

Metabolism of TMAO

The TMAO is an organic compound with the chemical formula of $(CH_3)_3NO$. It is commonly found in the tissues of marine organisms, where it mitigates the adverse effects of temperature, salinity, as well as high urea and hydrostatic pressure.[3] In humans, TMAO is produced by the oxidation of trimethylamine (TMA) and is absorbed directly from food. The TMAO is most abundant in fish and seafood.[4] Gut microbiota produce TMA from the dietary precursors: choline, L-carnitine, and betaine. These TMA precursors are most abundant in red meat and eggs.[5] The most recent research indicates that the intake of foods rich in TMA precursors does not translate directly into an increase in plasma TMAO level because it depends on individual metabolic features, such as hepatic enzymes activity and gut microbiota composition.[6,7]

Carnitine metabolism is key to human TMAO production and 3 major bacterial metabolic pathways leading to TMA synthesis from dietary precursors were described[8]:

a) anaerobic choline degradation by choline TMA-lyase;

b) hydroxylation of L-carnitine to TMA by carnitine oxidoreductase; and

c) conversion of L-carnitine to γ-butyrobetaine, which is then converted to TMA.

Trimethylamine produced by gut microbiota is excreted from the gut via 3 mechanisms: it can be absorbed to the circulation, excreted with stool or used by other bacteria in the process of syntrophy. Trimethylamine that has been absorbed to the circulation is oxidized to TMAO by hepatic flavin-containing monooxygenase (FMO).[5] Trimethylamine absorption occurs in the small intestine (Fig. 1).[9] After absorption, TMA is almost immediately oxidized to TMAO. Following the oral intake of phosphatidylcholine, the highest plasma and urinary TMAO levels are observed after 12 h and 24 h, respectively. After 48 h from intake, the plasma TMAO level returns to baseline.[9] The TMAO taken with food is absorbed through the intestinal barrier and is detectable in blood after 15 min. The maximum blood concentration is reached after 1 h and is maintained for approx. 6 h. After 24 h, 96% of the TMAO dose taken with food is excreted with urine, mostly in an unchanged form.[10]

To determine the concentration of TMAO, liquid chromatography coupled with tandem mass spectrometry and automated nuclear magnetic resonance spectrometry are most often used.[4] Due to the need of specialized equipment, reliable TMAO determination is possible in research or academic facilities; however, mentioned methods become more available.

The effect of TMAO on pathophysiological processes

The first studies reporting the negative effects of TMAO were conducted on an animal model and focused on atherogenesis. Initially, it was determined that TMAO accelerates the production of foam cells from macrophages. The TMAO was shown to promote the upregulation of the scavenger receptors CD36 and SR-A1[11] as well as induce inflammation[12] via the MAPK/JNP pathway, which regulates the synthesis of pro-inflammatory cytokines – tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and intercellular adhesion molecule 1 (ICAM-1). This leads to cholesterol overload in macrophage foam cells and their faster migration and adhesion to endothelial cells. A study on human umbilical vein endothelial cells (HUVECs) confirmed a link between high plasma TMAO levels and the development of atherosclerosis. Moreover, it indicated that TMAO impaired endothelial self-repair

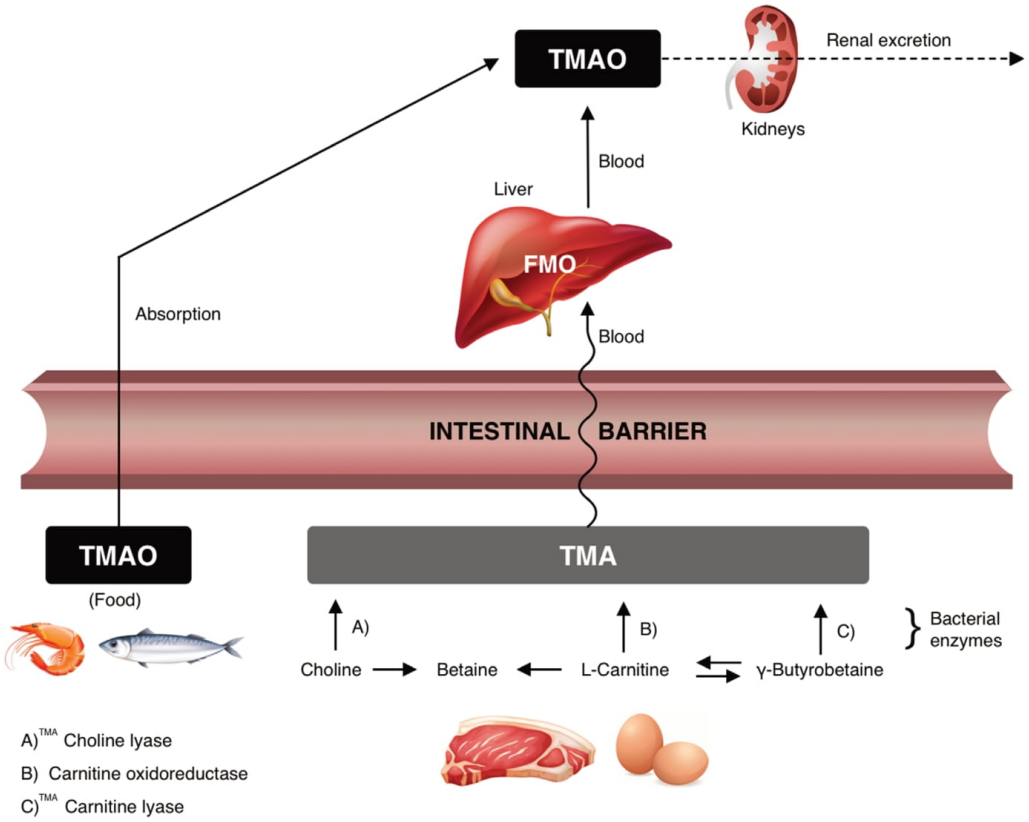


Fig. 1. Schematic presentation of the intestinal absorption of TMA and TMAO

TMA – trimethylamine; TMAO – trimethylamine N-oxide; FMO – flavin-containing monooxygenase.

already in the early stages of atherogenesis.[13] This is due to the inhibitory effect of TMAO on endothelial cell proliferation during the G0/G1 phase of the cell cycle[14] as well as its cytotoxic effect on circulating endothelial progenitor cells.[15] This cascade of events is accompanied by increased oxidative stress, another postulated effect of chronically elevated TMAO levels, observed also in healthy individuals.[16] The effect of TMAO on pathophysiological processes is depicted in Fig. 2.

At a systemic level, TMAO promotes atherogenesis by altering lipid metabolism. In a study on a mice model, Koeth et al. demonstrated that TMAO inhibited the expression of the Cyp7a1 enzyme and bile acid transport proteins.[17] The Cyp7a1 is responsible for bile acid synthesis and inhibition of cholesterol catabolism. The lack of Cyp7a1 leads to the reduced bile acid synthesis and secretion, resulting in atherosclerosis progression. At the same time, a reduction in the expression of bile acid transporter proteins in the liver negatively affects the major pathway of elimination of cholesterol from the body.[17]

Along with research on atherosclerosis, there have been studies investigating the prothrombotic effect of TMAO. Impaired intracellular calcium ion transport in platelets, heightened platelet reactivity and increased platelet adhesion to collagen fibers were reported.[18] In endothelial cells, an increased tissue factor synthesis and downregulation of thrombomodulin were described.[19]

Direct cardiotoxic and proarrhythmic effects of TMAO

The cardiotoxicity of TMAO was confirmed in morphological and functional studies, mainly in animal models. By activating the inflammatory pathways, TMAO promotes cardiac fibrosis, heart weight gain and cardiac remodeling.[20] At a cellular level, TMAO impairs the intracellular microtubule network and alters calcium concentration control in cardiac muscle cells. This leads to a decrease in contraction amplitude, longer time of peak and reduced

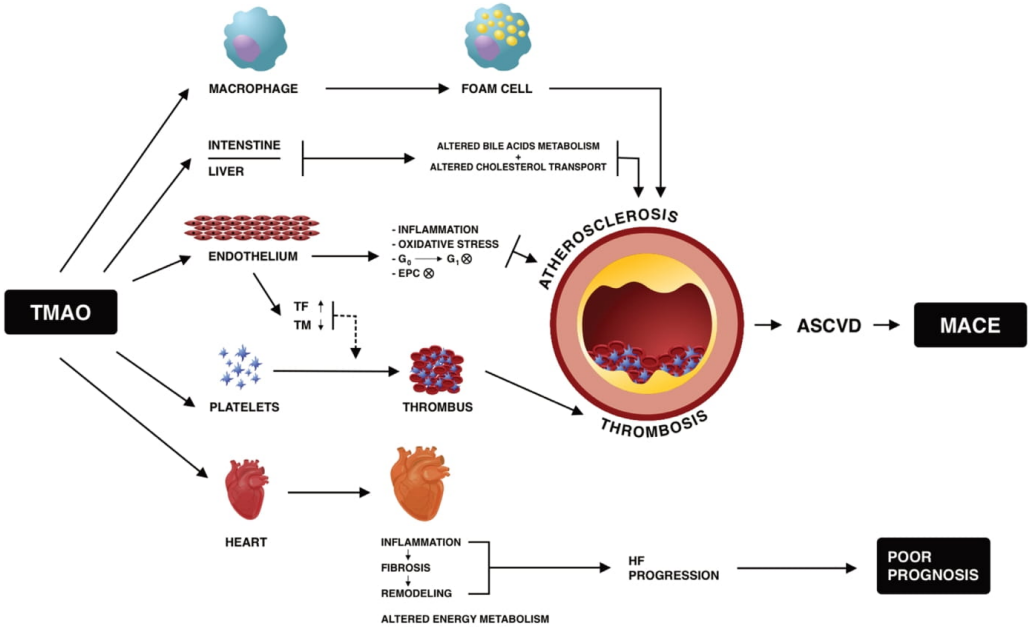


Fig. 2. Effect of TMAO on pathophysiological processes

TMAO – trimethylamine N-oxide; ASCVD – atherosclerotic cardiovascular disease; EPC – endothelial progenitor cells; HF – heart failure; MACE – major cardiovascular events; TF – tissue factor; TM – thrombomodulin.

DRUGS	SURGERY	DIET	GUT MICROBIOTA MODIFICATION
• STATIN [56] • ASPIRIN [57] • RIFAXIMIN [60] • RESVERATROL [64] • MELDONIUM [65]	• GASTRIC BYPASS [63] • DUODENAL SWITCH [63]	• DISCONTINUATION OF RED MEAT [5]	• Probiotics: -Saccharomyces Boulardi [60] -VSL#3 [61] • Fecal microbiota transplant [62]

Fig. 3. Therapeutic strategies affecting TMAO levels and metabolism in humans. Detailed information included in Table 3, with consistent reference numbers

TMAO – trimethylamine N-oxide.

synchronization.[21] Similar TMAO-induced abnormalities in contractility were also reported in ex-vivo human cardiac tissue.[22]

Results from studies on TMAO

Over the past 10 years, numerous studies investigating the prognostic value of TMAO have been conducted. The research included various populations of patients, both with acute and chronic illness. Most of those studies enrolled patients with coronary artery disease (CAD) and heart failure (HF). Selected studies are discussed below and summarized in Table 1.

The first meaningful study on the effect of TMAO in CAD was published in 2013.[23] Tang et al. demonstrated that higher plasma TMAO levels correlated with an increased risk of major adverse cardiovascular events (MACEs) during a 3-year follow-up in 4007 patients referred for elective coronary angiography.[23] The correlation was revealed even after adjustment for traditional risk factors. Similar findings were reported by Senthong et al.

in a study of 2235 patients with significant coronary artery stenosis receiving optimal treatment. Higher TMAO levels predicted mortality independent of traditional risk factors during a 5-year follow-up.[24] In a longitudinal study by Lee et al.,[25] a significant association between higher levels of TMAO and increased risk of incident and recurrent atherosclerotic CVD was shown.[25] Another large community-based study of middle-aged participants revealed that

Table 1. Results of studies on the association of TMAO with cardiovascular risk

Author	Type of study	Population characteristics	Follow-up	Study endpoint	Results HR/OR (95% CI)	TMAO [μmol/L], median (IQR)
Coronary artery disease						
Tang et al.[23]	prospective study	n = 4007; patients undergoing elective coronary angiography USA age: 63 ±11 male sex: 64%	3 years	MACE (death, myocardial infarction, stroke)	TMAO Q4 compared to Q1 MACE n = 513, HR 2.54 [1.96; 3.28]; p < 0.001 multivariate HR 1.43 [1.05; 1.94] death HR 3.37 [2.39; 4.75]; p < 0.001 nonfatal myocardial infarction or stroke HR 2.13 [1.48; 3.05]; p < 0.001	3.7 (2.4–6.20) MACE 5.0 (3.0–8.8) no MACE 3.5 μM (2.4–5.9); p < 0.001
Senthong et al.[24]	prospective study	n = 2235; patients with stable coronary artery disease who underwent elective coronary angiography USA age: 63 ±11 years male sex: 71%	5 years	death (all-cause)	TMAO Q4 compared to Q1 death n = 338 HR 3.90 [2.78; 5.48]; p < 0.0001	3.8 (2.5–6.5) Q4 > 6.5; 9.7 (7.7–14.9)
Lee et al.[25]	prospective multicenter community-based cohort study	n = 5580 USA age: 72 ±5.3 years male sex: 36% a) participants free of prevalent cardiovascular disease (n = 4131) age: 72.2 ±5.3 years male sex: 36% b) participants with prevalent cardiovascular disease (n = 1449) age: 73.6 ±5.8 years male sex = 53%	15 years	ASCVD defined as MI (fatal and nonfatal), fatal coronary heart disease, stroke (fatal and nonfatal), sudden cardiac death, and other atherosclerotic death	quintile 5 compared to quintile 1 multivariable HR 1.23 [1.04; 1.45]; p = 0.028 multivariable, diet and renal function adjusted HR 1.08 [0.91; 1.29]; p = 0.579 a) multivariable, diet and continuous eGFR, adjusted HR 1.07 [0.90; 1.27]; p = 0.516 b) multivariable HR 1.25 [1.01; 1.56]; p = 0.009 multivariable, diet and continuous eGFR, HR 1.10 [0.87; 1.39]; p = 0.179	4.7 (3.2–7.7) quintile 1 = 2.29 (1.84–2.61) quintile 5 = 13.2 (10.4–19.9) a) 4.72 (3.19–7.69) eGFR 70.1 (16.2) b) 5.43 (3.57–8.74) eGFR 63.8 (17.9)
Tang et al.[26]	nested case-control study	n = 2181; healthy individuals Europe age: 65 ±8 years male sex: 65%	8 years	CAD – hospital admission and/or death with CAD as underlying cause (ICD9 Code 410–414)	Q4 compared to Q1 n = 908 OR 1.86 [1.46; 2.37]; p < 0.001 adjusted for traditional risk factors OR 1.58 [1.21; 2.06]; p < 0.001	3.4 (2.3–5.7)
Suzuki et al.[27]	retrospective study	n = 1079; acute MI patients UK age: 67 (57–77) years male sex: 72%	2 years	all-cause mortality death/MI	TMAO T3 compared to T1 n = 292 events all-cause mortality n = 119 HR 1.21 [0.98; 1.48] p = 0.074 death/MI n = 232 HR 1.40 [1.26; 1.55] p < 0.0005 multivariate HR 1.21 [1.03; 1.4] p = 0.023	3.7 (4.6–6.4) T3 > 5.1; 8.5 (6.2–15.0)
Matsuzawa et al.[28]	observational study	n = 112; STEMI patients who underwent primary PCI Japan age: 63 (56–71) years male sex: 88%	median: 5.4 years	cardiovascular events	death n = 5 nonfatal myocardial infarction n = 5, unstable angina requiring revascularization n = 2 nonfatal stroke n = 5 TMAO >6.76 compared to <6.76 adjusted HR 6.21 [1.69; 30.285]; p = 0.005 adjusted HR for 0.1 increase in log TMAO 1.343 [1.122; 1.636]; p = 0.001	6.76 (3.82–12.53)

Table 1. Results of studies on the association of TMAO with cardiovascular risk – cont.

Author	Type of study	Population characteristics	Follow-up	Study endpoint	Results HR/OR (95% CI)	TMAO [μmol/L], median (IQR)
Li et al.[29]	prospective study	n = 530; patients presenting to the emergency department with chest pain of suspected cardiac origin (112 troponin T-positive) USA age: 62.4 ±13.9 years male sex: 57.5% n = 1683 who underwent coronary angiography for ACS Switzerland age: 63.9 ±12.4 years male sex: 77.8%	1 month, 6 months, 7 years, 1 year	MACE defined as a composite of MI, stroke, revascularization, or all-cause mortality (1 month, 6 months), all-cause mortality (7 years)	TMAO Q4 compared to Q1 MACE 1 month OR 6.30 [1.89; 21.0]; p < 0.01 MACE 6 months n = 220 (death n = 29) OR 5.65 [1.91; 16.7]; p < 0.01 mortality 7 years HR 1.81 [1.04; 3.15]; p < 0.05 MACE n = 119 (death n = 79) (1 year Q4 HR 1.57 [1.03; 2.41]; p < 0.05	4.28 (2.55–7.91) 2.87 (1.94–4.85)
Sheng et al.[30]	prospective observational study	n = 335; patients with STEMI China age: 58.7 ±12.1 years male sex: 80.6%	N/A	SYNTAX Score ≥23 presence of multivessel disease	adjusted OR 1.16 [1.06; 1.29]; p = 0.001 r = 0.237, p < 0.001 AUC 0.656 [0.591; 0.722]; p < 0.001 adjusted OR 1.15 [1.01; 1.32]; p = 0.035 r = 0.192, p < 0.001	2.18 (1.34–3.90)
Senthong et al.[31]	prospective cohort study	n = 353; stable patients with CAD detected by elective coronary angiography USA age: 65.0 ±11.0 years male sex: 79%	N/A	SYNTAX Score SYNTAX Score II presence of diffuse lesions	SYNTAX Score (r = 0.61) p < 0.0001 adjusted OR 4.82; p < 0.0001 SYNTAX Score II (r = 0.62) p < 0.0001 adjusted OR = 1.88; p = 0.0001 8.4 [5.7; 14.0] compared to 4.4 [5.2; 13.5] adjusted OR 2.05 [1.45; 2.90], p = 0.0001	5.5 mM (3.4–9.8)
Fu et al.[32]	observational study	n = 26 patients with CAD who underwent optical coherence tomography China age: 60 ±10 years male sex: 77% n = 12 – plaque rupture group n = 14 – non-plaque rupture group	N/A	TMAO concentration in rupture compared to non-rupture TMAO concentration and plaque composition	TMAO level – lipid arc (r = 0.43, p = 0.031), lipid volume index (r = 0.39, p = 0.048)	rupture compared to no rupture 8.6 ±4.8 compared to 4.2 ±2.4; p = 0.011
Tan et al.[33]	prospective observational study	n = 146; STEMI with pre-intervention optical coherence tomography China age: 57.0 ±11.0 years male sex: 82.2% n = 77 – plaque rupture n = 69 – plaque erosion	N/A	TMAO rupture compared to erosion	rupture adjusted OR 4.06 [2.38; 6.91]; p < 0.001 AUROC 0.89, 1.95 μM sensitivity = 88.3%, specificity = 76.8%	rupture compared to erosion 3.33 (2.48–4.57) compared to 1.21 (0.86–1.91); p < 0.001
Heart failure						
Tang et al.[37]	single-center prospective cohort study	n = 720; stable subjects with HF, patients with ACS within the preceding 30 days excluded USA age: 66 ±10 years male sex: 59%	5 years	all-cause mortality	death n = 207 Q4 compared to Q1 adjusted for traditional risk factors and BNP HR 2.2 [1.42; 3.43]; p < 0.001 adjusted for renal function HR 1.75 [1.07; 2.86]; p < 0.001	5.0 (3.0–8.5)
Trøseid et al.[38]	prospective observational study	n = 155; patients with stable HF for >6 months (NYHA class II–IV) Europe age: 57 ±11 years male sex: 83% n = 73 – CAD n = 75 – DCM n = 7 – other	median: 5.2 years	all-cause and anticipated mortality, i.e., HTx	death (n = 39) HTx (n = 16) T3 compared to T1 unadjusted HR 2.24 [1.28; 3.92]; p = 0.005 adjusted HR 1.79 [0.90; 1.79]; p = 0.097 NYHA class II/III/IV r = 0.15, p < 0.05	CAD – 12.1 ±19.5 DCM – 9.2 ±8.5 healthy control – 7.9 ±8.9

Table 1. Results of studies on the association of TMAO with cardiovascular risk – cont.

