**AGRICULTURAL UNIVERSITY OF ATHENS**
**SCHOOL OF FOOD AND NUTRITIONAL SCIENCES**
**DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION**
**LABORATORY OF DIETETICS AND QUALITY OF LIFE**

**PhD Dissertation**

Risk assessment of dietary nitrite and nitrate intakes in a representative sample of the Greek population and associations with health markers

**Sotiria N. Kotopoulou**

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**ATHENS 2023**

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Αξιολόγηση κινδύνου της διατροφικής πρόσληψης νιτρωδών και
νιτρικών αλάτων σε αντιπροσωπευτικό δείγμα ελληνικού πληθυσμού και
συσχετίσεις με παράγοντες υγείας

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**Risk assessment of dietary nitrite and nitrate intakes in a representative sample of the Greek population and associations with health markers**

*Laboratory of Dietetics and Quality of Life*
*Department of Food Science and Human Nutrition*

# Abstract

**Background:** Nitric compounds are highly controversial components in human nutrition and have been disputed for years as to their effects on CVD and cancer, the two main causes of mortality worldwide. Specific Acceptable Daily Intake (ADI) levels have been formulated based on current evidence; however, the evolving nutrition transition has increased processed meat consumption and thus nitric compound intake, as they are the main additives used in the meat industry, raising the risk of exceeding the ADIs set. Numerous studies have been conducted to estimate dietary nitrite and nitrate intakes and assess the relevant risk for consumers in other countries, but to our knowledge, to date, no exposure or risk assessment has been conducted in Greece. Furthermore, although many studies have explored the association of dietary nitrite and nitrate with various types of cancer as well as the association of processed meat with CVD, the relationship of processed meat constituents with significant risk factors for CVD, such as blood pressure (BP), remains unclear.

**Objectives:** The primary objective of this thesis was to investigate health risks and benefits linked with dietary nitrite and nitrate in order to address the data underlying the existing conundrum of being considered both healthy and unhealthy. The second objective was to estimate exposure to dietary nitrite and nitrate when used as additives, as well as to identify major food contributors, evaluate the relevant potential risk in the Greek population, and determine the population groups that may be at higher risk. The third objective was to examine the relationship between dietary nitrite and nitrate intake from processed meat and BP while controlling for major confounding factors that may play a role in CVD etiology. Ultimately, this thesis was set out to address the public and authority concerns based on estimated associations between dietary exposures and human health, to help raise public awareness of the potential adverse health effects and support competent authorities in formulating public health recommendations, if and where necessary.

**Methods:** A comprehensive review was conducted to provide an in-depth look at the current literature on the relationship between dietary nitrite and nitrate intake with health risks and benefits, with a focus on plant versus animal sources and drinking water. Based on the outcomes of our literature review, it was decided to proceed with the evaluation of the dietary exposure to nitrite and nitrate from processed meat products, as well as to investigate their relation to Blood Pressure (BP). To do so, individual consumption data from 2152 participant meat consumers (46.7% males) in the Hellenic National Nutrition and Health Survey (HNNHS) were linked with current Maximum Permitted Levels (MPLs) of nitric compounds content. Processed meat intakes were determined by combining data from 24h recalls and frequency of consumption reported in Food Propensity Questionnaires (FPQs) and the results were compared to ADI of nitrite (0.07 mg/kg bw/day) to assess the risk. Finally, data from 1774 adult processed meat consumers (≥18 years, 55.1% females) were used to evaluate the association of dietary nitrite and nitrate intake from processed meat, assessed as nitrite equivalent, with BP. To avoid selection and reverse causality bias,

associations with measured diastolic BP (DBP) and systolic BP (SBP) were considered instead of self-reported data of hypertension presence. Participants were divided by tertile of dietary nitrite intake and by level of dietary guideline adherence for sodium (<1500; 1500-2300; ≥2300 mg/day). Multiple regression models were used to examine associations with SBP and DBP, including an interaction term of nitrite with dietary sodium intake, for potential synergy.

**Results:** The results of the narrative review on dietary nitrite and nitrate and human health by intake source, highlighted the beneficial effects of nitrate and nitrite consumption from plant origin on CVD and, to date, no positive correlation has been reported with cancer. On the contrary, high intake of these compounds from processed animal-based foods is related to an increased risk of gastro-intestinal cancer, however no studies conducted to assess the association of nitrite and nitrate from processed meat with CVD were found. Nitrate in drinking water also raises some concern. Median exposure to dietary nitrite and nitrate (assessed as equivalent nitrite) ingested from processed meat products was estimated to be within safe levels for all population groups. However, 6.6% (N=143) of the consumers exceeded the Acceptable Daily Intake (ADI) of nitrite (0.07 mg/kg bw/day), of which 20.3% were children aged 0–9years (N=29) (15.3% of all children participants in the study, N=190). In total, pork meat was the major contributor (41.5%), followed by turkey meat (32.7%) and sausages (23.8%), although contribution variations were found among age groups. Finally, DBP increased by 3.05 mmHg (95% CI:0, 6.06), per tertile increase in nitrite intake and 4.41 mmHg (95% CI: 0.17, 8.66) per level increase in sodium intake, when the interaction effect between nitrite and total sodium intakes was accounted for. By considering the significant synergistic effect of the two factors, DBP finally increased by 0.94 mmHg overall and 2.24 mmHg for subjects in the third tertile compared to those in the first. Also, a rise in total sodium intake of approximately 800 mg, above 1500 mg, caused a 2.30 mmHg increase in DBP. No significant correlations were found with SBP.

**Conclusions:** The outcome of the risk assessment of nitrite and nitrate intake from processed meat and meat products if of important public health concern Especially, exposure among children needs to attract special attention and should be accounted for in public health campaigns. Finally, higher nitrite and nitrate intake from processed meats contributed to the increase of DBP, but the interaction effect with total sodium intake levels should have been accounted for to properly interpret the findings. The up-to-date debate on the role of nitrate and nitrite in human nutrition seems to be justified and more research is required to verify safe consumption. Future research is warranted to evaluate possible associations with health effects by using more refined occurrence data, if available. The concerns raised in this thesis are in line with the concerns of the European Commision, which has recently made the decision to modify the current use conditions of nitrite and nitrate as food additives by reducing their Maximum Permitted Limits in Regulation (EC) 2023/2108 of 6 October 2023.

**Scientific area:** Human Nutrition/Public Health Nutrition/Nutritional Epidemiology

**Keywords:** Cardiovascular disease; CVD; cancer; dietary exposure; dietary intake; nitric compounds; food additives; MPLs; risk; processed meat consumption; Greece; dietary nitrite; health benefit; health risk; hypertension; Systolic Blood Pressure; SBP; Diastolic Blood Pressure; DBP.

**Αξιολόγηση κινδύνου της διατροφικής πρόσληψης νιτρωδών και νιτρικών αλάτων σε αντιπροσωπευτικό δείγμα ελληνικού πληθυσμού και συσχετίσεις με παράγοντες υγείας**

*Εργαστήριο Διαιτολογίας και Ποιότητας Ζωής*
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# Περίληψη

**Γενική επισκόπηση:** Τα νιτρώδη και νιτρικά άλατα είναι ιδιαιτέρως αμφιλεγόμενα συστατικά στη διατροφή του ανθρώπου. Η διαχρονική διαμάχη υπέρ και κατά της πρόσληψής τους μέσω της διατροφής οφείλεται στη θετική έως τώρα συσχέτισή τους τους με καρδιαγγειακές παθήσεις και στην αρνητική με καρκίνο, τις δύο κύριες αιτίες θνησιμότητας παγκοσμίως. Επιπλέον, η διαρκώς εξελισσόμενη μεταβολή στις διατροφικές συνήθειες έχει οδηγήσει σε αύξηση της κατανάλωσης επεξεργασμένου κρέατος και συνεπώς σε αυξημένη πρόσληψη και των αλάτων αυτών, καθώς είναι τα κύρια πρόσθετα που χρησιμοποιούνται στη βιομηχανίας κρέατος. Σημαντική έρευνα έχει διεξαχθεί για την εκτίμηση της διατροφικής πρόσληψης νιτρωδών και νιτρικών και την αξιολόγηση του σχετικού κινδύνου για τους καταναλωτές σε άλλες χώρες, αλλά αντίστοιχη μελέτη εκτίμησης έκθεσης ή κινδύνου δεν είχε διεξαχθεί στη χώρα μας. Επιπλέον, αν και πολλές μελέτες έχουν διερευνήσει τη συσχέτιση των νιτρωδών και νιτρικών αλάτων μέσω της διατροφής με διάφορους τύπους καρκίνου, καθώς και τη συσχέτιση του επεξεργασμένου κρέατος με τις καρδιαγγειακές παθήσεις, η σχέση των συστατικών του επεξεργασμένου κρέατος με σημαντικούς παράγοντες κινδύνου για καρδιαγγειακές παθήσεις δεν είχε μελετηθεί επαρκώς και παρέμενε ασαφής.
**Στόχοι:** Ο πρωταρχικός στόχος της παρούσας έρευνας ήταν να διερευνήσει τους κινδύνους και τα οφέλη για την υγεία που συνδέονται με την πρόσληψη νιτρωδών και νιτρικών αλάτων, σε σχέση με την πηγή προέλευσής τους, προκειμένου να δοθεί, ει δυνατόν, τεκμηριωμένη απάντηση στο ερώτημα σχετικά με το αν η κατανάλωσή τους είναι τελικά ωφέλιμη ή επικίνδυνη. Ο δεύτερος στόχος ήταν να εκτιμηθεί η διατροφική έκθεση σε νιτρώδη και νιτρικά άλατα που χρησιμοποιούνται ως πρόσθετα στην παραγωγή επεξεργασμένων προϊόντων κρέατος, καθώς και να προσδιοριστούν οι τύποι των προϊόντων επεξεργασμένου κρέατος που αποτελούν τις κύριες πηγές έκθεσης, να αξιολογηθεί ο σχετικός δυνητικός κίνδυνος για τον ελληνικό πληθυσμό και να προσδιοριστούν οι ομάδες του πληθυσμού που διατρέχουν πιθανά μεγαλύτερο κίνδυνο. Ο τρίτος στόχος ήταν να αξιολογηθεί η σχέση μεταξύ της πρόσληψης νιτρωδών και νιτρικών αλάτων από επεξεργασμένο κρέας και της αρτηριακής πίεσης, λαμβάνοντας υπόψη συγχυτικούς παράγοντες που ενδέχεται να διαδραματίζουν ρόλο στην αιτιολογία της καρδιαγγειακής νόσου. Τελικά, η παρούσα διατριβή, λαμβάνοντας υπόψη τις χρόνιες ανησυχίες του κοινού και των αρχών, είχε ως στόχο να διερευνήσει τις συσχετίσεις μεταξύ της εκτιμώμενης διατροφικής έκθεσης σε νιτρικά και νιτρώδη άλατα και της ανθρώπινης υγείας, προκειμένου να ευαισθητοποιηθεί το κοινό σχετικά με τις πιθανές δυσμενείς επιπτώσεις στην υγεία και να υποστηριχθούν οι αρμόδιες αρχές στη διατύπωση συστάσεων για τη δημόσια υγεία, εάν και όπου είναι απαραίτητο.
**Μέθοδοι:** Έλαβε χώρα ανασκόπηση της τρέχουσας βιβλιογραφίας για να διερευνηθεί η συσχέτιση της πρόσληψης νιτρωδών και νιτρικών αλάτων με την υγεία, ανάλογα με την πηγή προέλευσης, ήτοι από τρόφιμα φυτικής (φυσικά συστατικά) προέλευσης, ζωικής προέλευσης και κυρίως επεξεργασμένο κρέας (πρόσθετα) και από το πόσιμο νερό (επιμολυντές). Με βάση τα αποτελέσματα της βιβλιογραφικής ανασκόπησης, αποφασίστηκε ο προσδιορισμός της διατροφικής έκθεσης σε νιτρώδη και νιτρικά