Author	Type of study	Population characteristics	Follow-up	Study endpoint	Results HR/OR (95% CI)	TMAO [μmol/L], median (IQR)
Zhou et al.[39]	prospective cohort study	n = 1208; patients with chronic HF after MI China age: 73 (64–80) years male sex: 68.5%	median: 1.84 years	MACE, all-cause mortality, HF rehospitalization, recurrent MI all-cause mortality	Q4 compared to Q1 MACE n = 507 death n = 56 readmitted with HF n = 384 recurrent MI n = 67 unadjusted HR 3.15 [2.09; 4.73]; p < 0.01 adjusted HR 2.31 [1.42; 3.59]; p < 0.01 all-cause mortality HR 2.15 [1.37; 3.24]; p < 0.01	Q4 > 7.92 Q1 < 2.83
Schuett et al.[40]	retrospective study	n = 2490; LURIC population Europe n = 823 – HF patients: HFrEF (n = 428) and HFpEF (n = 395)	mean: 9.7 years	all-cause mortality death due to cardiovascular causes	all patients T3 compared to T1 death n = 728 1.70 [1.41; 2.04]; p < 0.001 cardiovascular death n = 446 1.87 [1.48; 2.38] HFrEF T3 compared to T1 death 2.33 [1.67; 3.24]; p < 0.001 cardiovascular death 2.27 [1.52; 3.37] HFpEF – ns	T3 ≥ 5.92 T1 ≤ 3.90
Suzuki et al.[41]	retrospective study	n = 972; patients with acute HF UK age: 78 (69–84) years male sex: 61%	1 year	all-cause mortality (death) composite death or rehospitalization due to HF (death/HF)	death n = 268 T3 compared to T1 univariate HR 1.35 [1.21; 1.51]; p < 0.0005 n = 384 death/HF HR 1.33 [1.20; 1.46]; p < 0.0005 adjusted for renal function – ns	5.6 (3.4–10.5) T3 = 14.2 (8.2–151.5) T1 = 2.9 (0.5–4.0)
Israr et al.[42]	retrospective study	n = 806; patients with acute HF UK age: 78 (69–84) years male sex: 61%	1 year	death at 30 days n = 62 death at 1 year n = 213 death/HF at 30 days n = 98 death/HF at 1 year n = 313	T3 compared to T1 HR 1.39 [1.05; 1.84]; p = 0.022 HR 1.26 [1.08; 1.47] p = 0.004 HR 1.38 [1.10; 1.73] p = 0.006 HR 1.25 [1.09; 1.42] p = 0.001 adjusted for renal function – ns	10.2 (5.8–18.7)
Suzuki et al.[43]	multicenter prospective study (BIOSTAT-CHF)	n = 2234; patients with progressive worsening or new-onset symptoms of HF UK age: 70 (61–78) years male sex: 74%	3 years	all-cause mortality (3 years) composite event of mortality combined with rehospitalization due to HF (3 years)	unadjusted HR 2.27 [1.90; 2.72]; p < 0.001 adjusted |HR 1.42 [1.13; 1.80]; p = 0.003 unadjusted HR 1.93 [1.66; 2.23]; p < 0.001 adjusted HR 1.21 [1.00; 1.46]; p = 0.054	5.9 (3.6–10.8)
Wei et al.[44]	prospective study	n = 915; chronic HF patients with reduced ejection fraction China age: 57.1 ±14.1 years male sex: 69.9%	median: 33 months, max. 7 years	cardiovascular death or HTx (n = 314) recurrence of HF + first rehospitalization for cardiovascular causes	T3 compared to T1 HR 1.47 [1.13; 1.91]; p = 0.004 adjusted HR 1.33 [1.01; 1.74]; p = 0.039 high dose-dependent association: first rehospitalization for cardiovascular causes (p = 0.002) recurrence of HF (p = 0.003)	2.52 (1.20–4.76) T3 > 3.770 T1 ≤ 1.574
Atrial fibrillation						
Svingen et al.[47]	retrospective cohort study	n = 3797; patients with suspected stable angina Europe n = 3143; community-based control population Europe	median: 7.3 years community control 10.8 years	diagnosis of AF during hospitalization	m = 412 Q4 compared to Q1 adjusted HR 1.16 [1.05; 1.28]; p = 0.0009 community control n = 484 adjusted HR 1.10 [1.004; 1.19] per 1 standard deviation increase in log-transformed plasma TMAO	Q4 > 15.8 (11.9–23.5) Q1 < 2.7 (2.2–3.2)

Table 1. Results of studies on the association of TMAO with cardiovascular risk – cont.

Author	Type of study	Population characteristics	Follow-up	Study endpoint	Results HR/OR (95% CI)	TMAO [μmol/L], median (IQR)
Gong et al.[49]	prospective observational cohort study	n = 117; consecutive rheumatic heart disease patients with AF age: 57 (50–64) years male sex: 41% n = 25 – patients with cardiac thrombi n = 92 – patients without cardiac thrombi	N/A	comparison of TMAO concentration between 2 groups	TMAO, group I compared to group II: 4.55 [3.19; 4.83] compared to 3.53 [2.96; 4.25]; p = 0.01	N/A

ACS – acute coronary syndrome; DCM – dilated cardiomyopathy; MACE – major adverse cardiac event; TMAO – trimethylamine oxide; a – age; m – male; Q – quartile; T – tertile; HR – hazard ratio; OR – odds ratio; 95% CI – 95% confidence interval; IQR – interquartile range; AUROC – area under the receiver operating characteristic curve; ASCVD – atherosclerotic cardiovascular disease; CAD – coronary artery disease; MI – myocardial infarction; HF – heart failure; HFrEF – heart failure with reduced ejection fraction; HFpEF – heart failure with preserved ejection fraction; BNP – brain natriuretic peptide; HTx – heart transplantation; LURIC – Ludwigshafen Risk and Cardiovascular Health; PCI – percutaneous coronary intervention; SYNTAX – synergy between percutaneous coronary intervention with taxus and cardiac surgery score; STEMI – ST-segment elevation myocardial infarction; NYHA – New York Heart Association; eGFR – estimated glomerular filtration rate; N/A – not available/not applicable; AUC – area under the curve; AF – atrial fibrillation; N/A – not applicable; ns – nonsignificant.

higher TMAO levels are associated with the risk of CAD in previously healthy individuals.[26]

The prognostic value of TMAO was also assessed in patients with acute coronary syndrome (ACS). Increased TMAO levels showed an association with the risk of death or recurrent myocardial infarction (MI) during a 2-year follow-up,[27] as well as with future cardiovascular events during a 5-year follow-up.[28] Importantly, the risk persisted despite the improvement regarding traditional cardiovascular risk factors such as hypertension, dyslipidemia or diabetes.[28] In another interesting study, Li et al. showed that increased TMAO levels were a risk factor for MACEs at 30 days and 6 months in patients presenting with chest pain of suspected cardiac origin.[29] The strong prognostic significance of TMAO was observed irrespective of baseline troponin levels and the final diagnosis of ACS. In the same study, in an independent cohort of patients with ACS who underwent coronary angiography due to ACS, higher TMAO levels were associated with an increased risk of MACEs at 1 year, independent of traditional risk factors.

In addition to predicting future MACEs, plasma TMAO levels were shown to correlate with the extent of CAD and atherosclerotic plaque stability. Studies conducted to date have revealed an association between TMAO levels and the SYNTAX Score, multivessel CAD[30,31] and the risk of atherosclerotic plaque rupture.[32,33] Finally, it was reported that TMAO could act as a mediator of clopidogrel resistance and inhibit clopidogrel effects, which has significant implications for the medical treatment of CAD.[34]

In the context of atherosclerosis, TMAO has also been investigated in patients with chronic kidney disease (CKD).[35,36] As renal function deteriorates, TMAO level increases and correlates with coronary atherosclerosis burden,[35] gut microbiota alterations, increased intestinal permeability, chronic inflammation, and endothelial dysfunction.[36] Chronic kidney disease has complex pathogenesis and dynamics. The higher risk of death and the extent of atherosclerosis are therefore the cumulative effect of many processes. Accordingly, TMAO is one of the bystanders rather than the single and direct causative agent of vascular complications of CKD.

Heart failure

There is a considerable body of evidence on the role of TMAO as a prognostic marker in patients with HF. These associations were studied in the chronic and acute settings as well as depending on the preserved or reduced ejection fraction and the burden of symptoms.

A significant association between increased TMAO levels and mortality risk in patients with chronic HF (CHF) was first described in 2014.[37] Subsequent studies reported associations with the New York Heart Association (NYHA) functional class and CHF after MI.[38,39] Schuett et al. demonstrated that increased TMAO levels were a strong predictor of mortality in patients with HF with reduced ejection fraction, but not in those with preserved ejection fraction, over a mean follow-up of 9.7 years.[40]

Several studies reported data on the effect of increased TMAO levels on disease course and prognosis in patients with acute HF. Suzuki et al. showed that elevated TMAO levels were a strong predictor of mortality or rehospitalization at 1 year.[41] However, after adjustment for renal confounders, the correlation was no longer significant,[41] which is in line with the results of a more recent study by Israr et al.[42] In a multicenter study including patients with new-onset or progressive HF, higher TMAO levels were strongly associated with an increased risk of mortality and/or rehospitalization during 1-, 2- and 3-year follow-up. In line with previous studies, TMAO levels were not reduced by optimal treatment.[43]

The association between elevated TMAO levels and poor prognosis in patients with HF has not been fully elucidated

so far. Apart from gut microbiota, the role of FMO3 polymorphism has been postulated.[44] Moreover, Li et al. suggested a potential inhibitory effect of loop diuretics on renal excretion of TMAO, resulting in its retention in tissues.[45]

Atrial fibrillation

The role of TMAO as a risk factor or mediator of atrial fibrillation (AF) has not been determined so far.[46] A study on 2 Norwegian cohorts indicated that high TMAO levels were associated with a risk of incident AF, independent of traditional risk factors.[47] In the AF-RISK study, higher TMAO levels were associated with progression to permanent AF.[48] Finally, the proarrhythmic and prothrombotic effects of TMAO can result in increased risk of thrombus formation in patients with AF.[49]

Results from meta-analyses

There are several meta-analyses assessing TMAO levels as a predictor of mortality or other adverse events in patients with CVD.[50–55] The results strongly indicate that elevated TMAO levels are a significant risk factor for death and MACEs. Selected meta-analyses are summarized in Table 2.

Interventions aimed at reducing TMAO levels and toxicity

The knowledge of TMAO synthesis, metabolism and excretion pathways allows investigators to study interventions aimed at reducing TMAO toxicity. Some of the conclusions were formulated on the basis of random findings derived from other studies. Examples include the association between statin use and reduced TMAO levels[56] or between aspirin use and reduced TMA synthesis.[57] In both cases, the lowering effect was probably due to drug-induced alterations in gut microbiota.

A standard targeted approach is to reduce the dietary intake of TMA precursors and TMAO. The elimination of red meat from diet results in reduced TMAO levels after 4 weeks,[5] while vegetarians and vegans have lower circulating TMAO levels and a lower capacity to synthesize TMA, probably due to changes in gut microbiota.[17] The beneficial effect of Mediterranean diet on reducing TMAO levels is primarily due to the high intake of plant foods,[58] although the results of studies are equivocal.[59]

The use of broad-spectrum antibiotics was shown to suppress gut microbiota and reduce TMAO levels. However, antibiotic therapy does not offer satisfactory long-term outcomes and is associated with a high risk of side effects.[11] Rifaximin has been reported to be a safer alternative, but the results of the recent GUTHEART study are insufficient to confirm this suggestion.[60] Other interventions include supplementation with probiotics,[61] gut bacteria transplant from healthy donors,[62] bariatric surgery,[63] resveratrol,[64] and meldonium.[65] Current interventions are summarized in Table 3 and Fig. 3.

Review limitations and gaps in knowledge

The possibility of discussing all ongoing studies in microbiota biomarkers is beyond the scope of this review. For now, to the best of our knowledge, despite the extensive literature supporting its usefulness, TMAO has not been identified as an established biomarker in CAD or HF guidelines. Perhaps, the ongoing research will consolidate the use of TMAO and other gut microbiota metabolites such as bile acids and short chain fatty acids in everyday practice. Linking the metabolism of the gut

Table 2. Results of meta-analyses on the association of TMAO with cardiovascular risk

Author	Population (number of studies included)	Endpoints	Results RR/HR (95% CI)
Heianza et al.[50]	n = 19,256 (19)	MACCE, death	MACE: RR 1.62 [1.45; 1.80]; $p < 0.001$, $I^2 = 23.5\%$ death: RR 1.63 [1.36; 1.95]; $I^2 = 45.9\%$
Schiattarella et al.[51]	n = 26,167 (26)	MACCE, death	MACCE: RR 1.67 [1.33; 2.11]; $p < 0.00001$, $I^2 = 46\%$ death: RR 1.91 [1.40; 2.61]; $p < 0.0001$, $I^2 = 94\%$
Qi et al.[52]	n = 7716 (11)	cardiovascular events, death	cardiovascular events: RR 1.23 [1.07; 1.42]; $I^2 = 31.4\%$ death: RR 1.55 [1.19; 2.02]; $I^2 = 80.8\%$
Farhangi[53]	n = 31,230 (20)	death	death: RR 1.466 [1.291; 1.665]; $p < 0.001$, $I^2 = 81.9\%$
Li et al.[54]	n = 6879 (7)	MACE, death	MACE: T3 compared to T1: HR 1.68 [1.44; 1.96] death: T3 compared to T1: HR 1.67 [1.17; 2.38]
Guasti et al.[55]	n = 923 (3)	MACE, death	MACE: RR 2.05 [1.61; 2.61]; $I^2 = 50\%$ death: RR 3.42 [2.27; 5.15]; $I^2 = 0\%$

TMAO – trimethylamine oxide; MACE – major adverse cardiac events; MACCE – major adverse cardiac and cerebrovascular events; CVE – cardiovascular events; HR – hazard ratio; RR – risk ratio; T – tertile; CI – confidence interval.

Table 3. Results of studies assessing therapeutic strategies aimed at reducing TMAO levels

Author	Type of study	Population/ model	Intervention	Endpoint	Results	Comment
Li et al.[56]	retrospective study; 3-year follow-up	n = 4007; sequential patients undergoing elective diagnostic coronary angiography	statin use	MACE, defined as death, myocardial infarction, or stroke; reduction of TMAO concentration	n = 322 MACE by 3 years statin use associated with decreased MACE: HR 0.74, 95% CI: [0.60; 0.93]; p = 0.0089 plasma TMAO associated with increased MACE: HR 1.57, 95% CI: [1.40; 1.76]; p = 2.4e-14 statin use associated with decreased TMAO (3.9 compared to 4.3) p = 0.002	suspected mechanism: alteration in gut microbiome activity
Zhu et al.[57]	prospective study	healthy vegans/ vegetarians (n = 8); healthy omnivores (n = 10) orally supplemented with choline	aspirin 81 mg/day for 3 months	reduction of TMAO concentration	aspirin attenuated TMAO elevation TMAO: choline compared to choline + ASA 36.9 ±9.4 compared to 21.2 ±3.0; p = 0.009	suspected mechanism: alteration in gut microbiome activity
Wang et al.[5]	prospective study	n = 113; healthy adult participants all omnivores	discontinuation of red meat intake to non-meat or white meat	reduction of TMAO concentration	no meat – TMAO reduction; p < 0.0001 white meat – TMAO reduction; p < 0.0001	N/A
Awoyemi et al.[60]	prospective randomized, double-blind study	n = 151; patients with LVEF < 40%; NYHA class II–III despite optimal medical therapy	3 months: rifaximin, 550 mg twice daily, 250 mg 3 months: probiotic *Saccharomyces boulardii* NCM I-745 500 mg twice daily standard of care only	LVEF after 3 months of intervention baseline-adjusted NT-proBNP baseline-adjusted TMAO	LVEF: rifaximin compared to standard of care mean difference: −1.2 pp (3.2–0.7); p = 0.22 *Saccharomyces boulardii* −0.2 pp (2.2–1.9); p = 0.87 NT-proBNP: no significant effects rifaximin p = 0.28 *S. boulardii*: increase; p = 0.03 TMAO: no significant effects rifaximin; p = 0.8 *Saccharomyces boulardii* p = 0.16	patients low in baseline dysbiosis low dose of rifaximin
Boutagy et al.[61]	randomized double-blind, placebo-controlled	n = 19; healthy, non-obese males (18–30 years)	4-week hypercaloric (+1000 kcal day−1), high-fat diet (55% fat) + VSL#3 (900 billion live bacteria) orally placebo	reduction of TMAO concentration	plasma TMAO level increased significantly VSL#3 (89 ±66%); p < 0.05 placebo (115 ±61%); p < 0.05 VSL#3 compared to placebo: p > 0.05	VSL#3 does not influence plasma TMAO following a high-fat diet
Smits et al.[62]	double-blind randomized pilot study	n = 20; male patients with metabolic syndrome	vegan-donor FMT	conversion of choline and carnitine to TMA and TMAO fasting plasma TMAO level TMA/TMAO urinary excretion	no signifficant effect; p > 0.05	significant changes in intestinal microbiota composition did not affect TMAO metabolism; residual capacity to convert precursors to TMAO in vegans? short follow-up (2 weeks)
Trøseid et al.[63]	observational study	n = 34; obese patients (17 with and 17 without type 2 diabetes) undergoing bariatric surgery	bariatric surgery laparoscopic Roux-en-Y gastric bypass duodenal switch	abseline plasma TMAO level preoperatively (after 3 months of lifestyle intervention) 1 year after bariatric surgery	no significant effect of 3-month lifestyle intervention preoperatively; 1 year after bariatric surgery TMAO plasma levels more than doubled (HR 10.5, 95% CI: [7.5; 13.5]) compared to preoperative (HR 4.4, 95% CI: [2.8; 6.0]; p < 0.001) compared to baseline (HR 4.7, 95% CI: [3.7; 5.8]; p < 0.001), regardless of surgical method	mechanism: changes in gut microbiota profile

Table 3. Results of studies assessing therapeutic strategies aimed at reducing TMAO levels – cont.