άλατα από επεξεργασμένα προϊόντα κρέατος και η αξιολόγηση του σχετικού δυνητικού κινδύνου για τον ελληνικό πληθυσμό. Για το σκοπό αυτό, ανακτήθηκαν από την Πανελλαδική Μελέτη Διατροφής και Υγείας (ΠΑΜΕΔΥ) δεδομένα κατανάλωσης από 2152 συμμετέχοντες καταναλωτές επεξεργασμένου κρέατος (46,7% άνδρες), τα οποία και συνδυάστηκαν με τα τρέχοντα ανώτατα επιτρεπτά όρια (MPL) περιεκτικότητας σε νιτρώδη και νιτρικά άλατα. Η πρόσληψη επεξεργασμένου κρέατος προσδιορίστηκε συνδυάζοντας δεδομένα από ανακλήσεις 24 ωρών με τη συχνότητα κατανάλωσης που δηλώθηκε από τους συμμετέχοντες βάσει ερωτηματολογίων συχνότητας κατανάλωσης τροφίμων (Food Propensity Questionnaires, FPQs). Η πρόσληψη προσδιορίστηκε ως ισοδύναμο νιτρωδών αλάτων και το αποτέλεσμα συγκρίθηκε με το αντίστοιχο ADI (0.07 mg/kg bw/day) για την αξιολόγηση του κινδύνου. Στη συνέχεια διερευνήθηκε η συσχέτιση της πρόσληψης νιτρωδών και νιτρικών (ως ισοδύναμο νιτρωδών) με την αρτηριακή πίεση (ΑΠ). Για το σκοπό αυτό, αξιοποιήθηκαν δεδομένα κατανάλωσης από 1774 ενήλικες καταναλωτές επεξεργασμένου κρέατος (≥18 ετών, 55,1% γυναίκες). Για να αποφευχθεί η μεροληψία επιλογής και αντίστροφης αιτιότητας, εξετάστηκαν οι συσχετίσεις με τη μετρούμενη DBP και SBP. Οι συμμετέχοντες ταξινομήθηκαν βάσει του τριτημορίου της πρόσληψης νιτρωδών και ανάλογα με το επίπεδο τήρησης των διατροφικών κατευθυντήριων γραμμών για το νάτριο (<1500-, 1500-2300, ≥2300 mg/ημέρα). Χρησιμοποιήθηκαν μοντέλα πολλαπλής παλινδρόμησης για την εξέταση των συσχετίσεων με τη SBP και τη DBP, συμπεριλαμβανομένου ενός όρου αλληλεπίδρασης των νιτρωδών με τη διατροφική πρόσληψη νατρίου, για την εξέτασης πιθανής συνέργειας.

**Αποτελέσματα:** Η αφηγηματική ανασκόπηση ανέδειξε την ευεργετική επίδραση της κατανάλωσης νιτρωδών και νιτρικών αλάτων φυτικής προέλευσης στην καρδιαγγειακή νόσο και, μέχρι σήμερα, δεν έχει αναφερθεί συσχέτιση με καρκίνο. Αντίθετα, η υψηλή πρόσληψη αυτών των ενώσεων από επεξεργασμένα τρόφιμα ζωικής προέλευσης σχετίζεται με αυξημένο κίνδυνο εμφάνισης καρκίνου του γαστρεντερικού συστήματος, ωστόσο δεν βρέθηκαν μελέτες που να έχουν διεξαχθεί για την αξιολόγηση της συσχέτισης των νιτρωδών και των νιτρικών από επεξεργασμένο κρέας με τη καρδιαγγειακή νόσο. Τα νιτρικά και νιτρώδη άλατα από το πόσιμο νερό προκαλούν επίσης κάποια ανησυχία. Η διάμεση διατροφική έκθεση σε νιτρώδη και νιτρικά άλατα (εκτιμώμενη ως ισοδύναμο νιτρωδών αλάτων) από κατανάλωση επεξεργασμένου κρέατος εκτιμήθηκε ότι είναι εντός ασφαλών επιπέδων για όλες τις ομάδες πληθυσμού. Ωστόσο, το 6.6% (N=143) των καταναλωτών υπερέβη την Αποδεκτή Ημερήσια Πρόσληψη (ADI) νιτρωδών (0.07 mg/kg σωματικού βάρους/ημέρα), εκ των οποίων το 20.3% ήταν παιδιά ηλικίας 0-9 ετών (N=29) (15.3% όλων των παιδιών που συμμετείχαν στη μελέτη, N= 90). Συνολικά, το χοιρινό κρέας ήταν η κύρια πηγή έκθεσης (41.5%), ακολουθούμενο από το κρέας γαλοπούλας (32.7%) και τα λουκάνικα (23.8%), αν και διαπιστώθηκαν διαφορές στη συνεισφορά μεταξύ των ηλικιακών ομάδων. Αναφορικά με την επίδραση των νιτρωδών και νιτρικών αλάτων από επεξεργασμένο κρέας στην αρτηριακή πίεση, προσδιορίστηκε πως η DBP αυξήθηκε κατά 3.05 mmHg (95% CI: 0, 6.06), ανά τριτημόριο αύξησης της πρόσληψης νιτρωδών και 4.41 mmHg (95% CI: 0.17, 8.66) ανά επίπεδο αύξησης της πρόσληψης νατρίου. Λαμβάνοντας υπόψη τη σημαντική συνεργιστική επίδραση των δύο παραγόντων, νιτρωδών αλάτων και νατρίου, η DBP αυξήθηκε τελικά κατά 0.94 mm Hg συνολικά και κατά 2.24 mm Hg για τα άτομα στο τρίτο τριτημόριο σε σύγκριση με εκείνα στο πρώτο. Επίσης, η αύξηση της συνολικής πρόσληψης νατρίου κατά περίπου 800 mg, πάνω από τα 1500 mg, προκάλεσε αύξηση της DBP κατά 2.30 mm Hg. Δεν βρέθηκαν σημαντικές συσχετίσεις με τη SBP.

**Συμπεράσματα:** Τα αποτελέσματα της εκτίμησης του κινδύνου της πρόσληψης νιτρωδών και νιτρικών από επεξεργασμένο κρέας και προϊόντα κρέατος προκαλούν ανησυχία για τη δημόσια υγεία. Ειδικότερα, ιδιαίτερη προσοχή στα προγράμματα δημόσιας υγείας πρέπει να δοθεί στα παιδιά έως 9 ετών. Επίσης, υψηλότερη πρόσληψη νιτρωδών και νιτρικών από επεξεργασμένα κρέατα συνέβαλε στην αύξηση της DBP, αλλά η επίδραση αλληλεπίδρασης με τα επίπεδα συνολικής πρόσληψης νατρίου πρέπει να λαμβάνεται υπόψη για τη σωστή ερμηνεία των ευρημάτων. Η διαχρονική συζήτηση σχετικά με το ρόλο των νιτρικών και των νιτρωδών στη διατροφή του είναι δικαιολογημένη και περαιτέρω έρευνα, με περισσότερα και αναλυτικότερα δεδομένα, απαιτείται. Οι ανησυχίες που διατυπώνονται στην παρούσα διατριβή συνάδουν με τις ανησυχίες της Ευρωπαϊκής Επιτροπής, η οποία έλαβε πρόσφατα την απόφαση να τροποποιήσει τους ισχύοντες όρους χρήσης των νιτρωδών και των νιτρικών αλάτων ως πρόσθετων τροφίμων, μειώνοντας τα ανώτατα επιτρεπόμενα όρια στον κανονισμό (ΕΚ) 2023/2108 της 6ης Οκτωβρίου 2023.

**Επιστημονική περιοχή:** Διατροφή ανθρώπου/Διατροφή Δημόσιας Υγείας/Διατροφική Επιδημιολογία

**Λέξεις κλειδιά:** Καρδιαγγειακά νοσήματα, CVD, καρκίνος, διατροφική έκθεση, διατροφική πρόσληψη, νιτρικές ενώσεις, πρόσθετα τροφίμων, MPLs, κίνδυνος, κατανάλωση επεξεργασμένου κρέατος, Ελλάδα, νιτρώδη από τη διατροφή, όφελος για την υγεία, κίνδυνος για την υγεία, υπέρταση, συστολική αρτηριακή πίεση, SBP, διαστολική αρτηριακή πίεση, DBP.

**Declarations**

► The current thesis was conducted during a study leave granted by the Hellenic Food Safety Authority, spanning from September 15th, 2020 to September 15th, 2023.

► The Hellenic National Nutrition and Health Study (HNNHS) was co-funded by Greece and the European Union (European Social Fund) under the Operational Program "Human Resources Development 2007-2013". The research papers published through this thesis received no funding.

► With my permission, this paper was checked by the Examination Committee through the plagiarism detection software available at the AUA and its validity and originality was cross-checked.

**Δηλώσεις έργου**

► Η παρούσα διατριβή διεξήχθη κατά τη διάρκεια εκπαιδευτικής άδειας που χορηγήθηκε από τον Ενιαίο Φορέα Ελέγχου Τροφίμων, από τις 15 Σεπτεμβρίου 2020 έως τις 15 Σεπτεμβρίου 2023.

► Η ΠΑνελλήνια ΜΕλέτη Διατροφής & Υγείας (ΠΑΜΕΔΥ) συγχρηματοδοτήθηκε από την Ευρωπαϊκή Ένωση (Ευρωπαϊκό Κοινωνικό Ταμείο) στο πλαίσιο του Επιχειρησιακού Προγράμματος «Ανάπτυξη Ανθρώπινου Δυναμικού 2007-2013».

► Με την άδειά μου, η παρούσα εργασία ελέγχθηκε από την Εξεταστική Επιτροπή μέσα από λογισμικό ανίχνευσης λογοκλοπής που διαθέτει το ΓΠΑ και διασταυρώθηκε η εγκυρότητα και η πρωτοτυπία της.

**Acknowledgments**

How bizzare life can be… In 1998, so many years ago, upon embarking on my professional journey at the Agricultural University of Athens subsequent to the completion of my studies at the Department of Science and Technology of the AUA, I encountered some remarkable PhD thesis proposals. However, it was not meant to be then. I had to decline them, primarily due to my necessity of ensuring job stability. After a period exceeding two decades engaged in highly captivating and challenging professional domains, which diverged significantly from my academic pursuits, I eventually decided a carreer change. This decision led me to the Hellenic Food Authority (EFET), where I reconnected with my original area of study and my old dream of pusuing a PhD thesis. Furthermore, I was assigned a subject that immediately captivated my interest, that of risk assessment of contaminants and additives present in food products. This topic piqued my curiosity, prompting a desire to delve deeper and expand my knowledge in this field. I feel very lucky that this interest was shared by two great people and scientists who agreed to include me in their team and help me achieve my goal, Professor Antonis Zampelas and Assistant Professor Emmanouella Magriplis.

I would first like to express my sincere thanks and respect to my Supervisor and President of the Hellenic Food Safety Authority, Professor Antonis Zampelas, for giving me the opportunity to fulfill a long-standing aspiration that has persisted since my days as a student. I express my sincere gratitude to him for his trust and appreciation, his sharing of expertise, his constructive interventions and counseling whenever required and his overall support in everything I needed for our work to proceed effectively.

However, this thesis could not have produced the results presented in this manuscript without the valuable help of Assistant Professor Emmanuella Magriplis, member of my Supervising Committee. I would like to thank her warmly for always being by my side. I am grateful to her for engaging in fruitful discussions, along with my Supervisor, providing valuable advice, contributing insightful ideas, offering guidance throughout every step, making herself available, dedicating her time, and especially for her sharing of knowledge and expertise. There were many challenges that had to be overcome during the implementation of this thesis as its start coincided with the pandemic and lock

down period. The extent of the results would have been significantly diminished if she had not always been there to inspire me and find ways to keep going despite the adversity. I thank her for her trust as well as her love and deep commitment to her science and the students working with her. Keep up the good work my dearest! I believe we have a well-established, lifelong relationship, so you will always have a loyal companion and partner in whatever you need!

I would also like to express my thanks to Professor Eleftherios Drosinos, member of my Supervising Committee, for his assistance and immediate response to whatever was needed, scientifically and administratively. Additionally, I would like to acknowledge the other members of my committee Professor Konstantinos Tsioufis, Professor Androniki Naska, Professor Apostolos Vandarakis and Assistant Professor Kalliopi Karatzis. Thank you for the time spent on my study.

I owe special thanks to the officials at EFET for the educational leave granted to me as well as to my esteemed colleagues in the Risk Assessment and Nutrition Directorate who welcomed me in 2019, shared their passion for their work, kept on collaborating with me during the implementation of this thesis and supported me in every possible way.

Finally, a big thank you to my family, especially to my affectionate husband and my three incredible children, for their love, support and understanding. I am truly grateful for each one of you. I still can not believe we did this too, all of us together...

May you all be blessed!

**List of papers**

I. **Kotopoulou S**, Zampelas A, Magriplis E. Dietary nitrate and nitrite and human health: a narrative review by intake source. *Nutr Rev*. 2022 Mar 10;80(4):762-773. doi: 10.1093/nutrit/nuab113. PMID: 34919725.

II. **Kotopoulou S**, Zampelas A, Magriplis E. Risk Assessment of Nitrite and Nitrate Intake from Processed Meat Products: Results from the Hellenic National Nutrition and Health Survey (HNNHS). *Int J Environ Res Public Health*. 2022 Oct 6;19(19):12800. doi: 10.3390/ijerph191912800. PMID: 36232098; PMCID: PMC9565037.

III. **Kotopoulou S**, Zampelas A and Magriplis E (2023) Nitrite and nitrate intake from processed meat is associated with elevated Diastolic Blood Pressure (DBP). *Clinical Nutrition*: S0261561423000961. DOI: 10.1016/j.clnu.2023.03.015.

**Letter to the editor (presented in Appendix 1)**

**Kotopoulou S**, Zampelas A, Magriplis E (2023) Reply-Letter to the editor: "Nitrite and nitrate intake from processed meat is associated with elevated diastolic blood pressure (DBP)". *Clinical Nutrition* S0261561423001759. https://doi.org/10.1016/j.clnu.2023.06.008.

**Abstract/poster in conference (presented in Appendix 2)**

**Kotopoulou S**, Zampelas A, Magriplis E. Dietary exposure to nitrate and nitrite from processed meat and association with Blood Pressure: Results from the Hellenic National Nutrition and Health Survey (HNNHS). 10th National Gongress of the Hellenic Atherosclerosis Society. December 1-3, 2022. J Atherosclerosis Prev Treat. 2022 Supplement 1;13:121. E-poster: HAA94.

**Award**

"International Research Awards on Pediatrics, Perinatology and Child Health" schedule on 17th Edition of PPCH |16-18 October 2023 | Paris, France under the category of "Best Researcher Award" for Ass. Prof. Emm Magriplis on "Dietary nitrate and nitrite and human health: a narrative review by intake source".

**List of additional papers (presented in Appendix 4)**

I. Emmanuella Magriplis, Georgios Marakis, **Sotiria Kotopoulou**, Androniki Naska, George Michas, Renata Micha, Demosthenes Panagiotakos, Antonis Zampelas. Trans fatty acid intake increases likelihood of dyslipidemia especially among individuals with higher saturated fat consumption. Rev. Cardiovasc. Med. 2022, 23(4), 130. https://doi.org/10.31083/j.rcm2304130.

II. Georgios Marakis, **Sotiria Kotopoulou**, Charalampos Proestos, Stavroula Skoulika, Georgios Boukouvalas, Andreas Papaioannou, Zoe Mousia, Dimitra Papadimitriou, Eleni-Maria Katri, Androniki Naska, Michail Chourdakis, Antonis Zampelas, Emmanuella Magriplis. Changes of trans and saturated fatty acid content in savoury baked goods from 2015 to 2021 and their effect on consumers' intake using substitution models; a study conducted in Greece. The American Journal of Clinical Nutrition, 2023, ISSN 0002-9165, https://doi.org/10.1016/j.ajcnut.2023.08.014.

**Additional abstracts/presentations in conferences (presented in Appendix 5)**

I. G. Marakis, **S. Kotopoulou**, Ch. Proestos, Z. Mousia, D. Papadimitriou, A. Naska, A. Zampelas, E. Magriplis. Evaluation of the European Policy for the Reduction of Trans Fatty Acids (TFA) to protect public health. 6th Grespen Congress. 04-06 May 2023. Harokopio University of Athens. Oral presentation.

II. G. Marakis, A. Zampelas, **S. Kotopoulou**, A. Naska, Z. Mousia, E. Magriplis. Exposure Assessment of trans fatty acids among adults in Greece; results from the Hellenic National Nutrition and Health Survey (HNNHS). 9th Panhellenic Conference of the Greek Lipid Forum. 22-10-2021. Book of Abstracts p34. Oral presentation: O14.

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## Abbreviations

| | |
|---|---|
| **ACC** | American College Of Cardiology |
| **ADI** | Acceptable Daily Intake |
| **AESAN** | Spanish Agency for Food and Nutrition |
| **AGES** | Austrian Agency For Health And Food Safety |
| **AHA** | American Heart Association |
| **AICR** | American Institute For Cancer Research |
| **ANSES** | French Agency For Food, Environmental And Occupational Health Safety |
| **AOAC** | Association of Official Analytical Chemists |
| **BC** | Breast Cancer |
| **BE** | Barret's Esophagus |
| **BFR** | German Federal Institute For Risk Assessment |
| **BLC** | Bladder Cancer |
| **BMD** | Benchmark Dose |
| **BMDL** | Benchmark Dose Lower Confidence Limit |
| **BMI** | Body Mass Index |
| **BP** | Blood Pressure |
| **BT** | Brain Tumor |
| **CA** | Colorectal Adenoma |
| **CAPI** | Computer Assisted Personal Interview |
| **CHD** | Coronary Heart Disease |
| **CI** | Confidence Interval |
| **CRC** | Colorectal Cancer |
| **CRP** | C-Reactive Protein |
| **CVD** | Cardiovascular Disease |
| **CRA** | Cumulative Risk Assessment |
| **DASH** | Diet Approach To Stop Hypertension |
| **DBP** | Diastolic Blood Pressure |
| **DHQ** | Diet History Questionnaire |
| **E249** | Potassium Nitrite |
| **E250** | Sodium Nitrite |
| **E251** | Sodium Nitrate |
| **E252** | Potassium Nitrate |
| **EAC** | Esophageal Adenocarcinoma |
| **EC** | European Commission |
| **EC** | Esophageal Cancer |

| | |
|---|---|
| **EFSA** | European Food Safety Authority |
| **ENOC** | Endogenous N-Nitroso Compounds |
| **ESC** | European Society Of Cardiology |
| **ESCC** | Esophageal Squamous Cell Carcinoma |
| **ESH** | European Society Of Hypertension |
| **EU** | European Union |
| **FAME** | Fatty Acid Methyl Ester |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **FFQ** | Food Frequency Questionnaire |
| **FG** | Food Group |
| **FPQ** | Food Propensity Questionnaire |
| **FSAI** | Food Safety Authority Of Ireland |
| **GC** | Gastic Cancer |
| **GC** | Gas Chromatography |
| **GCA** | Gastric Cardia Adenocarcinoma |
| **GI** | Gastrointestinal Cancer |
| **GNCA** | Gastric Non-Cardia Adenocarcinoma |
| **HBGV** | Health-Based Guidance Value |
| **HDL** | High Density Lipoprotein |
| **HDPA** | Hellenic Data Protection Authority |
| **HI** | Hazard Index |
| **HN** | High Nitrate |
| **HNNHS** | Hellenic National Nutrition And Health Survey |
| **HTN** | Hypertension |
| **IARC** | International Agency For Research On Cancer |
| **IOM** | Institute Of Medicine Of The National Academies |
| **IOTF** | International Obesity Task Force |
| **IPAQ** | International Physical Activity Questionnaire |
| **IQR** | Interquartile Range |
| **i-TFA, ITFA** | Industrial Trans Fatty Acids |
| **JECFA** | Joint FAO/WHO Expert Committee on Food Additives |
| **kg** | kilogram |
| **LDL** | Low-Density Lipoprotein |
| **MC** | Mediterranean Countries |
| **MD** | Mediterranean Diet |
| **mg** | Milligram |

| | |
|---|---|
| **MOE** | Margin Of Exposure |
| **MPL** | Maximum Permitted Level |
| **MUFA** | Mono Unsaturated Fatty Acids |
| **NCD** | Non-Communicable Disease |
| **NDEA** | Nnitrosodiethylamine |
| **NDMA** | N-Nitrosodimethylamine |
| **NDSR** | Nutrition Data System For Research |
| **NHANES** | National Health And Nutrition Examination Survey |
| **NO** | Nitrite Oxide |
| **NOCS** | N-Nitroso Compounds |
| **OVC** | Ovarian Cancer |
| **PA** | Physical Activity |
| **PC** | Pancreatic Cancer |
| **PUFA** | Poly Unsaturated Fatty Acids |
| **RA** | Risk Assessment |
| **RCC** | Renal Cell Carcinoma |
| **R-TFA, rTFA** | Ruminant (Natural) Trans Fatty Acids |
| **SBG** | Savoury Baked Good |
| **SBP** | Systolic Blood Pressure |
| **SCF** | Scientific Committee On Food |
| **SD, sd** | Standard Deviation |
| **SFA** | Saturated Fatty Acids |
| **T2D** | Type 2 Diabetes |
| **T2DM** | Type 2 Diabetes Mellitus |
| **TC** | Thyroid Cancer |
| **TFA** | Trans Fatty Acids |
| **TMDI** | Theoretical Maximum Daily Intake |
| **TTHM** | Total Trihalomethanes |
| **USDA** | US Department Of Agriculture |
| **WCRF** | World Cancer Research Fund |
| **WHO** | World Health Organization |