Author	Type of study	Population/ model	Intervention	Endpoint	Results	Comment
Annunziata et al.[64]	double-blind, randomized, placebo-controlled study	n = 380; healthy individuals	grape pomace polyphenol nutraceutical (rich in resveratrol) 400 mg twice daily 4 weeks, 8 weeks	reduction of TMAO concentration	plasma TMAO reduction (–49.78%, $p < 0.0001$) 8 weeks – 75.85%; $p < 0.0001$	N/A
Dambrova et al.[65]	open label, interventional study	n = 8; healthy volunteers	meldonium orally, 500 mg twice daily, 7 days during TMAO-rich diet	reduction of TMAO concentration urine TMAO excretion	diet compared to diet + meldonium plasma: 81.5 ±8.6 mM compared to 43.0 ±3.8 mM; $p < 0.05$ excretion: 18.2 ±2.2 mmol/mg creatinine × 7 days compared to 24.3 ±1.5 mmol/mg creatinine × 7 days; $p < 0.05$	N/A

LVEF – left ventricular ejection fraction; MACE – major adverse cardiac event; N/A – not available/not applicable; NYHA – New York Heart Association functional classification; TMAO – trimethylamine oxide; NT-proBNP – N-terminal pro-B-type natriuretic peptide; FMT – fecal microbiota transplant; TMA – trimethylamine; ASA – acetylsalicylic acid (aspirin); HR – hazard ratio; 95% CI – 95% confidence interval.

microbiota to CVD is an attractive topic of ongoing research. It is worth mentioning the studies NCT04962763[66] and NCT02728154,[67] which will deepen the knowledge on the correlation of the intestinal microbiota with HF. The study NCT05014880 is going to assess the effectiveness of a dietary intervention reducing dietary TMAO levels during the rehabilitation of CAD patients.[68]

Conclusions

The TMAO is a biomarker that has been proven useful in a population of patients at higher cardiovascular risk. The use of TMAO in clinical practice requires confirmation in subsequent prospective interventional studies.

ORCID iDs

Radosław Andrzej Konieczny https://orcid.org/0000-0002-0949-4364
Wiktor Kuliczkowski https://orcid.org/0000-0001-6284-0820

References

1. Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organization and United Nations. *Int J Cardiol*. 2013;168(2):934–945. doi:10.1016/j.ijcard.2012.10.046
2. Gambardella J, Castellanos V, Santulli G. Standardizing translational microbiome studies and metagenomic analyses. *Cardiovasc Res*. 2021; 117(3):640–642. doi:10.1093/cvr/cvaa175
3. Velasquez MT, Ramezani A, Manal A, Raj DS. Trimethylamine N-oxide: The good, the bad and the unknown. *Toxins (Basel)*. 2016;8(11):326. doi:10.3390/toxins8110326
4. Lombardo M, Aulisa G, Marcon D, et al. Association of urinary and plasma levels of trimethylamine N-oxide (TMAO) with foods. *Nutrients*. 2021;13(5):1426. doi:10.3390/nu13051426
5. Wang Z, Bergeron N, Levison BS, et al. Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. *Eur Heart J*. 2019;40(7):583–594. doi:10.1093/eurheartj/ehy799
6. Hamaya R, Ivey KL, Lee DH, et al. Association of diet with circulating trimethylamine-N-oxide concentration. *Am J Clin Nutr*. 2020;112(6): 1448–1455. doi:10.1093/ajcn/nqaa225
7. Cho CE, Aardema NDJ, Bunnell ML, et al. Effect of choline forms and gut microbiota composition on trimethylamine-N-oxide response in healthy men. *Nutrients*. 2020;12(8):2220. doi:10.3390/nu12082220
8. Janeiro MH, Ramírez MJ, Milagro FI, et al. Implication of trimethylamine N-oxide (TMAO) in disease: Potential biomarker or new therapeutic target. *Nutrients*. 2018;10(10):1398. doi:10.3390/nu10101398
9. Stremmel W, Schmidt KV, Schuhmann V, et al. Blood trimethylamine-N-oxide originates from microbiota mediated breakdown of phosphatidylcholine and absorption from small intestine. *PLoS One*. 2017; 12(1):e0170742. doi:10.1371/journal.pone.0170742
10. Taesuwan S, Cho CE, Malysheva OV, et al. The metabolic fate of isotopically labeled trimethylamine-N-oxide (TMAO) in humans. *J Nutr Biochem*. 2017;45:77–82. doi:10.1016/j.jnutbio.2017.02.010
11. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472(7341): 57–63. doi:10.1038/nature09922
12. Geng J, Yang C, Wang B, et al. Trimethylamine N-oxide promotes atherosclerosis via CD36-dependent MAPK/JNK pathway. *Biomed Pharmacother*. 2018;97:941–947. doi:10.1016/j.biopha.2017.11.016
13. Ma G, Pan B, Chen Y, et al. Trimethylamine N-oxide in atherogenesis: Impairing endothelial self-repair capacity and enhancing monocyte adhesion. *Biosci Rep*. 2017;37(2):BSR20160244. doi:10.1042/BSR20160244
14. Seldin MM, Meng Y, Qi H, et al. Trimethylamine N-oxide promotes vascular inflammation through signaling of mitogen-activated protein kinase and nuclear factor-κB. *J Am Heart Assoc*. 2016;5(2):e002767. doi:10.1161/JAHA.115.002767
15. Chou RH, Chen CY, Chen IC, et al. Trimethylamine N-oxide, circulating endothelial progenitor cells, and endothelial function in patients with stable angina. *Sci Rep*. 2019;9(1):4249. doi:10.1038/s41598-019-40638-y
16. Brunt VE, Gioscia-Ryan RA, Casso AG, et al. Trimethylamine-N-oxide promotes age-related vascular oxidative stress and endothelial dysfunction in mice and healthy humans. *Hypertension*. 2020;76(1): 101–112. doi:10.1161/HYPERTENSIONAHA.120.14759
17. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013;19(5):576–585. doi:10.1038/nm.3145
18. Zhu W, Gregory JC, Org E, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell*. 2016; 165(1):111–124. doi:10.1016/j.cell.2016.02.011
19. Subramaniam S, Boukhlouf S, Fletcher C. A bacterial metabolite, trimethylamine N-oxide, disrupts the hemostasis balance in human primary endothelial cells but no coagulopathy in mice. *Blood Coagul Fibrinolysis*. 2019;30:324–330. doi:10.1097/MBC.0000000000000838
20. Li Z, Wu Z, Yan J, et al. Gut microbe-derived metabolite trimethylamine N-oxide induces cardiac hypertrophy and fibrosis. *Lab Invest*. 2019;99(3):346–357. doi:10.1038/s41374-018-0091-y

21. Jin B, Ji F, Zuo A, et al. Destructive role of TMAO in T-tubule and excitation-contraction coupling in the adult cardiomyocytes. *Int Heart J*. 2020;61(2):355–363. doi:10.1536/ihj.19-372
22. Oakley CI, Vallejo JA, Wang D, et al. Trimethylamine-N-oxide acutely increases cardiac muscle contractility. *Am J Physiol Heart Circ Physiol*. 2020;318(5):H1272–H1282. doi:10.1152/ajpheart.00507.2019
23. Tang WH, Wang Z, Levison BS, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013;368(17):1575–1584. doi:10.1056/NEJMoa1109400
24. Senthong V, Wang Z, Li XS, et al. Intestinal microbiota-generated metabolite trimethylamine-N-oxide and 5-year mortality risk in stable coronary artery disease: The contributory role of intestinal microbiota in a COURAGE-like patient cohort. *J Am Heart Assoc*. 2016;5(6): e002816. doi:10.1161/JAHA.115.002816
25. Lee Y, Nemet I, Wang Z, et al. Longitudinal plasma measures of trimethylamine N-oxide and risk of atherosclerotic cardiovascular disease events in community-based older adults. *J Am Heart Assoc*. 2021; 10(17):e020646. doi:10.1161/JAHA.120.020646
26. Tang WHW, Li XS, Wu Y, et al. Plasma trimethylamine N-oxide (TMAO) levels predict future risk of coronary artery disease in apparently healthy individuals in the EPIC-Norfolk prospective population study. *Am Heart J*. 2021;236:80–86. doi:10.1016/j.ahj.2021.01.020
27. Suzuki T, Heaney LM, Jones DJ, Ng LL. Trimethylamine N-oxide and risk stratification after acute myocardial infarction. *Clin Chem*. 2017; 63(1):420–428. doi:10.1373/clinchem.2016.264853
28. Matsuzawa Y, Nakahashi H, Konishi M, et al. Microbiota-derived trimethylamine N-oxide predicts cardiovascular risk after STEMI. *Sci Rep*. 2019;9(1):11647. doi:10.1038/s41598-019-48246-6
29. Li XS, Obeid S, Klingenberg R, et al. Gut microbiota-dependent trimethylamine N-oxide in acute coronary syndromes: A prognostic marker for incident cardiovascular events beyond traditional risk factors. *Eur Heart J*. 2017;38(11):814–824. doi:10.1093/eurheartj/ehw582
30. Sheng Z, Tan Y, Liu C, et al. Relation of circulating trimethylamine N-oxide with coronary atherosclerotic burden in patients with ST-segment elevation myocardial infarction. *Am J Cardiol*. 2019;123(6): 894–898. doi:10.1016/j.amjcard.2018.12.018
31. Senthong V, Li XS, Hudec T, et al. Plasma trimethylamine N-oxide, a gut microbe-generated phosphatidylcholine metabolite, is associated with atherosclerotic burden. *J Am Coll Cardiol*. 2016;67(22):2620–2628. doi:10.1016/j.jacc.2016.03.546
32. Fu Q, Zhao M, Wang D, et al. Coronary plaque characterization assessed by optical coherence tomography and plasma trimethylamine-N-oxide levels in patients with coronary artery disease. *Am J Cardiol*. 2016;118(9):1311–1315. doi:10.1016/j.amjcard.2016.07.071
33. Tan Y, Sheng Z, Zhou P, et al. Plasma trimethylamine N-oxide as a novel biomarker for plaque rupture in patients with ST-segment-elevation myocardial infarction. *Circ Cardiovasc Interv*. 2019;12(1):e007281. doi:10.1161/CIRCINTERVENTIONS.118.007281
34. Ma R, Fu W, Zhang J, et al. TMAO: A potential mediator of clopidogrel resistance. *Sci Rep*. 2021;11(1):6580. doi:10.1038/s41598-021-85950-8
35. Stubbs JR, House JA, Ocque AJ, et al. Serum trimethylamine-N-oxide is elevated in CKD and correlates with coronary atherosclerosis burden. *J Am Soc Nephrol*. 2016;27(1):305–313. doi:10.1681/ASN.2014111063
36. Al-Obaide MAI, Singh R, Datta P, et al. Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. *J Clin Med*. 2017;6(9):86. doi:10.3390/jcm6090086
37. Tang WH, Wang Z, Fan Y, et al. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: Refining the gut hypothesis. *J Am Coll Cardiol*. 2014;64(18):1908–1914. doi:10.1016/j.jacc.2014.02.617
38. Trøseid M, Ueland T, Hov JR, et al. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. *J Intern Med*. 2015;277(6): 717–726. doi:10.1111/joim.12328
39. Zhou X, Jin M, Liu L, et al. Trimethylamine N-oxide and cardiovascular outcomes in patients with chronic heart failure after myocardial infarction. *ESC Heart Fail*. 2020;7(1):188–193. doi:10.1002/ehf2.12552
40. Schuett K, Kleber ME, Scharnagl H, et al. Trimethylamine-N-oxide and heart failure with reduced versus preserved ejection fraction. *J Am Coll Cardiol*. 2017;70(25):3202–3204. doi:10.1016/j.jacc.2017.10.064
41. Suzuki T, Heaney LM, Bhandari SS, et al. Trimethylamine N-oxide and prognosis in acute heart failure. *Heart*. 2016;102(11):841–848. doi:10.1136/heartjnl-2015-308826
42. Israr MZ, Bernieh D, Salzano A, et al. Association of gut-related metabolites with outcome in acute heart failure. *Am Heart J*. 2021;234: 71–80. doi:10.1016/j.ahj.2021.01.006
43. Suzuki T, Yazaki Y, Voors AA, et al. Association with outcomes and response to treatment of trimethylamine N-oxide in heart failure: Results from BIOSTAT-CHF. *Eur J Heart Fail*. 2019;21(7):877–886. doi:10.1002/ejhf.1338
44. Wei H, Zhao M, Huang M, et al. FMO3-TMAO axis modulates the clinical outcome in chronic heart-failure patients with reduced ejection fraction: Evidence from an Asian population [published online ahead of print on June 22, 2021]. *Front Med*. 2021. doi:10.1007/s11684-021-0857-2
45. Li DY, Wang Z, Jia X, et al. Loop diuretics inhibit renal excretion of trimethylamine N-oxide. *JACC Basic Transl Sci*. 2021;6(2):103–115. doi:10.1016/j.jacbts.2020.11.010
46. Gawałko M, Linz D, Dobrev D. Gut-microbiota derived TMAO: A risk factor, a mediator or a bystander in the pathogenesis of atrial fibrillation? *Int J Cardiol Heart Vasc*. 2021;34:100818. doi:10.1016/j.ijcha.2021.100818
47. Svingen GFT, Zuo H, Ueland PM, et al. Increased plasma trimethylamine-N-oxide is associated with incident atrial fibrillation. *Int J Cardiol*. 2018;267:100–106. doi:10.1016/j.ijcard.2018.04.128
48. Nguyen BO, Meems LMG, van Faassen M, et al. Gut-microbe derived TMAO and its association with more progressed forms of AF: Results from the AF-RISK study. *Int J Cardiol Heart Vasc*. 2021;34:100798. doi:10.1016/j.ijcha.2021.100798
49. Gong D, Zhang L, Zhang Y, et al. Gut microbial metabolite trimethylamine N-oxide is related to thrombus formation in atrial fibrillation patients. *Am J Med Sci*. 2019;358(6):422–428. doi:10.1016/j.amjms.2019.09.002
50. Heianza Y, Ma W, Manson JE, et al. Gut microbiota metabolites and risk of major adverse cardiovascular disease events and death: A systematic review and meta-analysis of prospective studies. *J Am Heart Assoc*. 2017;6(7):e004947. doi:10.1161/JAHA.116.004947
51. Schiattarella GG, Sannino A, Toscano E, et al. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: A systematic review and dose-response meta-analysis. *Eur Heart J*. 2017;38(39):2948–2956. doi:10.1093/eurheartj/ehx342
52. Qi J, You T, Li J, et al. Circulating trimethylamine N-oxide and the risk of cardiovascular diseases: A systematic review and meta-analysis of 11 prospective cohort studies. *J Cell Mol Med*. 2018;22(1):185–194. doi:10.1111/jcmm.13307
53. Farhangi MA. Gut microbiota-dependent trimethylamine N-oxide and all-cause mortality: Findings from an updated systematic review and meta-analysis. *Nutrition*. 2020;78:110856. doi:10.1016/j.nut.2020.110856
54. Li W, Huang A, Zhu H, et al. Gut microbiota-derived trimethylamine N-oxide is associated with poor prognosis in patients with heart failure. *Med J Aust*. 2020;213(8):374–379. doi:10.5694/mja2.50781
55. Guasti L, Galliazzo S, Molaro M, et al. TMAO as a biomarker of cardiovascular events: A systematic review and meta-analysis. *Intern Emerg Med*. 2021;16(1):201–207. doi:10.1007/s11739-020-02470-5
56. Li DY, Wang Z, Li XS, et al. Relationship between statin use and trimethylamine n-oxide in cardiovascular risk assessment. *J Am Coll Cardiol*. 2018;71(11):A115. doi:10.1016/s0735-1097(18)30656-9
57. Zhu W, Wang Z, Tang WHW, Hazen SL. Gut microbe-generated trimethylamine N-oxide from dietary choline is prothrombotic in subjects. *Circulation*. 2017;135(17):1671–1673. doi:10.1161/CIRCULATIONAHA.116.025338
58. Guasch-Ferré M, Hu FB, Ruiz-Canela M, et al. Plasma metabolites from choline pathway and risk of cardiovascular disease in the PREDIMED (Prevention With Mediterranean Diet) study. *J Am Heart Assoc*. 2017;6(11):e006524. doi:10.1161/JAHA.117.006524
59. Pignanelli M, Just C, Bogiatzi C, et al. Mediterranean diet score: Associations with metabolic products of the intestinal microbiome, carotid plaque burden, and renal function. *Nutrients*. 2018;10(6):779. doi:10.3390/nu10060779
60. Awoyemi A, Mayerhofer C, Felix AS, et al. Rifaximin or *Saccharomyces boulardii* in heart failure with reduced ejection fraction: Results from the randomized GutHeart trial. *EBioMedicine*. 2021;70:103511. doi:10.1016/j.ebiom.2021.103511

61. Boutagy NE, Neilson AP, Osterberg KL, et al. Probiotic supplementation and trimethylamine-N-oxide production following a high-fat diet. *Obesity (Silver Spring)*. 2015;23(12):2357–2363. doi:10.1002/oby.21212
62. Smits LP, Kootte RS, Levin E, et al. Effect of vegan fecal microbiota transplantation on carnitine- and choline-derived trimethylamine-N-oxide production and vascular inflammation in patients with metabolic syndrome. *J Am Heart Assoc*. 2018;7(7):e008342. doi:10.1161/JAHA.117.008342
63. Trøseid M, Hov JR, Nestvold TK, et al. Major increase in microbiota-dependent proatherogenic metabolite TMAO one year after bariatric surgery. *Metab Syndr Relat Disord*. 2016;14(4):197–201. doi:10.1089/met.2015.0120
64. Annunziata G, Maisto M, Schisano C, et al. Effects of grape pomace polyphenolic extract (Taurisolo®) in reducing TMAO serum levels in humans: Preliminary results from a randomized, placebo-controlled, cross-over study. *Nutrients*. 2019;11(1):139. doi:10.3390/nu11010139
65. Dambrova M, Skapare-Makarova E, Konrade I, et al. Meldonium decreases the diet-increased plasma levels of trimethylamine N-oxide, a metabolite associated with atherosclerosis. *J Clin Pharmacol*. 2013;53(10):1095–1098. doi:10.1002/jcph.135
66. Correlation of Intestinal Flora and Metabolomics in Patients With Ischemic Heart Failure. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT04962763?term=gut&recrs=adf&cond=CVD&draw=2&rank=28. Accessed February 10, 2022.
67. The Role of Gut Microbiota in Heart Failure and Pre-Heart Failure With Preserved Ejection Fraction. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02728154. Accessed February 10, 2022.
68. Impact of Time Restricted Eating on Patients With Coronary Artery Disease (CAD) Undergoing Cardiac Rehabilitation. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT05014880. Accessed February 10, 2022.