**Terminology**

This section aims to provide a clear definition of the terminology used in this thesis, based on dictionaries from the Centers for Disease Control and Prevention (CDC) [1] and the European Food Safety Authority (EFSA) )[2], as the use of various terms in the fields of toxicity, microbiology, and nutrition may cause confusion.

| TERM | DEFINITION |
|---|---|
| **ACCEPTABLE-DAILY-INTAKE (ADI)** | An estimate of the quantity of a substance in food or drinking water (such as food additives, pesticide residues and veterinary drugs) that can be consumed daily over a lifetime without posing a significant risk to health (in milligrams of the substance per kilogram of body weight). |
| **ADVERSE-EFFECT** | A change in the health, growth, behavior or development of an organism that impairs its ability to develop or survive |
| **AGENT** | A factor, such as a microorganism, chemical substance, or form of radiation, whose presence, excessive presence, or (in deficiency diseases) relative absence is essential for the occurrence of a disease. |
| **ASSOCIATION** | Statistical relationship between two or more events, characteristics, or other variables. |
| **BENCHMARK-DOSE (BMD)** | The minimum dose of a substance that produces a clear, low level health risk, usually in the range of a 1-10% change in a specific toxic effect such as cancer induction. |
| **BIAS** | Deviation of results or inferences from the truth, or processes leading to such systematic deviation. Any trend in the collection, analysis, interpretation, publication, or review of data that can lead to conclusions that are systematically different from the truth. |
| **BODY-MASS-INDEX (BMI)** | A measurement that expresses the relationship between an individual's weight and height. BMI is calculated by dividing weight in kilograms by height in metres squared (i.e. height x height). Used to assess whether someone's weight is appropriate. |
| **BURDEN OF DISEASE** | How a disease affects a population in terms of ill-health, risk of death, financial cost of treatment or other recognised indicators. |
| **CASE** | In epidemiology, a countable instance in the population or study group of a particular disease, health disorder, or condition under investigation. Sometimes, an individual with the particular disease. |
| **CARCINOGENICITY** | Cancer-causing property of a substance when an animal or human is exposed to it. |
| **CASE-CONTROL STUDY** | A type of observational analytic study. Enrollment into the study is based on presence ("case") or absence ("control") of disease. Characteristics such as previous exposure are then compared between cases and controls. |

| TERM | DEFINITION |
|------|------------|
| **CAUSE OF DISEASE** | A factor (characteristic, behavior, event, etc.) that directly influences the occurrence of disease. A reduction of the factor in the population should lead to a reduction in the occurrence of disease. |
| **CHEMICAL HAZARD** | Health hazard resulting from exposure to a chemical; for example, irritation, burns, carcinogenicity. |
| **CHRONIC EXPOSURE** | A long-term constant or intermittent exposure to a substance which may have an impact on health over time. |
| **COHORT** | A well-defined group of people who have had a common experience or exposure, who are then followed up for the incidence of new diseases or events, as in a cohort or prospective study. A group of people born during a particular period or year is called a birth cohort. |
| **COHORT STUDY** | A type of observational analytic study. Enrollment into the study is based on exposure characteristics or membership in a group. Disease, death, or other health-related outcomes are then ascertained and compared. |
| **CONFIDENCE INTERVAL (CI)** | A statistical term to describe a range within a distribution where you would expect most of the data to lie; for example, expecting that 95% of adults will be between 1.4m and 1.9m tall. The specified probability is called the confidence level, and the end points of the confidence interval are called the confidence limits. |
| **CONSERVATIVE ASSUMPTION** | An estimate that tends to err on the side of caution or gives a 'worst case scenario'. Often used in risk assessment to ensure that as much risk as possible is taken into account. |
| **CONTROL** | In a case-control study, comparison group of persons without disease. |
| **CORRELATION** | A statistical term to describe the relationship between two variables (e.g. calcium intake and bone growth). |
| **CUMULATIVE-EFFECT** | A term used to describe how exposure to more than one chemical might affect the body. Used to explain long-term exposure to mixtures of chemicals, such as pesticides or additives. |
| **CUMULATIVE RISK ASSESSMENT** | A method of assessing risks to health or the environment posed by multiple substances such as chemicals. |
| **DEMOGRAPHIC INFORMATION** | The personal characteristics— e.g. age, sex, race, and occupation—of descriptive epidemiology used to characterize the populations at risk. |
| **DEPENDENT VARIABLE** | In a statistical analysis, the outcome variable(s) or the Variable(s) whose values are a function of other variable(s) (called independent variable(s) in the relationship under study). |
| **DIETARY EXPOSURE** | Measurement of the amount of a substance consumed by a person or animal in their diet that is intentionally added or unintentionally present (e.g. a nutrient, additive or pesticide). |

| TERM | DEFINITION |
|------|------------|
| **DISTRIBUTION** | In epidemiology, the frequency and pattern of health-related characteristics and events in a population. In statistics, the observed or theoretical frequency of values of a variable. |
| **DOSE RESPONSE** | The relationship between the amount of a substance to which an individual organism, population or ecosystem is exposed and the way in which it responds (e.g. in terms of toxicity). |
| **EPIDEMIC** | The occurrence of more cases of disease than expected in a given area or among a specific group of people over a particular period of time. |
| **E-NUMBER** | A number used in the European Union to identify permitted food additives. An E number means that an additive has passed safety tests and has been approved for use. |
| **ENDPOINT** | A physical or chemical outcome that can be assessed by a test; for example, blood pressure or levels of a potential toxin in the body. |
| **EPIDEMIOLOGY** | The study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to the control of health problems. |
| **EVALUATION** | A process that attempts to determine as systematically and objectively as possible the relevance, effectiveness, and impact of activities in the light of their objectives. |
| **NUTRIENT** | Any substance which a living organism must consume from the diet in order to support normal health, development and growth. |
| **EXOGENOUS** | Describes substances within the human body which have arisen from an external source in the diet or environment; for example, veterinary medicine residues. |
| **EXPOSURE** | Concentration or amount of a particular substance that is taken in by an individual, population or ecosystem in a specific frequency over a certain amount of time. |
| **EXPOSURE ASSESSMENT** | One of the key steps in risk assessment, this relates to a thorough evaluation of who, or what, has been exposed to a hazard and a quantification of the amounts involved. |
| **FREQUENCY DISTRIBUTION** | A complete summary of the frequencies of the values or categories of a variable; often displayed in a two column table: the left column lists the individual values or categories, the right column indicates the number of observations in each category. |
| **FOOD-ADDITIVE** | A substance deliberately added to foods or beverages for beneficial technological reasons (e.g. to preserve, flavour, colour or ensure a particular texture). Food additives are not normally consumed by themselves nor used as typical ingredients in food. |
| **FOOD SUPPLEMENT** | Foodstuff containing concentrated amounts of nutrients or other substances that are intended to supplement the normal diet. |

| TERM | DEFINITION |
|------|------------|
| **FOODBORNE DISEASE** | An illness caused by foods or drinks which have been contaminated by toxins or harmful microbes (e.g. bacteria, viruses). |
| **GENOTOXICITY** | When a substance is capable of damaging the DNA in cells. |
| **HAZARD** | A substance or activity which has the potential to cause adverse effects to living organisms or environments. |
| **HEALTH** | A state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity. |
| **HEALTH-BASED GUIDANCE VALUE** | Guidance on safe consumption of substances that takes into account current safety data, uncertainties in these data, and the likely duration of consumption. |
| **INDEPENDENT VARIABLE** | An exposure, risk factor, or other characteristic being observed or measured that is hypothesized to influence an event or manifestation (the dependent variable). |
| **INDIVIDUAL DATA** | Data that have not been put into a frequency distribution or rank ordered. |
| **INTERQUARTILE RANGE** | The central portion of a distribution, calculated as the difference between the third quartile and the first quartile; this range includes about one-half of the observations in the set, leaving one-quarter of the observations on each side. |
| **IN VITRO** | Research method which involves testing cells or tissues extracted from living organisms. |
| **IN VIVO** | Research method which involves testing individual live animals or populations of live animals. |
| **INCIDENCE** | The number of new events occurring within a specified time period within a defined geographical area; for example, the number of flu cases per year in Europe. |
| **INGREDIENT** | Any substance deliberately added to a foodstuff which will remain in the finished product, even in an altered form. |
| **INTAKE** | The amount of a substance (e.g. nutrient or chemical) that is ingested by a person or animal via the diet. |
| **MARGIN OF EXPOSURE (MOE)** | A tool used in risk assessment to explore safety concerns arising from the presence of a potentially toxic substance in food or animal feed. |
| **MARGIN OF SAFETY** | The gap between the actual intake of a substance by a given population and the estimated daily dose over a lifetime that experts consider to be safe. |
| **MAXIMUM PERMITTED LEVEL** | The maximum amount of a contaminant, naturally occurring toxin or nutrient allowed in foods or animal feeds. |
| **MEAN** | The measure of central location commonly called the average. It is calculated by adding together all the individual values in a group of measurements and dividing by the number of values in the group. |
| **MEDIAN** | The measure of central location which divides a set of data into two equal parts. |

| TERM | DEFINITION |
|---|---|
| **MORBIDITY** | Any departure, subjective or objective, from a state of physiological or psychological well-being. |
| **MORTALITY RATE** | A measure of the frequency of occurrence of death in a defined population during a specified interval of time. |
| **NO OBSERVED ADVERSE EFFECT LEVEL (NOAEL)** | The greatest concentration or amount of a substance at which no detectable adverse effects occur in an exposed population. |
| **NORMAL DISTRIBUTION** | The symmetrical clustering of values around a central location. The properties of a normal distribution include the following: (1) It is a continuous, symmetrical distribution; both tails extend to infinity; (2) the arithmetic mean, mode, and median are identical; and, (3) its shape is completely determined by the mean and standard deviation. |
| **NUTRIENT** | An element or compound needed for normal growth, development and health maintenance. Essential nutrients cannot be made by the body and must, therefore, be consumed from food. |
| **OBSERVATIONAL STUDY** | Epidemiological study in situations where nature is allowed to take its course. Changes or differences in one characteristic are studied in relation to changes or differences in others, without the intervention of the investigator. |
| **ODDS RATIO** | A measure of association which quantifies the relationship between an exposure and health outcome from a comparative study; also known as the cross-product ratio. |
| **OCCURRENCE** | The fact or frequency of something (e.g. a disease or deficiency in a population) happening. |
| **PERCENTILE** | A way of visualising the low, medium and high occurrences of a measurement (e.g. vitamin C intake) by splitting the whole distribution into one hundred equal parts. The set of numbers from 0 to 100 that divide a distribution into 100 parts of equal area, or divide a set of ranked data into 100 class intervals with each interval containing 1/100 of the observations. A particular percentile, say the 5th percentile, is a cut point with 5 percent of the observations below it and the remaining 95% of the observations above it. |
| **POPULATION** | The total number of inhabitants of a given area or country. In sampling, the population may refer to the units from which the sample is drawn, not necessarily the total population of people. |
| **PREVALENCE** | The proportion of a population found to have a condition. |
| **PROPORTION** | A type of ratio in which the numerator is included in the denominator. The ratio of a part to the whole, expressed as a "decimal fraction" (e.g., 0.2), as a fraction (1/5), or, loosely, as a percentage (20%). |
| **RANGE** | In statistics, the difference between the largest and smallest values in a distribution. In common use, the span of values from smallest to largest. |

| TERM | DEFINITION |
|---|---|
| RATE | An expression of the frequency with which an event occurs in a defined population. |
| RELATIVE RISK | A comparison of the risk of some health-related event such as disease or death in two groups. |
| REPRESENTATIVE SAMPLE | A sample whose characteristics correspond to those of the original population or reference population. |
| RISK | The probability that an event will occur, e.g. that an individual will become ill or die within a stated period of time or age. |
| RISK FACTOR | An aspect of personal behavior or lifestyle, an environmental exposure, or an inborn or inherited characteristic that is associated with an increased occurrence of disease or other health-related event or condition. |
| SAMPLE | A selected subset of a population. A sample may be random or non-random and it may be representative or non-representative |
| SENSITIVITY | The ability of a system to detect epidemics and other changes in disease occurrence. The proportion of persons with disease who are correctly identified by a screening test or case definition as having disease. |
| STANDARD DEVIATION | The most widely used measure of dispersion of a frequency distribution, equal to the positive square root of the variance. |
| STANDARD ERROR (OF THE MEAN) | The standard deviation of a theoretical distribution of sample means about the true population mean. |
| STATISTICAL SIGNIFICANCE | A measure of the likelihood that a result occurred based on statistics. |
| SUB-POPULATION, SUB-GROUP | An identifiable subdivision of a population; for example, infants. |
| TOXICITY | The potential of a substance to cause harm to a living organism. |
| TREND | A long-term movement or change in frequency, usually upwards or downwards. |
| UNCERTAINTY | Scientific concept used in risk assessment to describe all types of limitations in available knowledge at the time an assessment is conducted, with the agreed resources, that affect the probability of possible outcomes to the assessment. |
| UNCERTAINTY ANALYSIS | A method of identifying the sources of uncertainty in a risk assessment calculation and estimating their size and direction so that errors can be taken into account. |
| VALIDITY | The degree to which a measurement actually measures or detects what it is supposed to measure. |
| VARIABLE | Any characteristic or attribute that can be measured. |
| VARIANCE | A measurement of the spread between numbers in a data set. |
| VULNERABLE | Group of people needing specific consideration when |

| TERM | DEFINITION |
|---|---|
| **GROUP** | assessing the nutritional needs or health effects of substances; for example, pregnant women, infants and people exposed to higher doses of substances through their environment. |

*To my family, friends and colleagues…*

# A. BACKGROUND-LITERATURE REVIEW-THESIS OVERVIEW

# 1. GENERAL INTRODUCTION

## 1.1. NITRIC COMPOUND IN HUMAN DIET: A CONUNDRUM

Nitrate (NO3-) and nitrite (NO2-) are organic chemicals formed naturally in plants, soil, and water by the microbial oxidation of nitrogen [3]. Nitrate is the most prevalent source of nitrogen (N) in soils [4] and thus a common source of amino acid synthesis in plants and a crucial nutrient for proper plant growth and development [5]. Food and water are the principal sources of human exposure [6], as dietary nitrite and nitrate can be present (a) naturally in plant foods, (b) as additives in processed products, mainly as salts in meats, and (c) as contaminants in drinking water [7,8]. Nitrate is converted to nitrite through the process of nitric oxide (NO) oxidation facilitated by the presence of naturally occurring bacteria in both the oral cavity and the gastrointestinal tract [9].
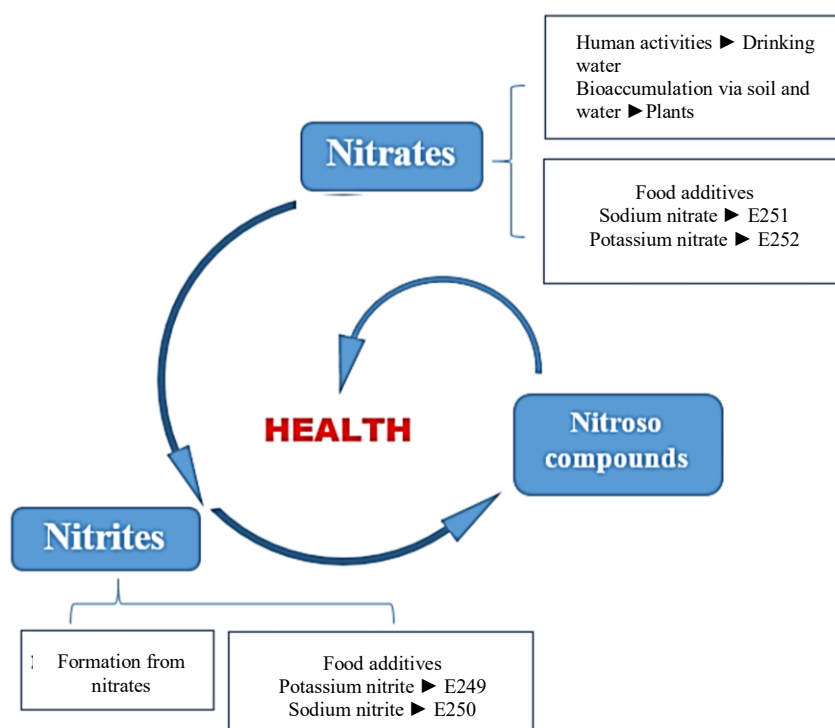


Figure 1. Compounds of interest in the perspective of human consumption *[10]*.

The effects of nitrite and nitrate ingestion through diet on human health and relevant controversies have been debated for years [11,12] with dietary nitrate being related to both numerous health benefits and risks [13–15]. In particular, extensive research has been conducted to investigate the therapeutic potential of nitrate in treating CVDs,

especially hypertension [16–18]. This research has focused on the nitrate derived from fruits, vegetables, and their combinations, which are nevertheless widely recognized as integral components of a healthy diet [19]. The findings have suggested that inorganic nitrate might be a simple and cost-effective approach for mitigating the CVD risk [20], by lowering BP and platelet aggregation [21]. In addition to favorable effects on vascular and metabolic health [22], there is evidence to suggest that the presence of nitrite and nitrate compounds consumed from fruits and vegetables may confer protective benefits against the development of cancer [23,24]

On the other hand, nitric compounds added in foods and those found in water, in comparison to inorganic nitrates, may have health risks [14,25]. There have been significant concerns regarding the potential health risks associated with the consumption of nitric compounds found in processed meat and have been found to be associated with the development of various types of cancer. Additionally, the presence of nitric compounds in drinking water can have acute effects on the human body, such as methemoglobinemia, as well as chronic effects, primarily manifested as an increased risk of cancer due to the formation of nitrosocompounds (NOCs) [26]. The physiological processes that may lead to the synthesis of NOCs endogenously have been exhaustively described previously [9,26–29]. In short, the human gastrointestinal tract easily absorbs nitrate and nitrite in food and drinking water, which is concentrated in the salivary glands and released in saliva, where oral bacteria convert it to nitrite. Nitrite causes the formation of methaemoglobin. Cytochrome b5 reductase converts methaemoglobin to haemoglobin, preventing oxygen delivery. High met-haemoglobin concentrations can impair cytochrome b5 reductase's regenerative ability. High methaemoglobin concentrations limit tissue oxygen delivery and may cause hypoxia. Furthermore, in the stomach's acidic environment, nitrite and protein secondary amines generate nitrosamines, including volatile N-nitrosodimethylamine (NDMA) and Nnitrosodiethylamine (NDEA), which are probable or highly likely carcinogenic to humans (IARC 2A; IARC 1987; EPA 2015). Such nitrosamines can also be generated in processed cured meat products during high-temperature [7]. Heme uses nitrate and nitrite ingested to promote the endogenous formation of nitroso compounds (NOCs) in red meat. However, the presence of nitrosation inhibitors, such as diverse polyphenols, as well as vitamins E and C, commonly present in fruits and vegetables, seem to mitigate this process [30,31].

## 1.2. REGULATORY FRAMEWORK FOR NITRITE AND NITRATE INTENDED FOR HUMAN CONSUMPTION

The association of nitrite and nitrate with adverse health effects has led to regulatory measures being enforced to regulate the concentrations of nitrate and nitrite by source of intake.

Member states should ensure that nitrate and nitrite quantities in the mains-supplied drinking water, spring water, and treated water comply with the parametric maximum values of 50 mg/l and 0.5 mg/l respectively, according to the Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption [32]. The natural mineral waters are regulated by Directives 2003/40/EC19 [33] and 2009/54/EC20 [34]. These directives establish the maximum allowable concentrations of nitrate and nitrite in natural mineral waters at 50 mg/l and 0.10 mg/l, respectively.

While nitrate is a naturally occurring compound in plants and non-toxic, its metabolites, nitrite, nitric oxide, and N-nitroso compounds, may pose health risks [35]. Due to the high levels of nitrate in plant foods, especially leafy vegetables like rucola, lettuce, and spinach, which may increase when grown in glass houses or in low-light conditions, regulatory thresholds for nitrate in plant-based foods have been set with Regulation (EU) No 1258/2011, amending previous Regulation (EC) No 1881/2006 [36]. The existing restrictions pertain to six distinct categories of plant products that are deemed to pose the highest risk in terms of nitrate consumption. These categories include fresh or preserved spinach, deep-frozen or frozen spinach, lettuce, iceberg lettuce, rucola (commonly known as rocket), as well as processed cereal-based foods and baby foods intended for infants and young children. Maximum levels range from 200 mg/kg in processed-cereal based foods and baby foods for infant and young children to 7000 mg/kg in Rucol harvested 1 October to 31 March. In contrast, there are no regulation limits for nitrites in the EU Regulation on Food Contaminants.

Regarding the use of nitrate and nitrite as additives in food, potassium or sodium nitrite (E249 or E250 respectively) and sodium or potassium nitrate (E251 or E252 respectively) are categorized within the "preservatives" class as defined in Regulation (EC) No 1333/2008 of the European Parliament and of the Council [8]. This class

encompasses substances that extend the shelf-life of food products by safeguarding them against deterioration caused by micro-organisms and/or inhibiting the growth of pathogenic micro-organisms. According to the EU regulation, nitrite salts are authorized for use in specific meat preparations as defined in Regulation (EC) No 853/200424 [37], as well as in both heat-treated and non-heat-treated meat products. Similarly, nitrate salts are allowed in certain fish varieties such as pickled herring and sprats, specific categories of cheese, and non-heat-treated meat products. Nitrate and nitrite compounds are permissible in certain traditional immersion-cured or dry-cured products as well, such as jellied veal and brisket, selský salám, saucissons etc. Regulation (EC) No 1333/2008 establishes prescribed limits, both for utilization and as remaining quantities, of nitrate and nitrite salts within the various authorized product classifications. Regarding processed meat products, Maximum Permitted Levels (MPLs) of nitrate and nitrite content up to 150 mg/kg have been established to ensure that the desired effect is accomplished without exceeding the levels that are safe for human health [8]. A maximum residual content of 250 mg/kg is allowed in traditional products, since they are produced in smaller quantities, resulting in lower consumer exposure. The residual content is influenced by various processing procedure parameters, such as temperature, pH, ascorbic acid, and other factors [10]. These limits were used to re-evaluate nitric compounds used as food additives in 2017, based on national food consumption data from 33 different dietary surveys carried out in 19 European countries; no need to change the current ADIs was identified [14,15]. The consumption of processed and packaged foods, however, has witnessed an uptrend due to shifting lifestyle patterns and dietary preferences [38]. This trend is particularly notable in the production and consumption of processed meat, which have experienced a significant rise over the past decades, with projections indicating a further 50% increase by the year 2050 [39]. The aforementioned trend, also referred to as nutrition transition [40], may necessitate regular re-evaluations of the ADIs for the use of nitric compounds as additives, alongside conducting risk assessments based on more recent consumption data.