9.2. Publikacja nr 2

Cardiology

Cardiovascular Prevention: Research Article

Cardiology 2022;147:443–452
DOI: 10.1159/000525972

Received: March 20, 2022
Accepted: June 15, 2022
Published online: August 15, 2022

All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease

Radosław Konieczny[a] Ewa Żurawska-Płaksej[b] Konrad Kaaz[c]
Hanna Czapor-Irzabek[d] Wojciech Bombała[e] Andrzej Mysiak[c]
Wiktor Kuliczkowski[c]

[a]Department of Gastroenterology and Hepatology, Mikulicz-Radecki University Clinical Hospital, Wroclaw Medical University, Wroclaw, Poland; [b]Department of Toxicology, Wroclaw Medical University, Wroclaw, Poland; [c]Institute for Heart Diseases, Wroclaw Medical University, Wroclaw, Poland; [d]Laboratory of Elemental Analysis and Structural Research, Wroclaw Medical University, Wroclaw, Poland; [e]Statistical Analysis Centre, Wroclaw Medical University, Wroclaw, Poland

What Is New?

- The predictive value of trimethylamine N-oxide (TMAO) levels is highly limited by other factors. Therefore, mortality in high-risk patients with cardiovascular disease (CVD) is determined by a combined effect of multiple factors that significantly affect not only the risk of death but also the TMAO levels themselves. This should be considered when assessing the prognosis of patients with CVD.

Keywords
Atrial fibrillation · Coronary artery disease · Heart failure · Trimethylamine N-oxide

Abstract
Introduction: Trimethylamine N-oxide (TMAO) is an organic compound with a well-established involvement in the pathogenesis of cardiovascular disease (CVD). However, data on the links between TMAO levels and cardiovascular mortality in Polish patients are lacking. ***Objectives:*** We aimed to assess the relationship between serum TMAO levels and 5-year mortality in Polish patients with CVD. ***Patients and Methods:*** We retrospectively assessed serum TMAO levels in 1,036 consecutive patients (median age, 62 years; men, 61%) hospitalized between 2013 and 2015. Correlations between TMAO levels and 5-year mortality as well as anthropometric and biochemical parameters were assessed for the whole population and the subgroups of patients with acute coronary syndrome, stable coronary syndrome (SCS), chronic heart failure (HF), and atrial fibrillation (AF). ***Results:*** In the univariate analysis, increased TMAO levels predicted 5-year mortality without clinically significant power (hazard ratio [HR], 1.01; 95% CI: 1.006–1.018; $p < 0.0001$). However, even this weak effect was lost in the multivariate analysis after adjustment for age, sex, comorbidities, and laboratory parameters. In the whole study group, TMAO levels in the fourth quartile of concentration (>6.01 μM) predicted 5-year mortality only in the univariate analysis (HR: 1.55; 95% CI: 1.34–1.79; $p < 0.0001$). In subgroup univariate analysis, TMAO lev-

Karger@karger.com
www.karger.com/crd

Correspondence to:
Radosław Konieczny, rkonieczny.med@gmail.com

Karger

DOI: 10.1159/000525972

Table 1. Characteristics of the whole study population and a comparison between deceased patients and survivors at 5-year follow-up

Parameter	Study population (n = 1,036)	Death at 5 years		p value[a]
		yes (n = 171)	no (n = 865)	
Age, years, mean (SD)	62.0 (14.1)	68.9 (11.1)	60.7 (14.3)	<0.0001
Male sex, %	61.1	73.0	58.8	<0.001
TMAO, μM	4.06 (2.79–6.01)	5.65 (3.48–8.94)	3.86 (2.70–5.62)	<0.0001
ACS, %	17.0	32.7	19.4	<0.001
SCS, %	42.5	57.1	40.0	<0.0001
Chronic HF, %	28.1	54.7	23.4	<0.0001
AF, %	26.7	43.2	23.4	<0.0001
Other types of arrhythmia, %	16.4	14.7	85.2	0.48
Conduction disorders, %	44.0	13.1	86.8	0.01
PCI during hospitalization, %	21.0	31.1	23.3	0.051
Previous PCI, %	14.4	25.7	16.9	0.017
Previous CABG, %	7.6	12.2	6.7	0.012
Diabetes, %	24.7	35.2	22.6	<0.001
Hypertension, %	71.7	81.8	69.7	0.0012
Smoking, %	23.2	30.0	21.9	0.026
NYHA class (% of patients with chronic HF)				
I	7.0	8.7	6.3	0.16
II	55.5	48.3	58.8	
III	34.0	37.7	32.3	
IV	3.3	5.3	2.6	
CCS, %				
I	10.2	6.0	11.3	0.65
II	54.3	57.5	53.5	
III	32.2	33.3	32.0	
IV	3.1	3.0	3.1	
LVEF, %	60 (50–65)	50 (37–65)	65 (55–66)	<0.0001
eGFR, mL/min	68 (55–80)	58 (44–72)	69 (58–81)	<0.0001
hsCRP, mg/L	3.47 (1.33–13.3)	7.79 (2.64–30.8)	3.11 (1.24–10.1)	<0.0001
HbA_{1c}, %	5.70 (5.40–6.20)	5.90 (5.50–6.65)	5.70 (5.40–6.10)	0.018
TC, mg/dL	182 (149–216)	171 (135–207)	184 (151–218)	0.0028
LDL-C, mg/dL	104 (80–136)	97 (33–249)	106 (83–136)	0.094
HDL-C, mg/dL	45 (37–55)	42 (34–51)	46 (38–55)	<0.001
Triglycerides, mg/dL	124 (95–169)	115 (89–155)	127 (96–172)	0.018
ASA, %	54.8	64.0	52.9	0.0085
Clopidogrel, %	29.4	28.1	35.1	0.075
OAC, %	28.9	43.4	26.0	<0.0001
ACEIs, %	70.2	76.1	69.0	0.066
ARBs, %	7.19	3.01	8.02	0.022
β-Blockers, %	81.2	92.2	79.0	<0.0001
Statins, %	79.1	89.2	77.0	<0.001
Loop diuretics, %	19.1	43.4	14.3	<0.0001

Data are presented as median (IQR) unless indicated otherwise. ACEI, angiotensin-converting enzyme inhibitor; ACS, acute coronary syndrome; AF, atrial fibrillation; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; CABG, coronary artery bypass grafting; CCS, Canadian Cardiovascular Society grading of angina pectoris; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated hemoglobin A_{1c}; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; hsCRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; OAC, oral anticoagulation; PCI, percutaneous coronary intervention; TC, total cholesterol; TMAO, trimethylamine N-oxide. [a] Deceased patients versus survivors at 5-year follow-up.

was 16.5% (171 deaths). Most clinical and biochemical parameters differed between deceased patients and survivors. Deceased patients were older, were more often male, and had higher levels of TMAO, high-sensitivity C-reactive protein, and glycated hemoglobin HbA_{1c}. Moreover, they had lower total cholesterol, high-density

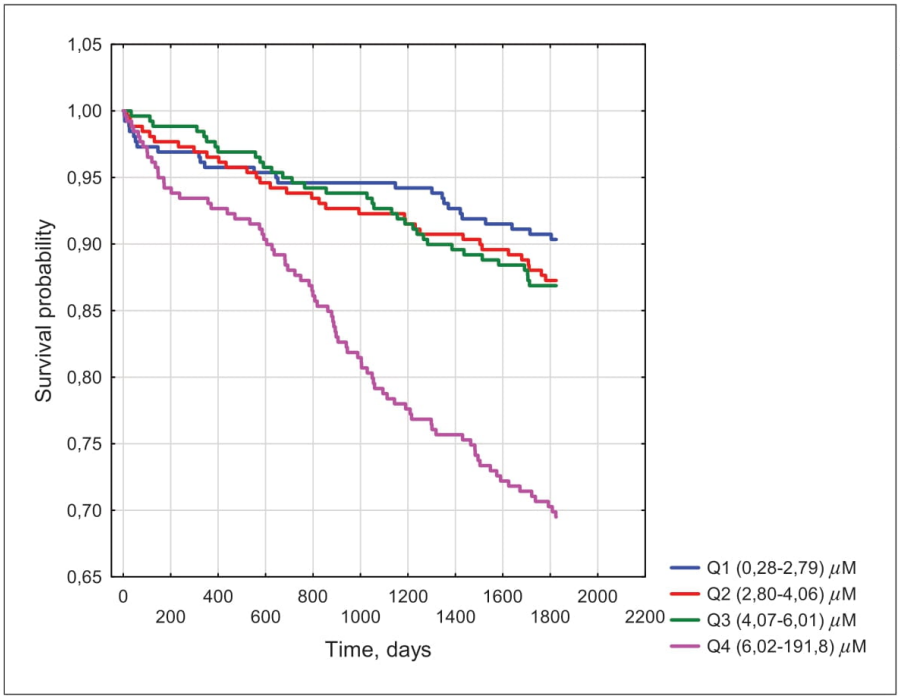


Fig. 1. Kaplan-Meier survival curves in study subgroups divided according to the quartiles of TMAO levels (Q1, Q2, Q3, and Q4 – quartiles 1, 2, 3, and 4, respectively).

lipoprotein, and triglyceride levels. They also had lower estimated glomerular filtration rate (eGFR) and left ventricular ejection fraction. They more often used acetylsalicylic acid, oral anticoagulation, β-blockers, statins, loop diuretics, and angiotensin receptor blockers and more often presented with ACS, SCS, chronic HF, AF, diabetes, hypertension, and a history of smoking than survivors. Finally, deceased patients more often had previous percutaneous coronary intervention and coronary artery bypass grafting.

Effect of TMAO Levels on Mortality in the Whole Study Population

The univariate Cox proportional hazards regression model revealed that higher TMAO levels predicted 5-year mortality without clinical significance (hazard ratio [HR], 1.01; 95% CI: 1.006–1.018; $p < 0.0001$). However, in the multivariate model adjusted for mortality risk factors listed in Table 1 (i.e., age, sex, comorbidities, and laboratory parameters), even this weak correlation was no longer significant (HR: 1.01; 95% CI: 0.99–1.04; $p = 0.19$).

For a more detailed analysis, TMAO levels were divided into quartiles and compared using the Kaplan-Meier method. A significant difference was noted in survival between patients with TMAO levels in the highest quartile versus those with TMAO levels in the remaining quartiles ($\chi^2 = 49.61$; $p < 0.00001$; Fig. 1).

A separate Cox proportional hazards regression analysis was performed for TMAO levels according to quartiles. In the univariate analysis, TMAO levels in the highest quartile were a significant predictor of 5-year mortality in the study population (HR: 1.55; 95% CI: 1.34–1.79; $p < 0.0001$). However, in the multivariate model adjusted for mortality risk factors listed in Table 1, the predictive value of TMAO levels in the highest quartile was no longer significant (HR: 1.13; 95% CI: 0.87–1.46; $p = 0.35$).

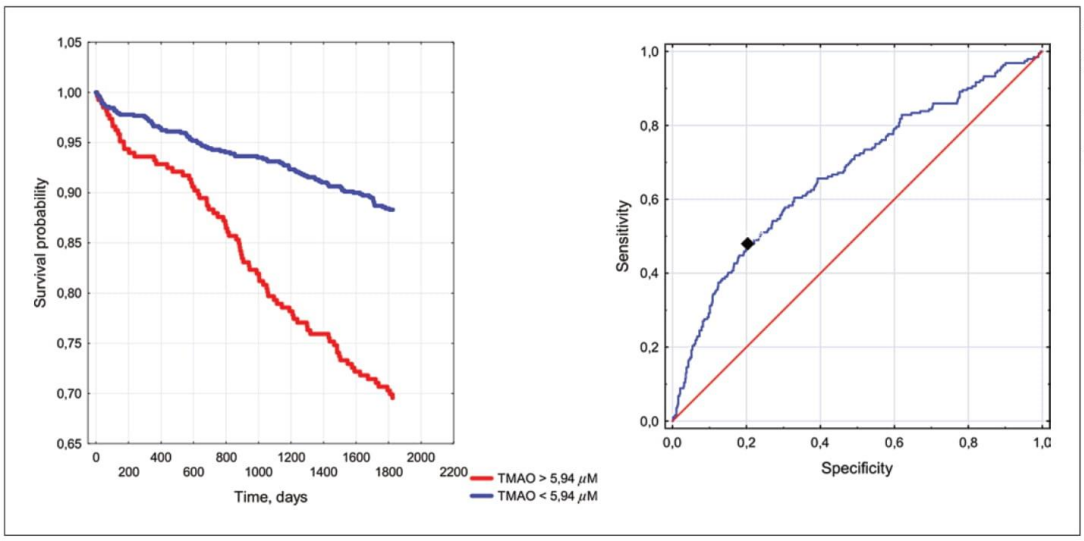


Fig. 2. Left-hand panel shows survival probability in the whole study group depending on the cutoff value for trimethylamine N-oxide (TMAO) levels of 5.94 μM (log-rank statistic = 7.07 at $p < 0.00001$). The right-hand panel presents the ROC curve analysis; the black diamond indicates the cutoff value for TMAO levels of 5.94 μM for predicting higher mortality risk at 5 years with a sensitivity of 48% and specificity of 80%.

Table 2. Levels of TMAO in study subgroups according to survival at 5 years

Subgroup	Whole subgroup	Death at 5 years		*p* value[a]
ACS (*n* = 177)	4.27 (2.87–5.92)	Yes (*n* = 37) 4.42 (3.31–7.45)	No (*n* = 140) 4.15 (2.83–5.71)	0.23
SCS (*n* = 441)	4.29 (2.94–6.67)	Yes (*n* = 96) 6.26 (2.70–10.1)	No (*n* = 345) 4.00 (2.84–5.88)	<0.00001
Chronic HF (*n* = 292)	4.91 (3.37–7.44)	Yes (*n* = 93) 5.84 (4.12–8.94)	No (*n* = 199) 4.65 (3.27–6.74)	<0.001
AF (*n* = 277)	4.86 (3.37–7.41)	Yes (*n* = 74) 6.61 (4.57–10.19)	No (*n* = 203) 4.46 (3.23–6.41)	<0.0001

Data are presented as median (IQR). Abbreviations: see Table 1. [a] Deceased patients versus survivors at 5-year follow-up.

In the ROC curve analysis for the whole population, TMAO levels higher than 5.94 μM identified patients at greater risk of death at 5 years, with low sensitivity of 48% and a specificity of 80% (area under the ROC curve: 0.67; 95% CI: 0.62–0.71; $p < 0.0001$; Youden index, 0.28; Fig. 2). For the cutoff value determined in the ROC curve analysis, the Kaplan-Meier survival probability curves were plotted with a significant difference of probability (log-rank test, 7.07; $p < 0.00001$; Fig. 2).

Effect of TMAO on Mortality in Study Subgroups

To obtain a more detailed insight into the effect of higher TMAO levels on mortality in patients with CVD, we divided patients into subgroups with ACS, SCS, chronic HF, and AF. The levels of TMAO in the study subgroups according to survival at 5 years are presented in Table 2. The univariate Cox proportional hazards analysis indicated a significant association between higher TMAO levels and mortality in patients with SCS, chronic HF, and AF. However, in the multivariate analysis ad-

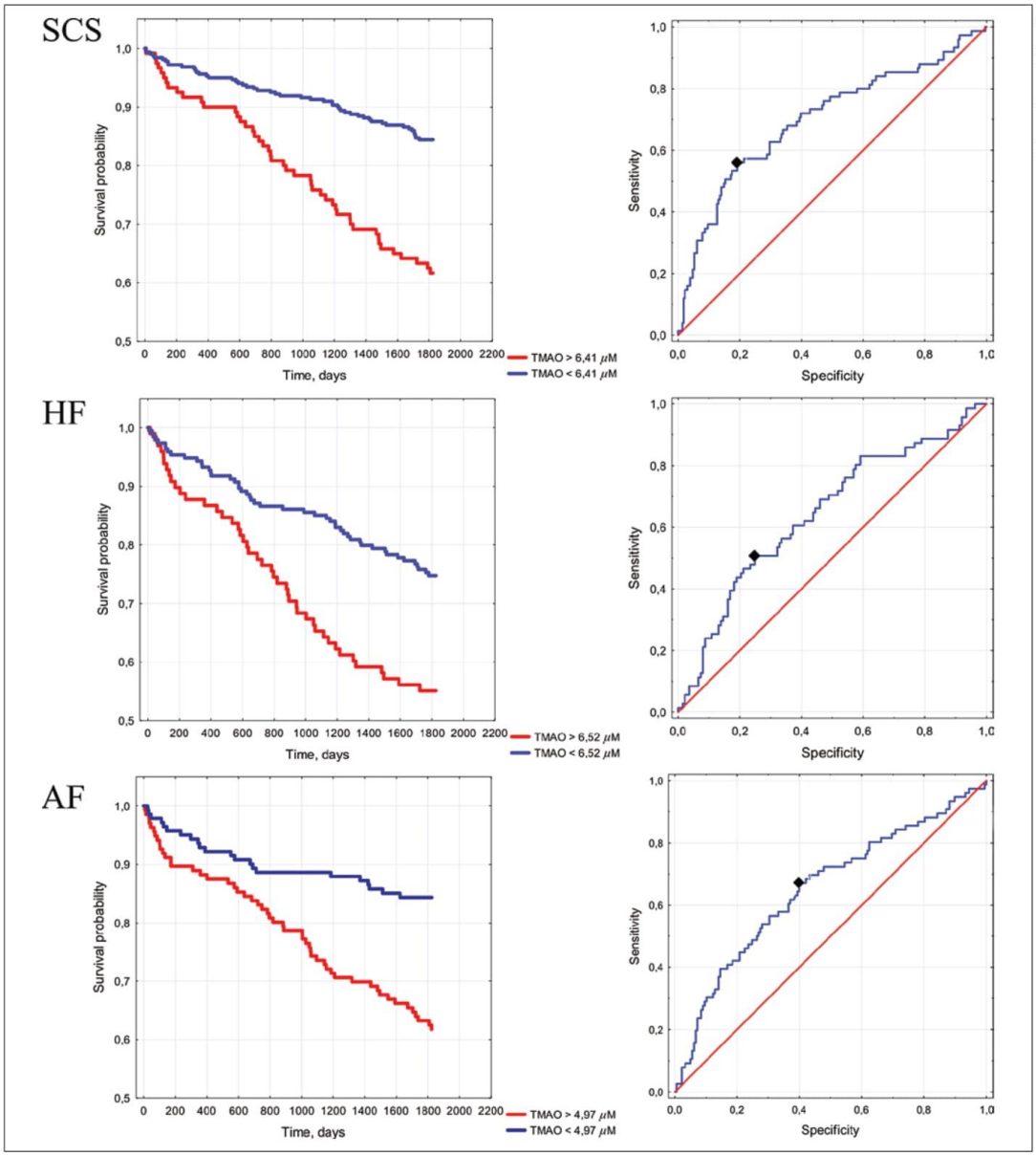


Fig. 3. Panels on the left-hand side show survival probability depending on the cutoff trimethylamine N-oxide (TMAO) levels. Stable coronary syndrome (SCS): log-rank statistic = 5.14; $p < 0.0001$; heart failure (HF): log-rank statistic = 3.49; $p < 0.001$; atrial fibrillation (AF): log-rank statistic = 4.12; $p < 0.0001$. Panels on the right-hand side present the results of the ROC curve analysis. The black diamond indicates the cutoff value for TMAO levels for predicting higher mortality risk in 5 years. SCS: 6.41 μM, sensitivity, 58%; specificity, 80%; HF: 6.52 μM; sensitivity, 50%; specificity, 75%; AF: 4.97 μM; sensitivity, 68%; specificity, 60%.

justed for mortality risk factors (Table 1), TMAO levels were no longer a significant predictor of 5-year mortality (Table 3).