## 1.3. NUTRITION TRANSITION AND NON-COMMUNICABLE DISEASES (NCDS)

Changing lifestyle and dietary trends have increased the consumption of processed and packaged foods [38]. Higher processed foods consumption, frequent dining out, utilization of edible oils, and increased intake of sugary beverages emerged as prevalent trends during the 1970s; however, the adverse effects of these trends were not widely acknowledged until the onset of global epidemics of diabetes, hypertension, and obesity in the 1990s, disproportionately affecting individuals with lower and middle incomes [41]. Research on defining and evaluating dietary habits has garnered significant attention, due to its established associations with NCDs [42,43].

The term NCDs relates to a cluster of conditions that are primarily not instigated by an acute infection, leading to persistent health implications and frequently necessitating prolonged medical intervention and attention [44]. NCDs have emerged as a significant public health issue across all nations, with a particular emphasis on low- and middle-income countries, which account for over three-quarters of total NCD deaths and amounts to 31.4 million deaths; in Greece, NCDs are responsible for 83% of deaths [45].

Cardiovascular diseases (CVDs) and cancer are categorized as NCDs or chronic illnesses, alongside chronic respiratory disease and Type 2 Diabetes Mellitus (T2D) [45]. Cardiovascular diseases (CVDs) are widely recognized as the leading cause of morbidity and mortality globally, representing 32% of all global deaths in 2019 [46], with the majority of which are attributed to specific health behaviors and factors [47]. Cancer, also attributed mainly to health related lifestyle habits, like tobacco use, physical activity, alcohol consumption and diet, is the second leading cause of death globally, accounting for nearly 10 million deaths in 2020, or nearly one in six deaths [48]. Specifically, 35.6% of all cancer-related deaths in the same year are attributed to gastrointestinal types [49].

Nutrition transition, which is characterized by a reduction in the consumption of plant-based foods and a corresponding increase in ultra-processed products of low nutritional value [40], has also led to an increase in the ingestion of various food additives [38]. Furthermore, in the past years, the decline in income has been associated with an increase in obesogenic diets and the intake of calorie-dense foods, and thus, more

people in the Mediterranean Countries (MC) appear to be abandoning the Mediterranean Diet (MD) due to financial constraints too [50]. This trend has been noticed among children and teenagers in the MC, who are increasingly adopting unhealthy lifestyle habits like a preference for "junk food" and sedentary activities (computer and TV use), leading to a Western diet high in saturated fat, refined grains, simple carbohydrates, and processed foods [51]. Greeks, in particular, have been reported to adhere to a diet that is vastly different from the MD, with a high consumption of red meat and fast food [52], as well as dairy, and alcohol among younger Greek adults, along with a low intake of fruits and vegetables [53]. This progressive adoption of unhealthy eating patterns appears to have accelerated since the beginning of the Greek debt crisis [54], creating an uncertainty with respect to their health effects.

Healthy dietary patterns, high on plantfood, seafood, whole grains and legumes, and low in sugary foods, processed meats and refined grains, have been proposed to reduce the CVD risk [55,56]. However, given the high degree of processing involved, even the growing popularity of plant-based alternatives in Western countries cannot adequately guarantee a diet that is both balanced and conducive to good health [57]. Ultra-processed foods consumption has been suggested to raise CVD risk [58] and overall mortality [59], with higher processed meat intake being specifically one of the main contributors to this finding [60–62], although questions remain as to the mechanism(s) involved.

Processed meat is any meat that has been seasoned, salted, cured, fermented, smoked or treated with other techniques to improve its durability, color or flavor [63]. Organizations such as the International Agency for Research on Cancer (IARC), the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) have linked processed meat consumption with human carcinogenesis [64], a fact evidenced by several epidemiological studies [22,28,65–68]. Following the above, the World Health Organization (WHO) recommends limiting or eliminating processed meat consumption, because no safe intake has been determined [69]. Greece has also adopted this recommendation [70]. Furthermore, CVD has been suggested to increase due to preservatives [71], such as sodium and additives, potentially nitrite [72]. Actually, nitrite (potassium nitrite-E249 and sodium nitrite-E250) and nitrate (sodium nitrate-E251 and potassium nitrate-E252)

are the most widely and legally used additives [8] to improve the visual appeal, taste, safety, and quality in processed meat products and their association with health has been debated for years [22]. The use of these agents as additives in processed meats have been discussed since the 1960s, mainly because of their link to the genesis of carcinogenic N-nitroso compounds (NOCs) when combined with amines or amides [25,73,74]. However, due to debatable evidence, their use is continued by the food industry with subsequent human consumption, warranting at the least a risk assessment based on population specific consumption data.

## 1.4. RISK ASSESSMENT

Risk assessment (RA) is a scientifically based process consisting of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization [75]. It constitutes one of the three interrelated components of risk analysis, alongside risk management and risk communication, as depicted in Figure 2.
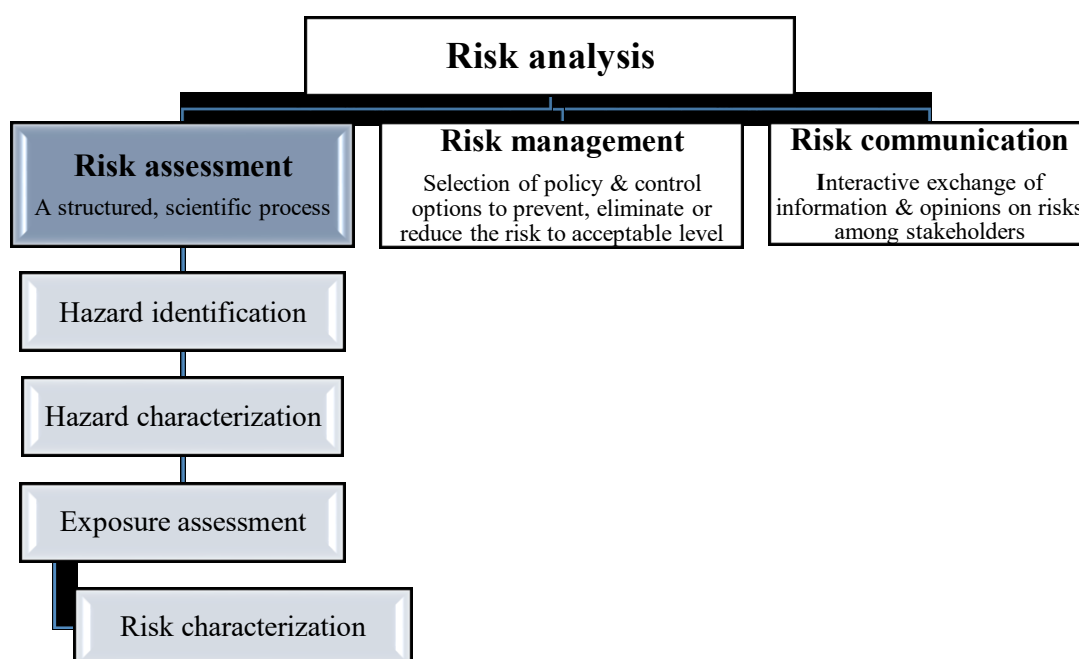


Figure 2. Components of risk analysis *[76]*.

RA provides a systematic framework for determining health protection measures, as presented in Figure 3 [75,77,78]. The primary goal of basic chemical-by-chemical risk

assessment is to answer the question "What is the appropriate concentration level of a chemical to ensure the protection of health outcomes?" [79].
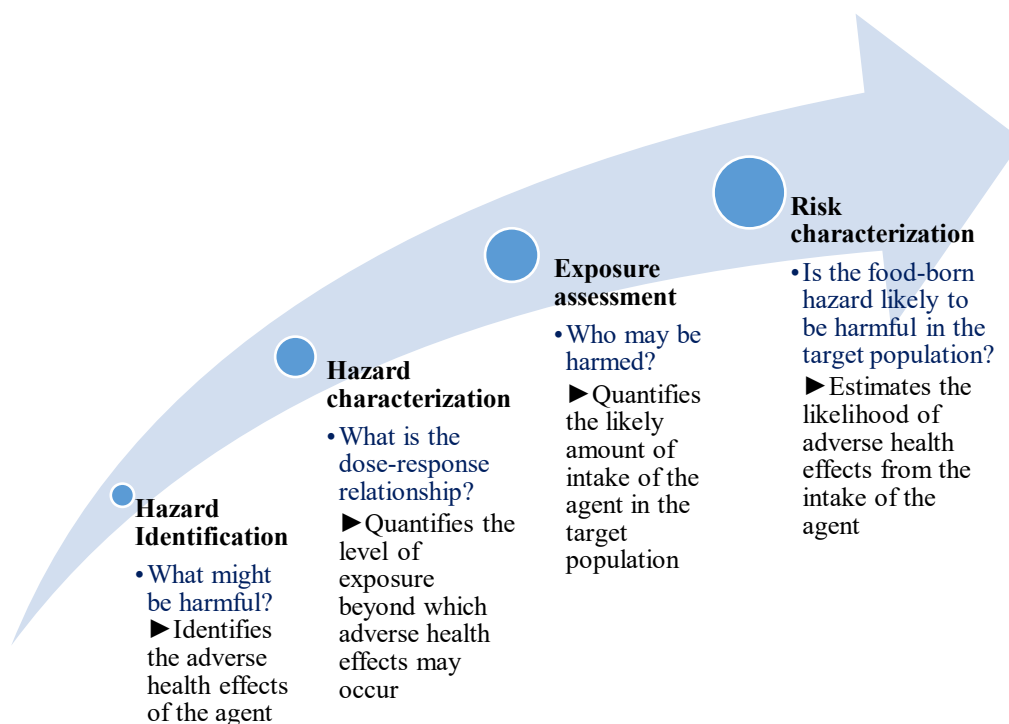


Figure 3. The 4 steps of risk assessment process *[75,77,78]*.

Qualitative risk assessment refers to the evaluation of risks that cannot be quantified precisely using numerical values, though remain valuable, especially when relying on expert knowledge and involves expressing risks in terms of categories such as high, medium, or low, or by making risk comparisons [80]. Quantitative risk assessment is based on numerical data and analysis and can be either deterministic (meaning single values like means or percentiles are used to describe model variables) or probabilistic (meaning that probability distributions are used to describe model variables) [81]. A quantitative risk assessment can answer risk management questions in greater depth than a qualitative risk assessment. Cumulative Risk Assessment (CRA) is a method used to evaluate the combined effects of multiple chemical and nonchemical stressors that have similar health outcomes or operate through similar mechanisms [79]. These stressors can originate from various sources or exposures, and may result in an exacerbation of symptoms or the occurrence of illnesses. The adoption of this approach

was motivated by the need to address the vulnerability of certain populations, such as infants and young children, who may encounter multiple stressors that can have detrimental consequences [82]. Political decision-makers can utilize this process as a means to mitigate consumers' exposure to risks within the food chain through the implementation of campaigns that provide guidance on diet and lifestyle choices, the enforcement of controls on food production of both of the aforementioned actions [78].