ROC Curve Analysis of Study Subgroups

The ROC curve analysis determined significant cutoff values for TMAO levels for the subgroups of patients with SCS, chronic HF, and AF, in which TMAO levels were shown to be a significant predictor of mortality in the univariate Cox proportional hazards analysis (Table 4; Fig. 3). For the cutoff values determined in the ROC curve analyses for these subgroups, the Kaplan-Meier survival probability curves were plotted with a significant difference of probability for a given cutoff value (Fig. 3).

Correlations between TMAO Levels and Clinical Parameters

For quantitative variables, TMAO levels were positively correlated ($p < 0.05$) with age ($r_s = 0.28$), the levels of high-sensitivity C-reactive protein ($r_s = 0.12$), HbA_{1c} ($r_s = 0.27$), and triglycerides ($r_s = 0.09$), while inversely with left ventricular ejection fraction ($r_s = -0.12$), eGFR ($r_s = -0.42$), and high-density lipoprotein levels ($r_s = -0.14$). For dichotomous variables, TMAO levels were positively correlated with the presence of diabetes ($r_s = 0.24$), SCS ($r_s = 0.1$), chronic HF ($r_s = 0.24$), arterial hypertension ($r_s = 0.17$), and AF ($r_s = 0.19$). The correlations were weak or very weak. The strongest correlation was an inverse correlation with eGFR.

Correlations between TMAO Levels and Multimorbidity

Considering the high prevalence of comorbidities in the study population, we investigated an association between TMAO levels and multimorbidity. We focused on such comorbidities as ACS, SCS, chronic HF, AF, arterial hypertension, and arrhythmias or conduction disorders. The presence of 1 comorbidity was noted in 130 patients; 2 comorbidities in 260 patients; 3 in 338 patients; 4 in 191 patients; 5 in 85 patients; and all 6 comorbidities were present in 32 patients. Multimorbidity was positively correlated with TMAO levels (Kendall Tau = 0.20; $p < 0.05$). There were also significant differences in TMAO levels between the subgroups, particularly between patients with 1 or 2 comorbidities and the remaining subgroups.

Table 3. Univariate and multivariate Cox proportional hazards model for the effect of TMAO levels on 5-year mortality in study subgroups

Subgroup	HR	95% CI	*p* value
Univariate analysis			
ACS (*n* = 177)	1.00	0.98–1.01	0.95
SCS (*n* = 441)	1.01	1.00–1.02	0.001
Chronic HF (*n* = 292)	1.01	1.00–1.03	0.033
AF (*n* = 277)	1.02	1.00–1.04	0.018
Multivariate analysis			
ACS (*n* = 180)	1.00	0.98–1.02	0.70
SCS (*n* = 441)	1.02	0.99–1.04	0.089
Chronic HF (*n* = 292)	1.00	0.98–1.02	0.56
AF (*n* = 277)	1.01	0.98–1.03	0.42

Abbreviations: see Table 1.

Discussion

The univariate analysis revealed that increased TMAO levels were associated with only 1% increase in the relative risk of death at 5-year follow-up. However, even this clinically insignificant predictive value was no longer statistically significant in the multivariate analysis. At the same time, patients with TMAO levels in the highest quartile showed a 55% higher risk of death at 5 years compared with those with TMAO levels in the lowest quartile, although the association was no longer significant in the multivariate analysis. The cutoff value of TMAO levels determined in the ROC curve analysis (5.94 μM) had a low sensitivity and an acceptable specificity for predicting a higher risk of death in the whole study population.

Table 4. Cutoff points for TMAO levels; results of the ROC curve analysis

Subgroup	Cutoff point (95% CI)	Youden index (95% CI)	AUC (95% CI)	*p* value
SCS	6.41 (3.80–6.98)	0.36 (0.22–0.45)	0.70 (0.62–0.77)	<0.0001
Chronic HF	6.52 (3.86–7.74)	0.25 (0.08–0.33)	0.64 (0.56–0.72)	<0.001
AF	4.97 (3.86–5.94)	0.27 (0.14–0.35)	0.65 (0.57–0.72)	<0.001

AUC, area under the receiver operating characteristic curve; others, see Table 1.

Compared with the results of meta-analyses [18, 19] on various populations of patients with higher cardiovascular risk, our univariate analysis showed clinically insignificant increase in the risk of mortality. However, for TMAO levels in the highest quartile, the relative risk of mortality in our study was similar to the results of meta-analyses that applied tertile, quartile, and quintile comparisons [18, 19]. The relative risk reported by meta-analyses ranged from 1.466 (95% CI: 1.291–1.665); $p < 0.001$ [18] to 1.91 (95% CI: 1.40–2.61); $p < 0.0001$ [19].

In our univariate analysis, higher TMAO levels were a significant predictor of mortality in patients with SCS, chronic HF, and AF. However, the increase in the relative risk of mortality by 1–2% in these subpopulations was no longer significant in the multivariate analysis adjusted for other mortality risk factors. In the ROC analysis, the cutoff values of TMAO levels showed low sensitivity and a maximum specificity of 80% for predicting mortality at 5 years.

In previous studies of patients with coronary artery disease, higher TMAO levels predicted mortality independently of traditional risk factors at 3-year [10] and 5-year follow-up [11]. The median TMAO levels were 3.7 μM (IQR, 2.4–6.20) [10] and 3.8 (IQR, 2.5–6.5) μM, respectively [11], which is lower than in our patients with SCS (4.29 μM [IQR, 2.94–6.67]). Additionally, in our study, median TMAO levels were higher in deceased patients with SCS (6.26 μM [IQR, 2.70–10.1]; $p < 0.00001$). At the same time, the cutoff value of 6.41 μM is close to the upper quartile reported by Senthong et al. [15]. Despite the higher TMAO levels than in the above studies [10, 11], there was clinically insignificant increase in the mortality risk of patients with SCS (1–2%), and after adjustment for other mortality risk factors, TMAO levels lost their statistical significance.

Considering the methodology adopted in our study, we cannot conclude that TMAO levels are an independent predictor of mortality in patients with SCS. However, in the analysis by the quartiles of TMAO levels, the Kaplan-Maier curves revealed significant differences in survival.

Importantly, the mortality rate in the study by Senthong et al. [11] was 15.1% as compared with 21% in our patients with SCS. This suggests the potential influence of other factors in our patients. Similarly, our results for the population of patients with ACS do not correspond with previous studies including patients with ST-segment elevation myocardial infarction [12] and acute myocardial infarction [13], which reported high TMAO levels to be an independent predictor of death.

In the multivariate analysis, increased TMAO levels were not significantly associated with mortality in patients with chronic HF, which is in line with the results reported by Troseid et al. [14]. Similar to our study, they included age, kidney function, and diabetes in the multivariate analysis, among other factors. Also, Tang et al. [15] revealed a significant association of TMAO levels with mortality, even after adjustment for eGFR. However, as the authors admit themselves, most of their patients had normal kidney function.

To our knowledge, no previous studies have assessed the effect of TMAO on mortality in patients with AF. In our study, deceased patients with AF showed significantly higher TMAO levels than survivors. However, TMAO levels were no longer an independent predictor in the multivariate analysis. Previous studies described a correlation between higher TMAO levels with thrombus formation in patients with AF [16]. Higher TMAO levels seem to be linked with a higher risk of thromboembolic events, but their ultimate effect on the risk of death may also depend on such factors as age, comorbidities, and concomitant medical therapy.

The strongest correlation between TMAO levels and clinical parameters was shown for eGFR, age, and the presence of diabetes. Previous studies investigated the cause-and-effect relationship between the increasing TMAO levels and kidney damage, including also in patients with diabetes [4]. This relationship probably works in both directions, and the possible underlying pathophysiological mechanism is the impaired renal clearance of TMAO as well as the common coexistence of chronic CVD and kidney disorders. It also seems reasonable to consider the deterioration of kidney function with age.

Lee et al. [5] provided a valuable insight into the relationship between TMAO levels, kidney function, and the risk of atherosclerotic CVD (ASCVD) [5]. The authors revealed that the risk of newly diagnosed ASCVD in healthy individuals at 15 years was greater only in patients with higher TMAO levels and impaired kidney function (eGFR <60 mL/min/1.73 m^2). In patients with preserved kidney function, the increase in the risk was not significant. However, in patients with previously diagnosed ASCVD, the risk of recurrent ASCVD increased with an increase in TMAO levels independently of kidney function.

We also observed the association between higher TMAO levels and multimorbidity in our subpopulations of patients with CVD. This is in line with a study by Montrucchio et al. [20], who revealed that higher TMAO levels were associated with multimorbidity in patients with HIV. While this is a different population with a different underlying disease, their findings underline the uncertainties as to the exact role of TMAO in CVD. The pathophysiologi-

cal processes associated with higher TMAO levels such as atherogenesis, cardiac remodeling, and arrhythmia may coexist at varied levels of severity and are influenced by numerous external time-related factors. Therefore, it is challenging to determine TMAO as either a causative factor of these conditions or merely a contributing factor.

Our study included a relatively large population of patients and had a long follow-up. Therefore, our results for the overall study group as well as patient subgroups may be considered as statistically representative of the population of patients with CVD. Moreover, as the study included consecutive patients, our findings may be extrapolated to the general population of patients treated at cardiac departments. Finally, as we assessed numerous biochemical and anthropometric parameters as well as data from medical records, we were able to perform a multivariate analysis including traditional risk factors.

Our study has several limitations. First, this was a single-center retrospective observational study. The retrospective design does not exclude the presence of other uncontrolled factors that might have affected clinical outcomes and the incidence of death, despite the use of multivariate regression. Second, data on the causes of death were lacking. As the study had a long follow-up, we may suspect that not all deaths were from cardiovascular causes. Third, the results are based on a single measurement of TMAO levels, which are liable to change over time and depend on multiple other factors. Most importantly, we did not have access to data on antibiotic therapy prior to serum collection, the levels of TMAO precursors, gut microbiota composition, and dietary habits of participants before hospitalization. During follow-up, we did not record data on subsequent hospitalizations, significant cardiovascular events, subsequent comorbidities, changes in medical therapy, and lifestyle modifications by patients. These factors might have affected TMAO levels and mortality rates after the index hospitalization. Finally, the lack of a control group made it impossible to compare the effect of higher TMAO levels between patients with CVD and healthy individuals. As our study included patients with high mortality risk due to the underlying CVD, the results cannot be generalized to a healthy population.

Conclusions

In conclusion, although our study revealed a very weak positive correlation between increased TMAO levels and a higher risk of mortality, the use of TMAO as an independent mortality predictor is limited, and numerous other factors should be included in the assessment of patient prognosis. Further research is needed to establish the exact role of TMAO as either a causative or a contributing factor of mortality in patients with high cardiovascular burden. Interventional studies assessing the benefits of reducing TMAO levels may provide additional important insights.

Acknowledgment

The samples were provided by the Biobank of Łukasiewicz Research Network – PORT Polish Center for Technology Development.

Statement of Ethics

The study protocol was approved by the local Ethics Committee of Wroclaw Medical University (No. 163/2019; February 28, 2019). The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

The study was funded by a statutory grant of the Institute for Heart Diseases.

Author Contributions

First author: Radosław Konieczny. Radosław Konieczny, Wiktor Kuliczkowski, and Andrzej Mysiak – conceived the concept for the study and designed the research. Radosław Konieczny, Ewa Żurawska-Płaksej, Konrad Kaaz, and Hanna Czapor-Irzabek – acquisition the data. Radosław Konieczny, Wiktor Kuliczkowski, Wojciech Bombała – analysis and interpretation of data. Radosław Konieczny, Wiktor Kuliczkowski, Ewa Żurawska-Płaksej, Konrad Kaaz, and Hanna-Czapor-Irzabek – drafting of the manuscript. Radosław Konieczny, Wiktor Kuliczkowski, Ewa Żurawska-Płaksej,Wojciech Bombała, and Andrzej Mysiak – revising for important intellectual content. All the authors have read and accepted the manuscript in its final form, including the authorship list.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References

1 Goel JC, Gaur A, Singhal V, Parakh N, Bhargava B, Sharma A. The complex metabolism of trimethylamine in humans: endogenous and exogenous sources-CORRIGENDUM. Expert Rev Mol Med. 2016;18:e19.

2 Wang Z, Bergeron N, Levison BS, Li XS, Chiu S, Jia X, et al. Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. Eur Heart J. 2019;40(7):583–94.

3 Romano KA, Vivas EI, Amador-Noguez D, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-Oxide. mBio. 2015;6(2):e02481.

4 Mueller DM, Allenspach M, Othman A, Saely CH, Muendlein A, Vonbank A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. Atherosclerosis. 2015;243(2):638–44.

5 Lee Y, Nemet I, Wang Z, Lai HTM, de Oliveira Otto MC, Lemaitre RN, et al. Longitudinal plasma measures of trimethylamine N-oxide and risk of atherosclerotic cardiovascular disease events in community-based older adults. J Am Heart Assoc. 2021;10(17):e020646.

6 Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.

7 Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. Cell. 2016;165(1):111–24.

8 Wilson A, McLean C, Kim RB. Trimethylamine-N-oxide; a link between the gut microbiome, bile acid metabolism, and atherosclerosis. Curr Opin Lipidol. 2016;27(2):148–54.

9 Li Z, Wu Z, Yan J, Liu H, Liu Q, Deng Y, et al. Gut microbe-derived metabolite trimethylamine N-oxide induces cardiac hypertrophy and fibrosis. Lab Invest. 2019;99(3):346–57.

10 Tang WHW, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.

11 Senthong V, Wang Z, Li XS, Fan Y, Wu Y, Tang WHW, et al. Intestinal microbiota-generated metabolite trimethylamine-N-Oxide and 5-year mortality risk in stable coronary artery disease: the contributory role of intestinal microbiota in a COURAGE-like patient cohort. J Am Heart Assoc. 2016;5(10):e004237.

12 Matsuzawa Y, Nakahashi H, Konishi M, Sato R, Kawashima C, Kikuchi S, et al. Microbiota-derived trimethylamine N-oxide predicts cardiovascular risk after STEMI. Sci Rep. 2019;9(1):11647.

13 Suzuki T, Heaney LM, Jones DJL, Ng LL. Trimethylamine N-oxide and risk stratification after acute myocardial infarction. Clin Chem. 2017;63(1):420–8.

14 Trøseid M, Ueland T, Hov JR, Svardal A, Gregersen I, Dahl CP, et al. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. J Intern Med. 2015;277(6):717–26.

15 Tang WHW, Wang Z, Fan Y, Levison B, Hazen JE, Donahue LM, et al. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: refining the gut hypothesis. J Am Coll Cardiol. 2014;64(18):1908–14.

16 Gong D, Zhang L, Zhang Y, Wang F, Zhao Z, Zhou X. Gut microbial metabolite trimethylamine N-oxide is related to thrombus formation in atrial fibrillation patients. Am J Med Sci. 2019;358(6):422–8.

17 Żurawska-Płaksej E, Płaczkowska S, Pawlik-Sobecka L, Czapor-Irzabek H, Stachurska A, Mysiak A, et al. Parameters of oxidative and inflammatory status in a three-month observation of patients with acute myocardial infarction undergoing coronary angioplasty: a preliminary study. Medicina. 2019;55(9):585.

18 Farhangi MA. Gut microbiota-dependent trimethylamine N-oxide and all-cause mortality: findings from an updated systematic review and meta-analysis. Nutrition. 2020;78:110856.

19 Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, et al. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. Eur Heart J. 2017;38(39):2948–56.

20 Montrucchio C, De Nicolò A, D'Ettorre G, D'Ascenzo F, Lazzaro A, Tettoni M, et al. Serum trimethylamine-N-oxide concentrations in people living with HIV and the effect of probiotic supplementation. Int J Antimicrob Agents. 2020;55(4):105908.

9.3. Publikacja nr 3

Original papers

Citrulline and long-term mortality in patients with cardiovascular disease

Radosław Andrzej Konieczny[1,A–F], Ewa Żurawska-Płaksej[2,B–D,F], Konrad Kaaz[3,B,C,F], Hanna Czapor-Irzabek[4,B,C,F], Wojciech Bombała[5,C,E,F], Andrzej Mysiak[3,A,C,F], Wiktor Kuliczkowski[3,A,C,D,F]

[1] Jan Mikulicz-Radecki University Teaching Hospital, Clinic of Gastroenterology and Hepatology, Wrocław, Poland
[2] Department of Toxicology, Wroclaw Medical University, Poland
[3] Institute of Heart Diseases, Wroclaw Medical University, Poland
[4] Laboratory of Elemental Analysis and Structural Research, Wroclaw Medical University, Poland
[5] Statistical Analysis Centre, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2022;31(10):1121–1128

Address for correspondence
Radosław Andrzej Konieczny
E-mail: rkonieczny.med@gmail.com

Funding sources
The study was funded by a statutory grant from the Institute of Heart Diseases, Wroclaw Medical University, Poland.

Conflict of interest
None declared

Acknowledgements
The samples were provided by the Biobank of Łukasiewicz Research Network – PORT Polish Center for Technology Development in Wrocław, Poland.

Received on March 25, 2022
Reviewed on May 19, 2022
Accepted on May 26, 2022

Published online on June 14, 2022

Cite as
Konieczny RA, Żurawska-Płaksej E, Kaaz K, et al. Citrulline and long-term mortality in patients with cardiovascular disease. *Adv Clin Exp Med*. 2022;31(10):1121–1128. doi:10.17219/acem/150475

DOI
10.17219/acem/150475

Abstract

Background. Cardiovascular disease (CVD) is associated with intestinal barrier dysfunction and increased intestinal permeability. Increased intestinal permeability to gut microbial metabolites may accelerate the progression of CVD. Plasma citrulline levels are a marker of functional enterocyte mass, and reduced citrulline levels indicate intestinal epithelial damage. Citrulline was reported as a useful prognostic marker in critically ill patients. However, data are lacking on the association of citrulline with long-term mortality in patients with CVD and with the levels of trimethylamine N-oxide (TMAO), a microbiota-derived metabolite which has been implicated in the pathogenesis of CVD.

Objectives. To assess the effect of citrulline levels, a marker of intestinal barrier disruption, on long-term mortality in patients with CVD. Moreover, the relationship between the concentrations of 2 biomarkers – citrulline and TMAO – was assessed.

Materials and methods. Serum citrulline levels were retrospectively assessed in 1036 consecutive patients with CVD (median age: 62 years; 61% men) hospitalized between 2013 and 2015. Associations of citrulline levels with 5-year mortality rates as well as anthropometric and biochemical parameters were evaluated for the entire study group and in subgroups of patients with acute coronary syndrome (ACS), chronic coronary syndrome, chronic heart failure (chronic HF), and atrial fibrillation (AF). Correlations between serum citrulline and TMAO levels were assessed.

Results. The median citrulline level in the study population was 22.5 μM (interquartile range (IQR): 17.8–27.9). Citrulline levels were not associated with 5-year mortality in patients with CVD (hazard ratio (HR) = 0.99; 95% confidence interval (95% CI): 0.97–1.00; p = 0.49). Median citrulline levels differed significantly between deceased patients and survivors at 5 years in patients with ACS (p = 0.025). There were no significant correlations between citrulline and TMAO levels (Kendall's tau = 0.027).

Conclusions. Decreasing citrulline levels do not predict long-term mortality of hospitalized patients with CVD. Moreover, they are not associated with the serum levels of TMAO in these patients.

Key words: cardiovascular diseases, intestinal barrier, citrulline, gut permeability, TMAO

Background

Cardiovascular disease (CVD) has been reported to be associated with intestinal barrier dysfunction and increased intestinal permeability.[1] Initial studies focused on altered intestinal function in patients with chronic heart failure (chronic HF).[2–4] However, a link between intestinal barrier dysfunction and coronary artery disease,[5,6] ST-segment elevation myocardial infarction[7,8] and arterial hypertension[9,10] has also been reported. A vicious cycle has been described wherein CVD impairs the intestinal barrier, making it more permeable to toxic substances that favor the progression of cardiovascular abnormalities.[1]

The assessment of the intestinal barrier competency is a complex task which can be achieved using a number of approaches.[11] One approach is to use biomarkers such as bacterial lipopolysaccharide (LPS),[11] zonulin,[12] claudins,[13] and intestinal fatty acid binding protein.[14] There are also tools for evaluating the function of intestinal barrier samples such as the Ussing chamber.[15] Depending on the method used, various structural and functional parameters can be measured. One available biomarker of intestinal barrier function is serum citrulline level, which allows for an indirect assessment of absorptive enterocyte mass.[1,16–18] Citrulline is an amino acid synthesized mainly by enterocytes in the proximal small bowel, in the middle and upper parts of intestinal villi.[17] Citrulline measurements have been reported to be a useful marker of acute mesenteric ischemia.[19] Moreover, their use as a marker of epithelial lining damage in a human model of small intestinal ischemia and reperfusion has been described.[20] Reduced citrulline levels also serve as a biomarker of intestinal barrier failure and are an independent prognostic factor in critically ill patients.[21–24]

A recent meta-analysis of 26 randomized controlled studies found that long-term citrulline supplementation significantly improves vascular endothelial function and reduces arterial stiffness.[25] As the authors point out, these effects are associated with a reduced risk of cardiovascular events. Other beneficial effects of citrulline supplementation include improved blood pressure, glucose and lipid profile, and the bioavailability of arginine and nitric oxide.[26]

So far, no studies have assessed the use of citrulline levels as a predictor of long-term mortality in patients with CVD. Moreover, data are lacking on the association between levels of citrulline and the microbiota-derived metabolite trimethylamine N-oxide (TMAO).[27,28] It was postulated that intestinal permeability can significantly affect the absorption of TMAO and its precursors.[29] The current study is a continuation of our previous research on the same population of patients (unpublished data). The previous study did not reveal a significant association between TMAO levels and long-term mortality in CVD patients. The assessment of intestinal permeability may shine a new light on our previous findings and guide the direction of future research.

Objectives

This study aimed to assess the effects of citrulline levels, a marker of enterocyte functional mass, on the long-term mortality of CVD patients. Moreover, the relationship between 2 biomarkers – citrulline and TMAO levels – was evaluated.

Materials and methods

Patients

The study included 1036 consecutive patients hospitalized between March 2013 and November 2015 in the Department of Cardiology at Wroclaw Medical University in Wrocław, Poland. All patients provided written informed consent to participate in the study and submitted blood samples for laboratory testing.

The exclusion criterion was the lack of patient's informed consent. Patients who did not want to consent or were unable to consent due to their clinical condition (i.e., unconscious or intubated) were excluded from the study. We routinely included patients on days 1 or 2 of the hospital stay in order to enroll patients with stable conditions who were willing to participate in the study.

Specimen characteristics

Blood samples were stored at a temperature of –80°C at the BioBank of the Łukasiewicz Research Network – PORT Polish Center for Technology Development in Wrocław, Poland.

Assay methods

Liquid chromatography with tandem mass spectrometry was used to determine serum L-citrulline and TMAO levels. Briefly, samples were thawed on ice and extracted by adding a mix of d4-citrulline and d9-TMAO (Cambridge Isotope Laboratories, Tewksbury, USA) in acetonitrile (final concentration 10 µM) at a ratio of 1:3 (v/v). The chromatographic separation was performed using a Luna Silica analytical column (3 µm, 100 Å, 150 × 2 mm; Phenomenex, Torrance, USA) and the UltiMate™ 3000 UPLC system (Dionex, Sunnyvale, USA). An isocratic elution of the mobile phase consisting of 0.1% formic acid in acetonitrile and water at a ratio of 60:40 (v/v) was applied at a flow rate of 0.3 mL/min (total run time: 5 min). A multiple reaction monitoring mode was selected for mass spectrometric detection using ESI-Q-TOF (Bruker Daltonics, Bremen, Germany) in a positive ion mode. A calibration curve was constructed at a range of 0.5–100 µM for L-citrulline (Sigma-Aldrich, St. Louis, USA) and 0.125–25 µM for TMAO.

This method has been validated according to the Food and Drug Administration (FDA) guidelines.[30] The linearity

was determined by using correlation coefficients (R^2) which were 0.9997 for both citrulline and TMAO levels. The sensitivity of the laboratory analysis was assessed based on the limit of detection and limit of quantification. These were 0.221 μM and 0.662 μM, respectively, for citrulline and 0.236 μM and 0.708 μM, respectively, for TMAO. The precision and accuracy of the measurements were assessed with the use of quality control samples and standard solutions of various concentrations. The calculated inter- and intra-assay coefficients of variation did not exceed 8% for any of the tested levels, and the accuracy was in the range of 95–105%. This is in line with the limits proposed for the validation of assay methods for biological samples.

All samples were measured in 2 biological and 2 technical replicates. The peak area ratio (analyte/internal standard) was used for the calculation of mean L-citrulline and TMAO levels in samples. Data on current diagnosis, comorbidities, anthropometric parameters, laboratory test results, and medication use were obtained from the medical records of the index hospitalization.

Study design

This is a retrospective observational study.

At the time of hospitalization, patients donated blood samples for future research. The protocol of sampling and biobank creation was approved by the local ethics committee of Wroclaw Medical University on February 9, 2011 (approval No. 73/2012). All patients consented to the storage and processing of samples for future studies.

The concentrations of the selected biomarkers were assessed retrospectively in collected samples. Mortality was analyzed using the registry of the Polish National Health Fund (as of February 19, 2020).

Statistical analyses were performed to assess the correlation of biomarker concentrations with mortality and the available clinical data obtained during the hospitalization of each patient.

The study protocol was approved by the local ethics committee of Wroclaw Medical University on February 28, 2019 (approval No. 163/2019). The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in the study.

Statistical analyses

The statistical analyses were performed on the whole population. Subgroups of patients were classified according to the main clinical diagnosis of CVD: 1) patients with acute coronary syndrome (ACS) during the index hospitalization; 2) patients with chronic coronary syndrome; 3) patients with chronic HF without ACS; and 4) patients with atrial fibrillation (AF).

Additionally, patients were classified according to the occurrence of death during the follow-up period, as shown in Table 1 for the whole study group, and within subpopulations, as shown in Table 2. Depending on data distribution, quantitative data were presented as means with standard deviations (SDs) and medians with interquartile ranges (IQRs). The rates of CVD and medication use were presented as percentages of patients. The normality of data distribution was assessed using the Kolmogorov–Smirnov and Lilliefors tests. The Kendall's tau test was used to assess the correlation between variables. The mean values of 2 independent variables were compared using the nonparametric Mann–Whitney test, while the Kruskal–Wallis analysis of variance (ANOVA) was used to compare more than 2 independent variables. The effect of continuous variables on mortality in subgroups was assessed using the Cox proportional hazards model. The proportional hazards assumptions were tested with proportional tests and presented graphically using the plots of scaled Schoenfeld residuals. The goodness-of-fit was tested by calculating the coefficient of determination (R^2). The Cox proportional hazards model with interactions was used to assess the independence of variables on mortality prediction. Survival curves for individual variables were generated using the Kaplan–Meier estimator. A value of $p < 0.05$ was considered statistically significant. All analyses were performed using STATISTICA v. 13 software (TIBCO Software Inc., Palo Alto, USA; https://www.tibco.com/).

Results

The characteristics of the study population are presented in Table 1. The study included a total of 1036 patients, including 177 (11.3%) patients with ACS, 441 (42.6%) patients with chronic coronary syndrome, 292 (28.2%) patients with chronic HF, and 277 (26.7%) patients with AF. The mean follow-up for the study group was 58.4 months. The 5-year mortality rate for the whole population was 16.5% (171 deaths). Most of the clinical and biochemical parameters differed between the deceased patients and the survivors (Table 1).

There were no significant differences in citrulline levels between subgroups divided according to survival (Table 1). Moreover, the univariate Cox proportional hazards analysis revealed no association between citrulline levels and death at 5 years (hazard ratio (HR) = 0.99; 95% confidence interval (95% CI): 0.97–1.00; p = 0.49). Therefore, a multivariate analysis was not performed.

For a more detailed analysis, citrulline levels were divided into quartiles. However, no differences were noted between the quartiles in terms of their effect on survival at 5 years (χ^2 test = 3.53; p = 0.31) (Fig. 1).

To obtain a more detailed insight into the effect of citrulline levels on mortality in patients with CVD, we performed a subgroup analysis of citrulline levels. The differences in citrulline levels in subgroups divided according to survival at 5 years are presented in Table 2. Significantly

Table 1. Study population characteristics

Parameter		Study population (n = 1036)	Death at 5 years		p-value*
			yes (n = 171)	no (n = 865)	
Age [years], mean (SD)		62.0 (14.1)	68.9 (11.1)	60.7 (14.3)	<0.0001
Male sex [%]		61.1	73.0	58.8	<0.001
TMAO [µmol/L]		4.06 (2.79–6.01)	5.65 (3.48–8.94)	3.86 (2.70–5.62)	<0.0001
Citrulline [µmol/L]		22.5 (17.8–27.9)	21.5 (16.5–27.6)	22.8 (18.0–28.0)	0.18
Acute coronary syndrome [%]		17.0	32.7	19.4	<0.001
Chronic coronary syndrome [%]		42.5	57.1	40.0	<0.0001
Chronic HF [%]		28.1	54.7	23.4	<0.0001
Atrial fibrillation [%]		26.7	43.2	23.4	<0.0001
Other types of arrhythmia [%]		16.4	14.7	85.2	0.48
Conduction disorders [%]		44.0	13.1	86.8	0.01
PCI during hospitalization [%]		21.0	31.1	23.3	0.051
Previous PCI [%]		14.4	25.7	16.9	0.017
Previous CABG [%]		7.6	12.2	6.7	0.012
Diabetes [%]		24.7	35.2	22.6	<0.001
Chronic kidney disease [%]		14.58	28.5	71.5	0.002
Dialysis patients [%]		1.45	27.67	73.33	0.001
Hypertension [%]		71.7	81.8	69.7	0.001
Smoking (current) [%]		23.2	30.0	21.9	0.026
NYHA (% of patients with chronic HF)	I	7.0	8.7	6.3	0.16
	II	55.5	48.3	58.8	
	III	34.0	37.7	32.3	
	IV	3.3	5.3	2.6	
CCS (% of patients with chronic coronary syndrome)	I	10.2	6.0	11.3	0.65
	II	54.3	57.5	53.5	
	III	32.2	33.3	32.0	
	IV	3.1	3.0	3.1	
LVEF [%]		60 (50–65)	50 (37–65)	65 (55–66)	<0.0001
eGFR [mL/min/1.73 m^2]		68 (55–80)	58 (44–72)	69 (58–81)	<0.0001
hsCRP [mg/L]		3.47 (1.33–13.3)	7.79 (2.64–30.8)	3.11 (1.24–10.1)	<0.0001
HbA_{1c} [%]		5.70 (5.40–6.20)	5.90 (5.50–6.65)	5.70 (5.40–6.10)	0.018
TC [mg/dL]		182 (149–216)	171 (135–207)	184 (151–218)	0.003
LDL-C [mg/dL]		104 (80–136)	97 (33–249)	106 (83–136)	0.094
HDL-C [mg/dL]		45 (37–55)	42 (34–51)	46 (38–55)	<0.001
Triglycerides [mg/dL]		124 (95–169)	115 (89–155)	127 (96–172)	0.018
ASA [%]		54.8	64.0	52.9	0.009
Clopidogrel [%]		29.4	28.1	35.1	0.075
OAC [%]		28.9	43.4	26.0	<0.0001
ACEIs [%]		70.2	76.1	69.0	0.066
ARBs [%]		7.19	3.01	8.02	0.022
β-blockers [%]		81.2	92.2	79.0	<0.0001
Statins [%]		79.1	89.2	77.0	<0.001
Loop diuretics [%]		19.1	43.4	14.3	<0.0001

Data are presented as median and interquartile range (IQR) unless indicated otherwise. ACEI – angiotensin-converting enzyme inhibitor; ARB – angiotensin receptor blocker; ASA – acetylsalicylic acid; CABG – coronary artery bypass grafting; CCS – Canadian Cardiovascular Society grading of angina pectoris; eGFR – estimated glomerular filtration rate; HbA_{1c} – glycated hemoglobin A_{1c}; HDL-C – high-density lipoprotein cholesterol; HF – heart failure; hsCRP – high-sensitivity C-reactive protein; LDL-C – low-density lipoprotein cholesterol; LVEF – left ventricular ejection fraction; NYHA – New York Heart Association Functional Classification; OAC – oral anticoagulation; PCI – percutaneous coronary intervention; SD – standard deviation; TC – total cholesterol; TMAO – trimethylamine N-oxide.

Table 2. Citrulline levels in subgroups of deceased patients compared to survivors at 5 years

Subgroup	Whole subgroup [μmol/L]	Death at 5 years		p-value
Acute coronary syndrome (n = 177)	23.3 (18.3–29.1)	yes (n = 37)	no (n = 140)	0.025
		20.9 (15.2–25.8)	24.0 (18.5–30.1)	
Chronic coronary syndrome (n = 441)	22.2 (17.5–28.1)	yes (n = 96)	no (n = 345)	0.12
		21.0 (15.7–21.3)	22.4 (18.0–28.4)	
Chronic HF (n = 292)	22.2 (17.9–28.2)	yes (n = 93)	no (n = 199)	0.50
		21.7 (16.6–28.2)	22.3 (18.3–28.3)	
Atrial fibrillation (n = 277)	22.2 (17.7–28.5)	yes (n = 74)	no (n = 203)	0.89
		22.9 (16.5–30.1)	22.1 (18.1–28.4)	

Data are presented as median and interquartile range (IQR). HF – heart failure.

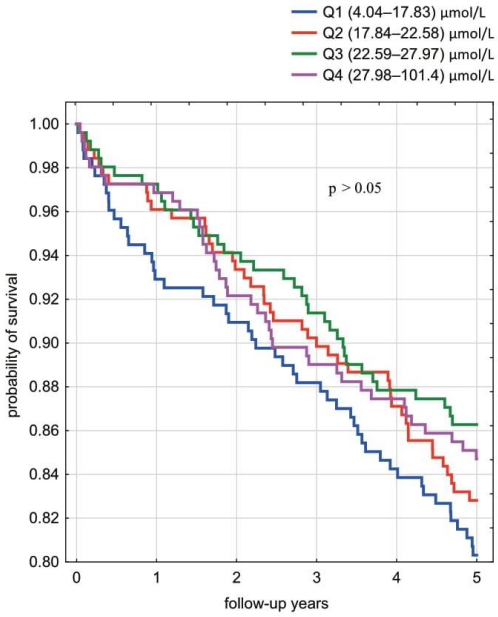


Fig. 1. Kaplan–Meier survival curves in subgroups divided according to quartiles of citrulline levels (Q1, Q2, Q3, and Q4 – quartiles 1, 2, 3, and 4, respectively).

lower citrulline levels were observed in deceased patients compared to survivors at 5 years in patients with ACS. However, the univariate Cox proportional hazards analysis did not reveal any significant associations between citrulline levels and the risk of death at 5 years in any of the subgroups, including patients with ACS. Therefore, multivariate analysis was not performed.

Our analysis revealed no significant correlations between citrulline levels and any of the recorded clinical parameters. Additionally, there were no significant correlations between citrulline levels and multimorbidity, defined as the number of comorbidities present in 1 patient, including ACS, stable coronary syndrome (SCS), chronic HF, AF, arterial hypertension, arrhythmias, and/or conduction disorders. Finally, no significant correlations were noted between TMAO and citrulline levels (Kendall's tau = 0.027).

Discussion

Our study indicates that serum citrulline levels are not correlated with long-term mortality in patients hospitalized due to CVD. While several markers of intestinal barrier dysfunction are available, this study was constructed to assess a single parameter. We decided on citrulline because there is limited research available on the association between citrulline levels and long-term outcomes in CVD patients.

When investigating a single marker of intestinal permeability, the interpretation of the results must account for some inherent limitations. One of the most common markers of an impaired intestinal barrier is LPS.[11] However, LPS assessment in peripheral blood is relatively difficult and often produces false positive results. Moreover, similarly to anti-endotoxin antibodies, it serves only as an indirect marker of increased intestinal permeability.[11] Another known biomarker is intestinal fatty acid-binding protein. However, there are limited data supporting its use in the assessment of chronic intestinal barrier disruption.[31] As for zonulin, previous studies have used commercial enzyme-linked immunosorbent assays (ELISAs) for zonulin measurements. However, these assays do not reflect the actual levels of zonulin but rather the levels of a structurally similar haptoglobin.[12] The reliability of studies on zonulin is further limited by the fact that zonulin is not expressed in mice. Thus, the results obtained actually reflect the levels of unknown proteins rather than zonulin.[12] Finally, claudins are a highly diverse class of 27 proteins present in numerous tissues.[13] They have not only low tissue specificity but also opposing functions, making it difficult to choose a specific protein and interpret the results.[13]

The usefulness of citrulline for the direct functional assessment of enterocyte mass has been confirmed in previous research studies.[16,32] According to the literature, citrulline cutoff values have a sensitivity of 80% and

a specificity of 84% for diagnosing intestinal dysfunction. The most commonly used threshold for low citrulline levels is 20 μmol/L,[16] which is in line with our findings. According to other investigators, a citrulline threshold of 10 μmol/L indicates a significant loss of enterocyte mass.[32] Finally, it has been reported that citrulline levels ranging from 10 μmol/L to 20 μmol/L are a grey zone for interpretation in critically ill patients.[33] Citrulline level of 40 ±10 μmol/L is considered normal.[32] The abnormal citrulline levels in our population may indirectly suggest intestinal epithelial damage. However, several other factors need to be considered when interpreting these results.

Despite high susceptibility to blood supply disorders, high regenerative capacity of the intestinal epithelial lining after ischemia and reperfusion has been reported.[20] So far, citrulline assays have been used mainly for the assessment of critically ill patients.[22–24,34,35] Changes in citrulline levels have been associated with patient prognosis[24] and enteral nutrition.[35] However, these studies were limited by a shorter duration of follow-up compared with our current research.