Finally, each stage of the risk assessment process introduces uncertainty [83], and should be carried out in a transparent manner, with data, conditions, assumptions, and uncertainties well-documented and scientifically explained [84].

### 1.4.1. Hazard identification

Risk assessment begins by assessing scientific data to identify a potential hazard—a chemical, biological, or physical agent in food that may harm health—and its sources [75,85]. Thus, the first step of the risk assessment process is to determine the toxicity of the agent.

| The hazard of nitrite and nitrate |
|---|
| The International Agency for Research on Cancer (IARC) classified nitrate or nitrite (after ingestion) in Group 2A as probably carcinogenic to humans, due to animal studies suggesting nitrite is harmful when paired with amines or amides [3]. Despite its association with GC, the IARC found limited evidence that dietary nitrite is carcinogenic in humans [3]. |
| The available in vivo data on nitrite do not suggest the presence of genotoxicity [14], whereas the existing data on nitrate do not indicate any genotoxic potential [15]. After oral exposure, 75% of absorbed dietary nitrate is eliminated in the urine, and 25% is released by the salivary glands into the mouth cavity where bacteria convert some to nitrite [10]. The deleterious effects of nitrate are ascribed to its transformation into nitrite and the inherent capacity for endogenous generation of N-nitroso compounds (NOCs), nitrosamines, and nitrosamides under favorable environmental conditions (e.g., pH, reactant concentration) [86]. Multiple research studies suggest that NOCs present in various foods possess genotoxic properties and are anticipated to be potential |

human carcinogens. [28,87–92]. Insufficient data exists regarding the identification and quantification of exogenous, in the food matrix, and endogenous, in human body, NOCs [10].

## 1.4.2. Hazard characterization

This step in RA process involves the identification of potential adverse outcomes associated with the hazardous agent being evaluated, as well as the establishment of an upper threshold for the dosage at which those outcomes may occur [93]. The critical effect refers to the point at which harmful consequences begin to manifest following a specific level of dosage or exposure [94]. In situations where it is assumed that the toxic effect of a substance has a threshold level, a Health-Based Guidance Value (HBGV) is defined as a scientifically-based recommendation for the maximum safe consumption established after considering the currently available safety data, possible uncertainties encompassed, and the probable time span of consumption [2,94]. The Acceptable Daily Intake (ADI), for example, is an HBGV used to depict the permissible quantities of food additives, pesticide residues, and veterinary medications that can be consumed daily throughout a lifetime without creating a significant health risk [2,95] and it is usually expressed as milligrams of the substance per kilogram of body weight per day [96]. Establishing the HBGV is not part of performing a risk assessment. The Scientific Panels and Units of EFSA do so on a regular basis as part of their evaluation of regulated products in the food and feed industries for which a scientific risk assessment is necessary prior to their authorization on the EU market [96]. Dietary nitrite and nitrate exposure may also be reported as a percentage contribution to ADI (EFSA, 2017). This is called Hazard Index (HI). If the HI is less than 100% then there is no harm from exposure to the additive. Characterization focuses on the consumers and especially the extreme consumers or vulnerable sub-groups that they might be at risk [97].

| HBGV for nitrite and nitrate |
| --- |
| Nitrate and nitrite have ADIs determined by the EFSA for the human body at 3.7 [15] and 0.07 [14] mg/kg of body weight per day (mg/kg of bw/day), expressed as ions, respectively. |

The derivation of an ADI for nitrate was based on the process of methaemoglobin formation, which occurs after the conversion of nitrate, excreted in the saliva, into nitrite. Nevertheless, significant discrepancies were observed in the dataset pertaining to the conversion of nitrate to nitrite in human saliva. Hence, the Panel of EFSA deliberated that the derivation of a singular value for the ADI based on the existing data was not feasible, since even when employing the most conservative nitrate-to-nitrite conversion factor, the levels of methaemoglobin generated as a result of nitrite derived from this conversion would not have any clinical significance. Additionally, the estimated production of endogenous N-nitroso compounds (ENOC) resulting from this process would be at levels that are of minimal concern. Therefore, despite the inherent uncertainty surrounding the acceptable daily intake (ADI) determined by the Scientific Committee on Food (SCF), the EFSA Panel has reached the conclusion that there is presently an insufficient amount of evidence to warrant the withdrawal of this ADI [15].

The ADI for nitrite, as determined by the Scientific Committee on Food (SCF) in 1997 and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002, were found to range from 0 to 0.06 mg/kg body weight per day and 0 to 0.07 mg/kg bw/day, respectively. In general, an ADI for nitrite alone could be established based on the existing studies on the repeated exposure to nitrite in animals, taking into account the absence of carcinogenic effects. The Panel reached the determination that the observed elevation in methaemoglobin levels, both in humans and animals, constituted a pertinent outcome for the establishment of the ADI. The EFSA Panel employed a Benchmark Dose (BMD) modeling approach to calculate an ADI for nitrite of 0.07 mg/kg bw/day. The evaluation conducted focused on the intrinsic generation of nitrosamines from nitrite. This was achieved through the utilization of theoretical calculations to determine the amount of N-nitrosodimethylamine (NDMA) produced upon the consumption of nitrite at the ADI level. The resulting margin of exposure (MoE) was estimated to be greater than 10,000. The Panel has determined that the MoE to exogenous nitrosamines in meat products is estimated to be less than 10,000 across all age groups under conditions of high level exposure. Epidemiological studies have provided indications of a potential association

between (i) the consumption of dietary nitrite and the occurrence of gastric cancers, as well as (ii) the combined intake of nitrite and nitrate from processed meat and the development of colorectal cancers. There exists evidence establishing a correlation between preformed NDMA and the occurrence of colorectal cancers.[14].

### 1.4.3. Exposure assessment

The third step of the risk assessment process pertains to determining the probability of being exposed to biological, chemical, or physical agents via food or other relevant sources and quantifying such exposure [98]. This phase entails ascertaining a consumer's chemical consumption by combining information on the substance's concentration in food and beverages with the amount of those commodities ingested [96]. Comprehensive and high-quality data on food consumption gathered at the individual level is necessary for assessing the exposure to potential risks in the food chain [99].

European Commission recommended a stepwise method to estimate the additive intakes [100]. The first tier (Tier 1) uses the budget method and concerns all the additives for which an acceptable daily intake (ADI) and maximum permitted level (MPL) are established [101,102]. It estimates the theoretical maximum daily intake (TMDI) by combining the maximum quantity of food and drinks that an individual consumes with the MPLs of the additive. When the TMDI exceeds the ADI, the second tier (Tier 2) is carried out by using actual national food consumption data and MPLs [103]. For the additives that exceed the ADI, a third tier (Tier 3) is performed using individual food consumption data and additives' measured concentrations [104]. Since measured concentration data are in many cases difficult to obtain, other authors have suggested assuming the presence of the additive at the MPL only when reported in the label of the food product and combining it with the actual national food consumption data. This approach is defined as the Tier 2a [105].

| Additional factors to consider for evaluating nitrite and nitrate exposure |
|---|
| While nitrate can be converted to nitrite in the human body, their risk assessment is usually based on single substance exposure due to different regulatory |

frameworks [7]. However, these chemical substances can be treated as a group by applying a common risk assessment principle (assessment group) in a component-based concentration addition approach [106].

Additionally it is well known that nitrate is transformed into nitrite in the body, mainly in the mouth cravity [107]. FAO/WHO suggested that 5-7% of nitrate consumed is converted to nitrite by bacteria in the mouth in healthy adults [108], however, a conversion rate up to 20% has also been reported [35]. Therefore, although the data on the nitrate-to-nirite conversion in the saliva in humans vary greatly, based on the nitrate secretion rates (20–25%) in the saliva and the range of nitrate to nitrite conversion rates (5–36%) in the mouth, an overall conversion percentage of 1–9% has been projected [15]. Based on these projections and recommendations, researchers should account for conversion of nitrates to nitrites although do not.

### 1.4.4.  Risk characterization

Characterizing the risk, which involves estimating the likelihood that negative health impacts would occur in a certain population, is the last step in the risk assessment process [2,93]. *Risk* is defined as the relationship between the likelihood and seriousness of a health consequence as a result of a hazard [75]. This phase allows for both qualitative and quantitative descriptions of risk (see Section 1.4) as depicted in Figure 4 and offers a basis for determining whether risks are acceptable by directly comparing the exposure assessment results with HGBV, as defined in the preceding steps of the process [83].
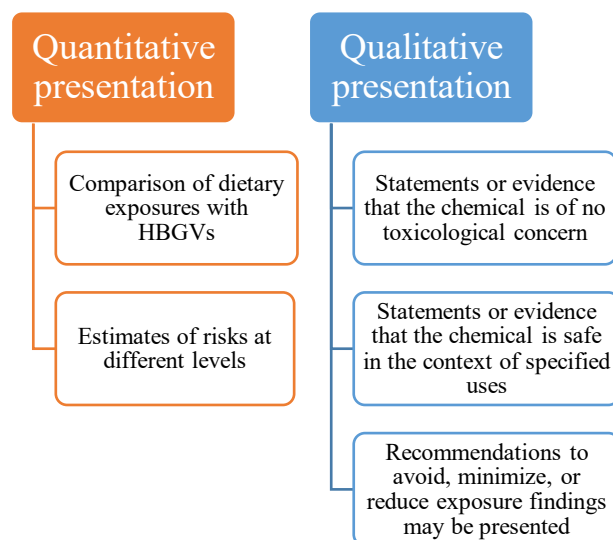
Figure 4. Quantitative and qualitative way of presenting risk assessment findings *[94]*

The process of risk characterization places emphasis on the consumer population, with particular attention given to sub-groups or extreme consumers who might be at risk (EFSA, 2017).

Therefore, this risk assessment step determines whether there is a risk and describes its nature and degree under certain conditions, providing the best available science-based evidence to support food safety management with enough knowledge to explore appropriate solutions [95,109,110]. Uncertainties, randomness, variability, and their differentiation must be included in risk evaluations since they affect risk estimate confidence and risk management decisions [111].

## 1.5. RE-EVALUATIONS OF NITRITE AND NITRATE USED AS ADDITIVES

The opinions pertaining to the re-evaluation of the nitric compounds when used as additives were released by the European Food Safety Authority (EFSA) in June 2017, following relevant requests from the European Commission [14,15]. The re-evaluations took place as foreseen in the programme set under the Regulation (EU) No 257/2010, within the overall deadline of 31.12.2008 [112]. The results ultimately demonstrated that the current levels of added nitrite and nitrate when used as additives, mainly in processed meat products, effectively ensure the protection of consumers.

According to the findings of the experts [15], the utilization of nitrate salts as food additives resulted in consumer exposure that accounted for less than 5% of the overall exposure to nitrate in food. Furthermore, this level of exposure did not surpass the ADI of 3.7mg/kg bw/day for nitrate.