Considering the currently available literature, the fact that the diagnostic and prognostic values of citrulline are higher in acute states than in chronic ones cannot be excluded. At the same time, reduced citrulline levels are typically observed in chronic diseases associated with intestinal epithelial loss, such as Crohn's disease, celiac disease and short bowel syndrome.[16] It is possible that changes in citrulline levels during the course of chronic CVD result in different dynamics and are induced by different mechanisms. This seems to be supported by the lack of correlation between citrulline levels and multimorbidity in our study. A significant difference in citrulline levels between deceased patients and survivors was noted only in patients with ACS, the only subgroup with an acute cardiovascular condition in our study. Previous studies on patients with myocardial infarctions reported abnormal levels of other markers indicating impaired intestinal barrier, such as LPS, D-lactate, zonulin, and endotoxin.[7,8,36] Moreover, Zhou et al. reported that LPS and D-lactate were associated with a higher risk of mortality at 3 years.[8] In our study, citrulline was not related to mortality risk at 5 years even in patients with ACS. The discrepancy between our study and the study by Zhou et al. may result from differences in the type of biomarkers and frequency of measurements. Zhou et al. assessed the levels of LPS and D-lactate at 2 time points over consecutive days.[8] Our study, on the other hand, involved a single measurement of citrulline levels.

Serial measurements have advantages over a single assessment. Previous studies assessing changes in citrulline levels have revealed correlations between citrulline and the clinical status of patients.[24,35] In an experimental study by Park et al., serial measurements reduced the potential confounding effect of circadian variation and fluctuations in citrulline levels.[37]

Our study did not reveal any correlation between citrulline levels and clinical parameters such as estimated glomerular filtration rate (eGFR) or C-reactive protein (CRP) levels. This is surprising because kidney disorders and enhanced inflammatory processes may induce changes in citrulline levels.[32] Citrulline is converted to arginine in the kidneys. Arginine is then used in nitric oxide synthesis, for example, in response to inflammation.[32] Rare metabolic disorders and partial small bowel resections resulting in short bowel syndrome can affect citrulline levels, but these conditions were excluded from our study population. Only 2 patients had Crohn's disease and there were no cases of celiac disease or short bowel syndrome in the population examined in our study. Therefore, citrulline levels were not affected by these factors in our patients.

In our opinion, this study increases the current knowledge on the absorption of gut microbial metabolites. A previous prospective study by Kitai et al. revealed a significant association between worse prognosis and increased intestinal permeability assessed using the lactulose/rhamnose permeability test.[38] However, the sample size was relatively small (29 patients with HF) and the follow-up was shorter than in our study (median: 56 days). The increased intestinal permeability was not associated with levels of TMAO and intestinal fatty acid binding protein.[38] The lack of correlation between citrulline and TMAO levels may be explained by a yet unknown mechanism of TMAO uptake through the intestinal wall, independent of the number of enterocytes. However, this would be surprising because enterocyte mass reduction should result in the disruption of organic cation transporters mediating TMAO uptake.[39,40]

Limitations

The major limitation of our study is that only a single measurement of citrulline was performed due to the retrospective study design and available biologic material. Optimally, the assessment of several different markers indicating intestinal barrier function performed at different timepoints depending on the duration of short-term and long-term follow-up needs to be conducted. A histopathological examination of an intestinal mucosa biopsy or the functional assessment of gastrointestinal absorption might also provide additional valuable data.

The strength of our study was its large sample size, the use of multiple clinical parameters, and the long-term follow-up. Additionally, the samples were obtained in a daily clinical practice setting and the citrulline levels were assessed using validated and reproducible methods.

Conclusions

Despite the limitations inherent to its retrospective design, our study provides novel insights into intestinal barrier dysfunction and fills the existing gap in the literature.

Decreased citrulline levels do not predict long-term mortality of hospitalized patients with CVD. Moreover, they are not associated with serum TMAO levels in these patients. We believe that these findings will provide a basis for future research into the links between intestinal permeability and the prognosis of CVD patients. The use of citrulline levels in conjunction with morphological and functional assessments of intestinal biopsies in CVD patients would allow for the assessment of potential new correlations. Perhaps serial measurements of citrulline concentrations during hospitalization and shorter follow-up will allow to capture the dynamics of the processes affecting the intestinal barrier and prognosis of patients with CVD.

ORCID iDs

Radosław Andrzej Konieczny https://orcid.org/0000-0002-0949-4364
Ewa Żurawska-Płaksej https://orcid.org/0000-0001-8566-3943
Konrad Kaaz https://orcid.org/0000-0001-5227-0027
Hanna Czapor-Irzabek https://orcid.org/0000-0001-8689-8314
Andrzej Mysiak https://orcid.org/0000-0002-4728-2565
Wiktor Kuliczkowski https://orcid.org/0000-0001-6284-0820

References

1. Lewis CV, Taylor WR. Intestinal barrier dysfunction as a therapeutic target for cardiovascular disease. *Am J Physiol Heart Circ Physiol*. 2020; 319(6):H1227–H1233. doi:10.1152/ajpheart.00612.2020
2. Sandek A, Bauditz J, Swidsinski A, et al. Altered intestinal function in patients with chronic heart failure. *J Am Coll Cardiol*. 2007;50(16): 1561–1569. doi:10.1016/j.jacc.2007.07.016
3. Sandek A, Bjarnason I, Volk HD, et al. Studies on bacterial endotoxin and intestinal absorption function in patients with chronic heart failure. *Int J Cardiol*. 2012;157(1):80–85. doi:10.1016/j.ijcard.2010.12.016
4. Sandek A, Swidsinski A, Schroedl W, et al. Intestinal blood flow in patients with chronic heart failure: A link with bacterial growth, gastrointestinal symptoms, and cachexia. *J Am Coll Cardiol*. 2014; 64(11):1092–1102. doi:10.1016/j.jacc.2014.06.1179
5. Li C, Gao M, Zhang W, et al. Zonulin regulates intestinal permeability and facilitates enteric bacteria permeation in coronary artery disease. *Sci Rep*. 2016;6:29142. doi:10.1038/srep29142
6. Sanchez-Alcoholado L, Castellano-Castillo D, Jordán-Martínez L, et al. Role of gut microbiota on cardio-metabolic parameters and immunity in coronary artery disease patients with and without type-2 diabetes mellitus. *Front Microbiol*. 2017;8:1936. doi:10.3389/fmicb.2017.01936
7. Carrera-Bastos P, Picazo Ó, Fontes-Villalba M, et al. Serum zonulin and endotoxin levels in exceptional longevity versus precocious myocardial infarction. *Aging Dis*. 2018;9(2):317–321. doi:10.14336/AD.2017.0630
8. Zhou X, Li J, Guo J, et al. Gut-dependent microbial translocation induces inflammation and cardiovascular events after ST-elevation myocardial infarction. *Microbiome*. 2018;6(1):66. doi:10.1186/s40168-018-0441-4
9. Kim S, Goel R, Kumar A, et al. Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clin Sci (Lond)*. 2018;132(6):701–718. doi:10.1042/CS20180087
10. Santisteban MM, Qi Y, Zubcevic J, et al. Hypertension-linked pathophysiological alterations in the gut. *Circ Res*. 2017;120(2):312–323. doi:10.1161/CIRCRESAHA.116.309006
11. Galipeau HJ, Verdu EF. The complex task of measuring intestinal permeability in basic and clinical science. *Neurogastroenterol Motil*. 2016;28(7):957–965. doi:10.1111/nmo.12871
12. Massier L, Chakaroun R, Kovacs P, Heiker JT. Blurring the picture in leaky gut research: How shortcomings of zonulin as a biomarker mislead the field of intestinal permeability. *Gut*. 2021;70(9):1801–1802. doi:10.1136/gutjnl-2020-323026
13. Scalise AA, Kakogiannos N, Zanardi F, Iannelli F, Giannotta M. The blood–brain and gut–vascular barriers: From the perspective of claudins. *Tissue Barriers*. 2021;9(3):1926190. doi:10.1080/21688370.2021.1926190
14. Kastl SP, Krychtiuk KA, Lenz M, et al. Intestinal fatty acid binding protein is associated with mortality in patients with acute heart failure or cardiogenic shock. *Shock*. 2019;51(4):410–415. doi:10.1097/SHK.0000000000001195
15. Hempstock W, Ishizuka N, Hayashi H. Functional assessment of intestinal tight junction barrier and ion permeability in native tissue by using chamber technique. *J Vis Exp*. 2021;2021:71. doi:10.3791/62468
16. Fragkos KC, Forbes A. Citrulline as a marker of intestinal function and absorption in clinical settings: A systematic review and meta-analysis. *United European Gastroenterol J*. 2018;6(2):181–191. doi:10.1177/2050640617737632
17. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. *Clin Nutr*. 2008;27(3):328–339. doi:10.1016/j.clnu.2008.02.005
18. Vancamelbeke M, Vermeire S. The intestinal barrier: A fundamental role in health and disease. *Expert Rev Gastroenterol Hepatol*. 2017; 11(9):821–834. doi:10.1080/17474124.2017.1343143
19. Kulu R, Akyildiz H, Akcan A, Oztürk A, Sozuer E. Plasma citrulline measurement in the diagnosis of acute mesenteric ischaemia. *ANZ J Surg*. 2017;87(9):E57–E60. doi:10.1111/ans.13524
20. Schellekens DH, Hundscheid IH, Leenarts CA, et al. Human small intestine is capable of restoring barrier function after short ischemic periods. *World J Gastroenterol*. 2017;23(48):8452–8464. doi:10.3748/wjg.v23.i48.8452
21. Piton G, Capellier G. Biomarkers of gut barrier failure in the ICU. *Curr Opin Crit Care*. 2016;22(2):152–160. doi:10.1097/MCC.0000000000000283
22. Fagoni N, Piva S, Marino R, et al. The IN-PANCIA study: Clinical evaluation of gastrointestinal dysfunction and failure, multiple organ failure, and levels of citrulline in critically ill patients. *J Intensive Care Med*. 2020;35(3):279–283. doi:10.1177/0885066617742594
23. Blaser A, Padar M, Tang J, Dutton J, Forbes A. Citrulline and intestinal fatty acid-binding protein as biomarkers for gastrointestinal dysfunction in the critically ill. *Anaesthesiol Intensive Ther*. 2019;51(3):230–239. doi:10.5114/ait.2019.86049
24. Piton G, Manzon C, Monnet E, et al. Plasma citrulline kinetics and prognostic value in critically ill patients. *Intensive Care Med*. 2010; 36(4):702–706. doi:10.1007/s00134-010-1751-6
25. Smeets ETHC, Mensink RP, Joris PJ. Effects of L-citrulline supplementation and watermelon consumption on longer-term and postprandial vascular function and cardiometabolic risk markers: A meta-analysis of randomized controlled trials in adults. *Br J Nutr*. 2021;2021:1–34. doi:10.1017/S0007114521004803
26. Flores-Ramírez AG, Tovar-Villegas VI, Maharaj A, Garay-Sevilla ME, Figueroa A. Effects of L-citrulline supplementation and aerobic training on vascular function in individuals with obesity across the lifespan. *Nutrients*. 2021;13(9):2991. doi:10.3390/nu13092991
27. Stremmel W, Schmidt KV, Schuhmann V, et al. Blood trimethylamine-N-oxide originates from microbiota mediated breakdown of phosphatidylcholine and absorption from small intestine. *PLoS One*. 2017; 12(1):e0170742. doi:10.1371/journal.pone.0170742
28. Farhangi MA. Gut microbiota-dependent trimethylamine N-oxide and all-cause mortality: Findings from an updated systematic review and meta-analysis. *Nutrition*. 2020;78:110856. doi:10.1016/j.nut.2020.110856
29. Ufnal M, Pham K. The gut–blood barrier permeability: A new marker in cardiovascular and metabolic diseases? *Med Hypotheses*. 2017; 98:35–37. doi:10.1016/j.mehy.2016.11.012
30. United States Department of Health and Human Services – Food and Drug Administration (FDA) and Center for Drug Evaluation and Research (CDER). *Bioanalytical Method Validation Guidance for Industry Biopharmaceutics. Bioanalytical Method*. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf.
31. Bischoff SC, Barbara G, Buurman W, et al. Intestinal permeability: A new target for disease prevention and therapy. *BMC Gastroenterol*. 2014;14:189. doi:10.1186/s12876-014-0189-7
32. Maric S, Restin T, Muff JL, et al. Citrulline, biomarker of enterocyte functional mass and dietary supplement: Metabolism, transport, and current evidence for clinical use. *Nutrients*. 2021;13(8):2794. doi:10.3390/nu13082794

33. Piton G, Capellier G. Plasma citrulline in the critically ill: Intriguing biomarker, cautious interpretation. *Crit Care*. 2015;19:204. doi:10.1186/s13054-015-0881-1
34. Reintam Blaser A, Padar M, Mändul M, et al. Development of the Gastrointestinal Dysfunction Score (GIDS) for critically ill patients: A prospective multicenter observational study (iSOFA study). *Clin Nutr*. 2021;40(8):4932–4940. doi:10.1016/j.clnu.2021.07.015
35. Padar M, Starkopf J, Starkopf L, et al. Enteral nutrition and dynamics of citrulline and intestinal fatty acid-binding protein in adult ICU patients. *Clin Nutr ESPEN*. 2021;45:322–332. doi:10.1016/j.clnesp.2021.07.026
36. Carnevale R, Sciarretta S, Valenti V, et al. Low-grade endotoxaemia enhances artery thrombus growth via Toll-like receptor 4: Implication for myocardial infarction. *Eur Heart J*. 2020;41(33):3156–3165. doi:10.1093/eurheartj/ehz893
37. Park CJ, Shaughnessy MP, Armenia SJ, Cowles RA. Serum citrulline levels exhibit circadian variation and fluctuations in relation to food intake in mice. *Gastroenterology Res*. 2019;12(2):88–92. doi:10.14740/gr1146
38. Kitai T, Nemet I, Engelman T, et al. Intestinal barrier dysfunction is associated with elevated right atrial pressure in patients with advanced decompensated heart failure. *Am Heart J*. 2022;245:78–80. doi:10.1016/j.ahj.2021.11.014
39. Samodelov SL, Kullak-Ublick GA, Gai Z, Visentin M. Organic cation transporters in human physiology, pharmacology, and toxicology. *Int J Mol Sci*. 2020;21(21):E7890. doi:10.3390/ijms21217890
40. Teft WA, Morse BL, Leake BF, et al. Identification and characterization of trimethylamine-N-oxide uptake and efflux transporters. *Mol Pharm*. 2017;14(1):310–318. doi:10.1021/acs.molpharmaceut.6b00937

8. Oświadczenia współautorów publikacji na podstawie których oparta jest rozprawa doktorska

Wrocław, 02.11.2022

Lek. Radosław Konieczny
Klinika Gastroenterologii i Hepatologii
Uniwersytecki Szpital Kliniczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R. A., Kuliczkowski, W. (2022). Trimethylamine N-oxide in cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(8), 913–925.

mój udział polegał na zaplanowaniu i projekcie publikacji, przeglądzie literatury, zebraniu, analizie i interpretacji danych, stworzeniu manuskryptu publikacji.

Podpis

Wrocław, 02.11.2022

Dr hab. n. med. Wiktor Kuliczkowski
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R. A., Kuliczkowski, W. (2022). Trimethylamine N-oxide in cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(8), 913–925.

mój udział polegał na analizie i interpretacji danych, weryfikacji pracy, ostatecznej akceptacji manuskryptu.

Podpis

Wrocław, 02.11.2022

Lek. Radosław Konieczny
Klinika Gastroenterologii i Hepatologii
Uniwersytecki Szpital Kliniczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na zaplanowaniu i projekcie badania, zebraniu danych klinicznych z hospitalizacji badanej populacji, stworzeniu bazy danych, analizie i interpretacji uzyskanych danych laboratoryjnych, interpretacji analiz statystycznych, zebraniu wyników, przeprowadzeniu analizy wyników dotychczasowych badań w ramach dyskusji, zredagowaniu publikacji.

Podpis

Wrocław, 02.11.2022

Dr Ewa Żurawska- Płaksej
Katedra i Zakład Toksykologii
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na przygotowaniu metod laboratoryjnych oznaczenia stężenia TMAO w próbkach surowicy, wykonaniu analiz laboratoryjnych, zebraniu uzyskanych danych laboratoryjnych, redagowaniu publikacji w części odpowiadającej opisowi metodyki laboratoryjnej.

Podpis

Wrocław, 02.11.2022

Dr n. med. Konrad Kaaz
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na zbieraniu danych klinicznych pacjentów podczas hospitalizacji.

Podpis

Wrocław, 02/11/2022

Dr Hanna Czapor-Irzabek
Pracownia Analizy Elementarnej i Badań Strukturalnych
Wydział Farmaceutyczny; Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na przygotowaniu metod laboratoryjnych oznaczenia stężenia TMAO w próbkach surowicy, wykonywaniu oznaczeń stężenia TMAO w surowicy oraz zebraniu uzyskanych danych laboratoryjnych.

Hanna Czapor-Irzabek
Podpis

Wrocław, 02.11.2022 r.

Mgr Wojciech Bombała
Centrum Analiz Statystycznych
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na wyborze metod statystycznych, interpretacji uzyskanych wyników analiz statystycznych, redagowaniu części pracy dotyczącej metodyki analiz statystycznych.

Podpis
Wojciech Bombała

Wrocław, 02.11.2022

Prof. dr hab. n. med. Andrzej Mysiak
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na zaplanowaniu i przygotowaniu projektu badania, akceptacji ostatecznej wersji manuskryptu.

Podpis

Wrocław, 02.11.2022

Dr hab. n. med. Wiktor Kuliczkowski
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). All-Cause Mortality and Trimethylamine N-Oxide Levels in Patients with Cardiovascular Disease. *Cardiology*, *147*(4), 443–452.

mój udział polegał na zaplanowaniu i projektowaniu badania, analizie i interpretacji danych oraz redagowaniu publikacji.

Podpis

Wrocław, 02.11.2022

Lek. Radosław Konieczny
Klinika Gastroenterologii i Hepatologii
Uniwersytecki Szpital Kliniczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128

mój udział polegał na zaplanowaniu i projekcie badania, zebraniu danych klinicznych z hospitalizacji badanej populacji, stworzeniu bazy danych, analizie i interpretacji uzyskanych danych laboratoryjnych, interpretacji analiz statystycznych, zebraniu wyników, przeprowadzeniu analizy wyników dotychczasowych badań w ramach dyskusji, zredagowaniu publikacji.

Podpis

Wrocław, 02.11.2022

Dr Ewa Żurawska- Płaksej
Katedra i Zakład Toksykologii
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na przygotowaniu metod laboratoryjnych oznaczenia stężenia cytruliny i TMAO w próbkach surowicy, wykonaniu analiz laboratoryjnych, zebraniu uzyskanych danych laboratoryjnych, redagowaniu publikacji w części odpowiadającej opisowi metodyki laboratoryjnej.

Podpis

Wrocław, 07.11.2022

Dr n. med. Konrad Kaaz
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na zbieraniu danych klinicznych pacjentów podczas hospitalizacji.

Podpis

Wrocław, 02/11/2022

Dr Hanna Czapor-Irzabek
Pracownia Analizy Elementarnej i Badań Strukturalnych
Wydział Farmaceutyczny; Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na przygotowaniu metod laboratoryjnych oznaczenia stężenia cytruliny i TMAO w próbkach surowicy, wykonywaniu oznaczeń stężenia cytruliny i TMAO w surowicy oraz zebraniu uzyskanych danych laboratoryjnych.

Hanna Czapor-Irzabek
Podpis

Wrocław, 02.11.2022r.

Mgr Wojciech Bombała
Centrum Analiz Statystycznych
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na wyborze metod statystycznych, interpretacji uzyskanych wyników analiz statystycznych, redagowaniu części pracy dotyczącej metodyki analiz statystycznych.

Podpis
Wojciech Bombała

Wrocław, 02.11.2022

Prof. dr hab. n. med. Andrzej Mysiak
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

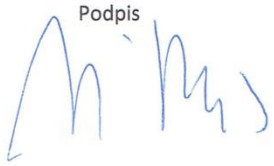
OŚWIADCZENIE

Oświadczam, że w pracy

Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na zaplanowaniu i przygotowaniu projektu badania, akceptacji ostatecznej wersji manuskryptu.

Podpis



Wrocław, 02.11.2022

Dr hab. n. med. Wiktor Kuliczkowski
Instytut Chorób Serca
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy Konieczny, R. A., Żurawska-Płaksej, E., Kaaz, K., Czapor-Irzabek, H., Bombała, W., Mysiak, A., & Kuliczkowski, W. (2022). Citrulline and long-term mortality in patients with cardiovascular disease. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*, *31*(10), 1121–1128.

mój udział polegał na Zaplanowaniu i projektowaniu badania, analizie i interpretacji danych oraz redagowaniu publikacji.

Podpis

Streszczenie

Wstęp N-Tlenek trimetyloaminy (TMAO) powstaje w ludzkim organizmie na drodze enzymatycznego utlenienia metabolitu mikrobioty jelitowej – trimetyloaminy oraz jest wchłaniany bezpośrednio z pokarmu. TMAO może mieć wpływ na przebieg i rokowanie u pacjentów z chorobami układu krążenia. Stopień wchłaniania TMAO i jego prekursora wiąże się z przepuszczalnością bariery jelitowej, która może się zwiększyć w chorobach układu krążenia. Biomarkerem przepuszczalności bariery jelitowej jest cytrulina - aminokwas syntetyzowany przez enterocyty.

Cel pracy: Ocena przydatności klinicznej stężenia TMAO jako niezależnego czynnika rokowniczego u polskich pacjentów obciążonych chorobami układu krążenia w obserwacji długoterminowej, ocena przydatności klinicznej stężenia cytruliny jako niezależnego czynnika rokowniczego u tych pacjentów oraz ocena wzajemnego powiązania stężenia TMAO i uszkodzenia bariery jelitowej ocenionego stężeniem cytruliny.

Materiał i metody

W pierwszej pracy wchodzącej w skład rozprawy dokonano przeglądu literatury dotyczącej metabolizmu TMAO oraz wyników dotychczasowych badań nad wpływu tej cząsteczki na przebieg i rokowanie u pacjentów z chorobami układu krążenia.

Materiał kliniczny: Retrospektywnie oceniono stężenie TMAO oraz cytruliny w surowicy 1036 kolejnych pacjentów (mediana wieku 62, mężczyźni 61%) hospitalizowanych w Oddziale Kardiologicznym Uniwersyteckiego Szpitala Klinicznego we Wrocławiu w latach 2013-2015. Oceniono wpływ stężenia TMAO i cytruliny na śmiertelność długoterminową (obserwacja 5-letnia) oraz zbadano korelacje stężenia TMAO oraz cytruliny z parametrami antropometrycznymi i biochemicznymi. Analizę wykonano dla całej badanej populacji oraz dla podgrup pacjentów z ostrym zespołem wieńcowym (ACS) n=177, stabilnym zespołem wieńcowym (SCS) n=441, przewlekłą niewydolnością serca (HF) n=292 oraz migotaniem przedsionków (AF) n=277. Stężenie cytruliny skorelowano ze stężeniem TMAO w surowicy

Wyniki: Na podstawie przeglądu literatury wykazano, że TMAO jest użytecznym markerem ryzyka sercowo naczyniowego.

W obserwacji 5-letniej stwierdzono 171 zgonów. W analizie jednoczynnikowej wyższe stężenie TMAO w całej grupie badanej było istotnym predykatorem śmiertelności 5-letniej (HR 1,01 przy 95% CI 1,006-1,018 i p<0,0001) jednak zależność ta traciła istotność statystyczną w analizie wieloczynnikowej. Dodatkowo stwierdzając istotny wzrost ryzyka zgonu u pacjentów w górnym kwartylu stężeń TMAO. Analiza ROC pozwoliła wyznaczyć punkt odcięcia stężenia TMAO wynoszący 5,94 μM powyżej którego z 48% czułością i 80% specyficznością ryzyko zgonu było istotnie wyższe w porównaniu z pozostałą grupą. W grupie pacjentów z przewlekłym zespołem wieńcowym, przewlekłą niewydolnością serca i migotaniem przedsionków wykazano istotne różnice stężenia TMAO pomiędzy pacjentami zmarłymi, a przeżywającymi obserwację 5-letnią.

Nie stwierdzono istotnej różnicy stężenia cytruliny pomiędzy grupą pacjentów zmarłych oraz przeżywających 5-letni okres obserwacji. Nie wykazano także statystycznie istotnego wpływu stężenia cytruliny na śmiertelność pacjentów w obserwacji 5-letniej HR 0.99 (95% CI 0.97-1.00 p=0,49). Jedynie w podgrupie pacjentów z ostrym zespołem wieńcowym obserwowano istotnie niższe stężenie cytruliny w grupie pacjentów, którzy zmarli w trakcie obserwacji 5-letniej w porównaniu z pacjentami, którzy przeżyli: 20,9 μM (15,2-25,8) vs 24,0 μM (18,5-30,1) p=0,025. Nie wykazano istotnych statystycznie korelacji stężenia cytruliny z danymi biochemicznymi, antropometrycznymi ani wielochorobowością w badanej grupie. Nie wykazano istotnej korelacji pomiędzy stężeniami TMAO i cytruliny (korelacja tau Kendala = 0,027).

Wnioski:

1.W analizie jednoczynnikowej stężenie TMAO jest czynnikiem rokowniczym zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia. W analizie wieloczynnikowej zależność ta traci istotność statystyczną.

2.Uszkodzenie bariery jelitowej, mierzone stężeniem cytruliny w surowicy nie jest czynnikiem ryzyka zgonu w obserwacji 5-letniej pacjentów obciążonych chorobami układu krążenia.

3.Nie występuje korelacja pomiędzy stężeniem TMAO oraz cytruliny w surowicy.

Streszczenie w języku angielskim

Introduction Trimethylamine N-oxide (TMAO) is formed in the human body by enzymatic oxidation of the gut microbiota metabolite- trimethylamine and is absorbed directly from food. TMAO may affect the clinical course and prognosis of patients with cardiovascular disease. The degree of absorption of TMAO and its precursor is related to intestinal barrier permeability, which may be increased in cardiovascular disease. A biomarker of intestinal barrier permeability is citrulline, an amino acid synthesised by enterocytes.

Objectives: To assess the clinical usefulness of TMAO levels as an independent prognostic factor in Polish patients with cardiovascular disease in long-term follow-up. Moreover, to evaluate the clinical usefulness of citrulline levels as an independent prognostic factor in these patients, and to assess the interrelationship between TMAO levels and intestinal barrier damage assessed by citrulline levels.

Material and methods

The first paper included the literature review on TMAO metabolism and the results of previous studies on the influence of this molecule on the course and prognosis of patients with cardiovascular diseases.

Clinical material: TMAO and citrulline concentrations were retrospectively assessed in serum of 1036 consecutive patients (median age 62, men 61%), hospitalised in the Cardiology Department of Jan Mikulicz-Radecki University Teaching Hospital**,** Wroclaw, between 2013 and 2015. The effect of TMAO and citrulline concentrations on long-term mortality (5-year follow-up) was assessed. Correlations of TMAO and citrulline concentrations with anthropometric and biochemical parameters were investigated. The analysis was performed for the whole study population and for subgroups of patients with acute coronary syndrome (ACS) n=177, stable coronary syndrome (SCS) n=441, chronic heart failure (HF) n=292 and atrial fibrillation (AF) n=277. Citrulline concentration was correlated with serum TMAO concentration.

Results:

Based on the literature review, TMAO was shown to be a useful marker of cardiovascular risk.

There were 171 deaths at 5-year follow-up. In univariate analysis, higher TMAO levels in the entire study group were a significant predictor of 5-year mortality (HR 1.01 with 95% CI 1.006-1.018 and $p<0.0001$); however, this relationship lost statistical significance in multivariate analysis. In addition, we found a significant increase in the risk of death in patients in the upper quartile of TMAO concentrations. ROC analysis determined a TMAO concentration cut-off point of 5.94 μM with 48% sensitivity and 80% specificity, above which, the risk of death was significantly higher compared with the rest of the group. In the group of patients with chronic coronary syndrome, chronic heart failure and atrial fibrillation, significant differences in TMAO concentration were found between patients who died and 5-year survivors.

No significant difference in citrulline concentration was found between the group of patients who died and those surviving a 5-year follow-up. There was also no statistically significant effect of citrulline concentration on patient mortality at 5-year follow-up HR 0.99 (95% CI 0.97-1.00 $p=0.49$). Only in the subgroup of patients with acute coronary syndrome were significantly lower citrulline concentrations observed in patients who died during the 5-year follow-up compared to patients who survived: 20.9 μM (15.2-25.8) vs 24.0 μM (18.5-30.1) $p=0.025$. There was no statistically significant correlation of citrulline concentration with biochemical, anthropometric or multimorbidity data in the study group. There was no significant correlation between TMAO and citrulline concentrations (Kendal's tau correlation = 0.027).

Conclusions:

1.In univariate analysis, TMAO concentration is a prognostic factor for death in the 5-year follow-up of patients with cardiovascular disease. In multivariate analysis, this relationship loses statistical significance.

2.Intestinal barrier damage, as measured by serum citrulline concentration, is not a risk factor for death in the 5-year follow-up of patients with cardiovascular disease.

3.There is no correlation between TMAO and serum citrulline concentrations.

Piśmiennictwo

[1]Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organization and United Nations. *Int J Cardiol*. 2013;168(2):934–945. doi:10.1016/j.ijcard.2012.10.046

[2] Masenga, S.K., Hamooya, B., Hangoma, J. *et al.* Recent advances in modulation of cardiovascular diseases by the gut microbiota. *J Hum Hypertens* (2022). https://doi.org/10.1038/s41371-022-00698-6

[3] J. Chhibber-Goel, A. Gaur, V. Singhal, N. Parakh, B. Bhargava, and A. Sharma, "The complex metabolism of trimethylamine in humans: endogenous and exogenous sources," Expert Rev. Mol. Med., Apr. 2016, doi: 10.1017/erm.2016.6.

[4] Z. Wang et al., "Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease," Nature, vol. 472, no. 7341, pp. 57–65, Apr. 2011, doi: 10.1038/nature09922.

[5] V. E. Brunt et al., "Trimethylamine-N-Oxide Promotes Age-Related Vascular

Oxidative Stress and Endothelial Dysfunction in Mice and Healthy Humans," Hypertension, vol. 76, no. 1, pp. 101–112, Jul. 2020, doi: 10.1161/HYPERTENSIONAHA.120.14759.

[6] W. Zhu et al., "Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk," Cell, vol. 165, no. 1, pp. 111–124, Mar. 2016, doi: 10.1016/j.cell.2016.02.011.

[7] Z. Li et al., "Gut microbe-derived metabolite trimethylamine N-oxide induces cardiac hypertrophy and fibrosis," Lab. Invest., vol. 99, no. 3, pp. 346–357, Mar. 2019, doi: 10.1038/S41374-018-0091-Y.

[8] Y. Lee et al., "Longitudinal Plasma Measures of Trimethylamine N-Oxide and Risk of Atherosclerotic Cardiovascular Disease Events in Community-Based Older Adults," J. Am. Heart Assoc., vol. 10, no. 17, 2021, doi: 10.1161/jaha.120.020646.

[9] M. Trøseid et al., "Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure," J. Intern. Med., vol. 277, no. 6, pp. 717–726, 2015, doi: 10.1111/joim.12328.

[10] D. Gong, L. Zhang, Y. Zhang, F. Wang, Z. Zhao, and X. Zhou, "Gut Microbial Metabolite Trimethylamine N-Oxide Is Related to Thrombus Formation in Atrial Fibrillation Patients," Am. J. Med. Sci., vol. 358, no. 6, pp. 422–428, Dec. 2019, doi:10.1016/j.amjms.2019.09.002.

[11] M. Ufnal and K. Pham, "The gut-blood barrier permeability – A new marker in cardiovascular and metabolic diseases?," *Med. Hypotheses*, vol. 98, pp. 35–37, Jan. 2017, doi: 10.1016/j.mehy.2016.11.012.

[12] W. Stremmel *et al.*, "Blood trimethylamine-n-oxide originates from microbiota mediated breakdown of phosphatidylcholine and absorption from small intestine," *PLoS One*, vol. 12, no. 1, Jan. 2017, doi: 10.1371/journal.pone.0170742.

[13] K. Jaworska *et al.*, "Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite," *PLoS One*, vol. 12, no. 12, Dec. 2017, doi: 10.1371/journal.pone.0189310.

[14] K. Jaworska, K. Bielinska, M. Gawrys-Kopczynska, and M. Ufnal, “TMA (trimethylamine), but not its oxide TMAO (trimethylamine-oxide), exerts haemodynamic effects: implications for interpretation of cardiovascular actions of gut microbiome,” *Cardiovasc. Res.*, vol. 115, no. 14, pp. 1948–1949, Dec. 2019, doi: 10.1093/CVR/CVZ231.

[15] K. Jaworska *et al.*, “Trimethylamine But Not Trimethylamine Oxide Increases With Age in Rat Plasma and Affects Smooth Muscle Cells Viability,” *Journals Gerontol. Ser. A*, vol. 75, no. 7, pp. 1276–1283, Jun. 2020, doi: 10.1093/GERONA/GLZ181.

[16] T. Kitai *et al.*, “Intestinal barrier dysfunction is associated with elevated right atrial pressure in patients with advanced decompensated heart failure,” *Am. Heart J.*, vol. 245, pp. 78–80, Mar. 2022, doi: 10.1016/J.AHJ.2021.11.014.

[17] C. V. Lewis and W. Robert Taylor, “Intestinal barrier dysfunction as a therapeutic target for cardiovascular disease,” *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 319, no. 6, pp. H1227–H1233, Nov. 2020, doi: 10.1152/AJPHEART.00612.2020/ASSET/IMAGES/LARGE/AJ-AHRT200027F001.JPEG.

[18] A. Sandek *et al.*, “Altered Intestinal Function in Patients With Chronic Heart Failure,” *J. Am. Coll. Cardiol.*, vol. 50, no. 16, pp. 1561–1569, Oct. 2007, doi: 10.1016/j.jacc.2007.07.016.

[19] A. Sandek *et al.*, “Studies on bacterial endotoxin and intestinal absorption function in patients with chronic heart failure,” *Int. J. Cardiol.*, vol. 157, no. 1, pp. 80–85, May 2012, doi: 10.1016/J.IJCARD.2010.12.016.

[20] C. Li *et al.*, “Zonulin Regulates Intestinal Permeability and Facilitates Enteric Bacteria Permeation in Coronary Artery Disease,” *Sci. Reports 2016 61*, vol. 6, no. 1, pp. 1–10, Jun. 2016, doi: 10.1038/srep29142.

[21] P. Carrera-Bastos *et al.*, “Serum Zonulin and Endotoxin Levels in Exceptional Longevity versus Precocious Myocardial Infarction,” *Aging Dis.*, vol. 9, no. 2, pp. 317–321, Apr. 2018, doi: 10.14336/AD.2017.0630.

[22] X. Zhou *et al.*, “Gut-dependent microbial translocation induces inflammation and cardiovascular events after ST-elevation myocardial infarction,” *Microbiome*, vol. 6, no. 1, p. 66, Apr. 2018, doi: 10.1186/S40168-018-0441-4.

[23] S. Kim *et al.*, “Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure,” *Clin. Sci.*, vol. 132, no. 6, pp. 701–718, Mar. 2018, doi: 10.1042/CS20180087.

[24] M. M. Santisteban *et al.*, “Hypertension-Linked Pathophysiological Alterations in the Gut,” *Circ. Res.*, vol. 120, no. 2, pp. 312–323, Jan. 2017, doi: 10.1161/CIRCRESAHA.116.309006.

[25] P. Crenn, B. Messing, and L. Cynober, “Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction,” *Clin. Nutr.*, vol. 27, no. 3, pp. 328–339, 2008, doi: 10.1016/j.clnu.2008.02.005.

[26] H. J. Galipeau and E. F. Verdu, “The complex task of measuring intestinal permeability in basic and clinical science,” *Neurogastroenterology and Motility*, vol. 28, no. 7. Blackwell Publishing Ltd, pp. 957–965, 01-Jul-2016, doi: 10.1111/nmo.12871.

[27] K. C. Fragkos and A. Forbes, “Citrulline as a marker of intestinal function and absorption in clinical settings: A systematic review and meta-analysis,” *United Eur. Gastroenterol. J.*, vol. 6, no. 2, pp. 181–191, 2018, doi: 10.1177/2050640617737632.

[28] R. Kulu, H. Akyildiz, A. Akcan, A. Oztürk, and E. Sozuer, “Plasma citrulline measurement in the diagnosis of acute mesenteric ischaemia,” *ANZ J. Surg.*, vol. 87, no. 9, pp. E57–E60, Sep. 2017, doi: 10.1111/ans.13524.

[29] D. H. S. M. Schellekens *et al.*, “Human small intestine is capable of restoring barrier function after short ischemic periods,” *World J. Gastroenterol.*, 2017, doi: 10.3748/wjg.v23.i48.8452.

[30] A. R. Blaser, M. Padar, J. Tang, J. Dutton, and A. Forbes, “Citrulline and intestinal fatty acid-binding protein as biomarkers for gastrointestinal dysfunction in the critically ill,” *Anaesthesiology Intensive Therapy*, vol. 51, no. 3. Termedia Publishing House Ltd., pp. 230–239, 2019, doi: 10.5114/ait.2019.86049

[31] N. Fagoni *et al.*, “The IN-PANCIA Study: Clinical Evaluation of Gastrointestinal Dysfunction and Failure, Multiple Organ Failure, and Levels of Citrulline in Critically Ill Patients.,” *J. Intensive Care Med.*, vol. 35, no. 3, pp. 279–283, Mar. 2020, doi: 10.1177/0885066617742594.

[32] .G. Piton and G. Capellier, “Biomarkers of gut barrier failure in the ICU,” *Curr. Opin. Crit. Care*, vol. 22, no. 2, pp. 152–160, Apr. 2016, doi: 10.1097/MCC.0000000000000283.

[33] G. Piton *et al.*, “Plasma citrulline kinetics and prognostic value in critically ill patients,” *Intensive Care Med.*, vol. 36, no. 4, pp. 702–706, Apr. 2010, doi: 10.1007/s00134-010-1751-6.

Wykaz tabel, wykresów i rycin

Tabele

Wykresy

Ryciny