In relation to nitrite [14], the experts concluded that the exposure levels were below the acceptable thresholds for all demographic groups. The EFSA panel emphasized that nitrite has the potential to surpass the ADI of 0.07mg/kg bw/day for individuals of all age groups when accounting for all sources of dietary exposure, including natural presence in food, environmental contamination, and use as additives. This conclusion was based on the assumption of medium to high levels of exposure; actually, under conditions of high exposure nitrite levels could surpass the ADI for all age groups. Finally, specific age groups such as infants, young children, and children may be at risk of exceeding the ADI for nitrite even under conditions of low exposure.

## 1.6. LITERATURE GAP

The health effects of nitrite and nitrate consumption, both in terms of origin and amount, remain extremely disputed, since these compounds have been directly or indirectly associated with both positive effects on CVD and negative effects on cancer, the two leading global causes of death. Therefore, the review of evidence regarding their effect on human health by source of origin necessitates further exploration. The following questions needed to be addressed:

1. What should a literature review focus on?
2. Which are the effects of dietary nitrite and nitrate on human health by source of origin?
3. Which sources are the most important from a public health perspective and dietary nitrite and nitrate intakes should be risk assessed?

## 1.7. AIM OF THE LITERATURE REVIEW OF THE THESIS

The first aim of the current PhD thesis was to review the data regarding the health effects of dietary nitrite and nitrate by source of intake (plant foods, animal based foods and water).

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# Dietary nitrate and nitrite and human health: a narrative review by intake source

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*Nitrate and nitrite are plant nutrients that, although ubiquitous in plant foods, are highly controversial substances in human nutrition because they are also used as additives in processed foods and may be found as contaminants in drinking water. The aim for this narrative review is to provide a thorough insight into the current literature on the relationship between dietary nitrate and nitrite and the health risks and benefits by source of intake. The results highlight beneficial effects of nitrate and nitrite consumption from plant origin on cardiovascular disease and, to date, no positive correlation has been reported with cancer. On the contrary, high intake of these compounds from processed animal-based foods is related to an increased risk of gastro-intestinal cancer. Nitrate in drinking water also raises some concern, because it appears to be related to adverse health effects. The up-to-date debate on the role of nitrate and nitrite in human nutrition seems to be justified and more research is required to verify safe consumption.*

## INTRODUCTION

Nitrate and nitrite are natural products formed in plants, soil, or water from nitrogen oxidation by microorganisms.[1] Nitrate is the most plentiful source of nitrogen in soils[2] and thus a common source of amino acid production in plants, serving as a key nutrient that coordinates optimal plant growth and development.[3] Dietary exposure to nitrate and nitrite happens through (1) fruits, vegetables, and their products as natural derivatives; (2) processed foods that contain nitrate and nitrite salts as food additives, specifically sodium and potassium nitrate (E251 and E252, respectively), and sodium and potassium nitrite (E250 and E249, respectively), and (3) drinking water in which the components are present as contaminants.[4,5] Metabolism of nitrogen compounds to nitrosamine has been associated with the risk of gastrointestinal cancer,[6] whereas nitric oxide (NO) produced endogenously from nitrate, a substance found naturally in plants, may help control blood pressure. The latter, therefore, has been associated with improved cardiovascular health.[6,7] Ingestion of inorganic nitrate has a positive effect on endothelial function because it significantly decreases cardiovascular disease (CVD) risk factors through a reduction in platelet aggregation and reduction in arterial rigidity.[8] CVD[9] and cancer[10] are the main causes of death globally, with colorectal cancer (CRC) being 1 of the top causes of cancer globally, being the third most prevalent cancer in men and the second most frequent in women.[11] In 2016, approximately 17.9 million people died of CVD; more recent data attribute 10 million deaths in 2020 to cancer.[10] Specifically, 35.6% of all cancer-related deaths in 2020 are attributed to gastrointestinal types.[12]

Fruits, vegetables, and fruit and vegetable combinations are considered to be a significant component of a

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healthy diet.[13] Nitrate and nitrite naturally derived from fruits and vegetables are considered to have beneficial effects against cancer[14,15] and for vascular and metabolic health.[16] The nitrite–NO pathway has been shown to have antihypertensive actions.[7] Therefore, although a great quantity of nitrate is consumed from vegetable origin,[17] its intake is unlikely to be considered problematic.[18] Moreover, recently it has been suggested by the International Olympic Committee that nitrate supplementation may directly enhance athletic performance.[19] On the contrary, concerns have been raised about oral exposure to nitrate and nitrite from processed meat.[17] Several epidemiological studies provide evidence that consumption of processed animal products may cause cancer.[6,16,18,20–22] In particular, nitrate and nitrite from processed meat have been linked with CRC, both colon and rectum types, whereas dietary nitrite has been associated with gastric cancer (GC), gastric cardia adenocarcinoma and gastric noncardia adenocarcinoma.[23] Use of nitrate and nitrite salts in processed meats has been discussed since the 1960s, mainly because of their association with the genesis of carcinogenic *N*-nitroso compounds (NOCs).[24,25] Heme promotes NOC synthesis in red meat endogenously from the ingestion of nitrate and nitrite, but nitrosation inhibitors including various polyphenols, and vitamins E and C found in fruits and vegetables appear to mitigate it.[26,27] The physiological processes that may lead to the synthesis of NOCs endogenously have been exhaustively described previously.[6,28–31] In addition, consumption of drinking water with elevated nitrate concentrations has been found to affect the human body acutely and chronically. The former is usually seen as methemoglobinemia, and the latter as cancer occurrence due to organism's nitrosamine exposure.[31]

Thus, it is evident that the safety of long-term intake of nitrate and nitrite is of critical importance because the health effects in terms of type and amount of consumption remain highly controversial and have been linked to the 2 leading causes of death worldwide, CVD and cancer. In this study, we aimed to address data behind the enigma of the health risks and benefits linked with dietary nitrate and nitrite, focusing on plant vs animal sources, as well as water, by conducting a thorough review of the evidence.

This literature review was conducted by searching the PubMed database to identify eligible publications worldwide up to December 2020. English-language restrictions were applied, and relevant scientific studies were identified by combining the following Medical Subject Heading keywords: nitrate, nitrite, health, neoplasms, vegetables, fruit, drinking water, food additives, meat, and dietary exposure. Moreover, an extra search of the references of related articles was carried out.

Published data as well as reports, opinions, and related documents from the European Food Safety Authority (EFSA), World Health Organization, and Joint Food and Agriculture Organization of the United Nations–World Health Organization Expert Committee Report on Food Additives were also included. The screening process resulted in our review of 21 dietary exposure assessment studies, 22 epidemiological observational studies, 14 cross-over studies, 4 meta-analyses, 4 systematic reviews, 16 reviews, and 1 re-analysis (Table S1 in the Supporting Information online). The reviews and meta-analyses considered in this article have been published in the last decade (2010 and forward). To evaluate consistency of the evidence, exposure, outcomes, and confounders, variables were extracted from each study and tabulated. The key elements of the epidemiological and clinical studies that were reviewed are listed in Tables S2–S5 in the Supporting Information online. Information on variables accounted for in models used in cohort studies are also reported in Table S6 in the Supporting Information online.

## DIETARY EXPOSURE

### Sources of nitrate and nitrite in human nutrition

Nitrate food sources are mostly derived from vegetables and vegetable products,[4,21,32–38] whereas nitrite is produced endogenously via NO oxidation and nitrate reduction by commensal bacteria in the mouth and gastrointestinal tract.[29]

The contribution of vegetables and vegetable products to the combined exposure of nitrate and nitrite has been estimated in several studies: approximately 34%–41% in the Netherlands[4] (60% for a subgroup of highly trained Dutch athletes)[38]; 51.2% in Iran (another 30.7% of exposure coming from grains)[32]; 81%–83% in Australia[33]; 88% in Poland[35]; 80% in Belgium[36]; and 24%–27% of the acceptable daily intake (ADI) in France.[37] Many studies have been conducted on the variables that determine the concentration of nitrate in vegetables.[20,34,39–41] Fruit contribution has also been estimated in Australia as approximately 6%–7% of total dietary nitrate intakes.[33] The current maximum levels of nitrate permitted in vegetable foodstuffs in the European Union (EU) were set in Regulation (European Commission [EC])[42] and its amendment.[43] These were expressed as weight (in milligrams) per kilogram of fresh produce weight.

Nitrate salts are used in preserved meat products, sausages, and cheese, making these foods the most important exposure contributors for the consumption of nitrate salts as food additives in the EU,[44] whereas

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preserved meats and sausages are the main contributors to the exposure of nitrite salts used as food additives.[23,45] The foodstuffs that may contain nitrate salts as food additives with corresponding maximum permitted levels in the EU being set in the main and amended Regulation (EC/EU).[5,46] Mainly, nitrate salts are added as a precursor of nitrite salts, which have a beneficial impact on the visual appeal, flavor security, and quality of cured meats.[16] In particular, food additives contributed 8%–9% to the combined exposure in the Netherlands[4] and approximately 1% (processed meats) in Australia.[33] The main dietary sources for nitrite were estimated in Poland and these consisted of cold and processed meats, both at 77%.[35,45]

Finally, nitrate and nitrite may also be present in drinking water.[47] Drinking water was found to contribute 3%–19% to the combined exposure to nitrate and nitrite in the Netherlands[4] and 5%–15% of the ADI in France.[37] Nitrate in drinking water has been established at a maximum of 50 mg/L (Council Directive 98/83/EC).[48] In the United States, the maximum contaminant level for nitrate-nitrogen concentration in drinkable water has been set at 10 mg/L.[49]

Recently, Kalaycıoğlu and Erim[21] investigated and published analytical data of nitrate and nitrite food-residue levels of fruits, vegetables, other natural foods, meat, dairy, and baby and infant food. They suggested the data collected could be used either to update dietary guidelines or as an effective resource in risk-benefit assessments for the food industry and clinical trials. In addition, Blekkenhorst et al[50] created a database to be used as a reference for determining nitrate concentrations in a variety of vegetables, which takes into account several processing aspects that may alter nitrate concentrations in vegetables, including various preservation and cooking procedures. Finally, the EFSA presented summary data on levels of nitrate and nitrite salts used as food additives reported by the food industry (2000–2014) as well as analytical results of nitrate and nitrite from natural sources or contamination in foods reported by Member States (2000–2015).[23,44] Based on these data, exposure assessments have been performed.

### Evaluation of dietary exposure to nitrate and nitrite

Different approaches for assessing the ingestion of food additives and contaminants can be used and, therefore, the results can vary significantly.[41] Babateen et al[51] pointed out differences in dietary assessment methodologies and food-composition tables use, and underlined the necessity of obtaining accurate estimates of daily consumption. Most studies reviewed in the present article investigated combined exposure to different foods, including fresh and/or processed, drinking water, and

beverages (Table S1 in the Supporting Information online). A few studies focused on estimating the intake only from processed foods—mainly meat products,[45,52–54]—and only 1 study evaluated nitrite intake from processed vegetables, cheese, and meat.[36] Finally, 3 studies focused exclusively on exposure via the intake of vegetables and vegetable products (Table S1 in the Supporting Information online). Actually, the data on dietary exposure that are currently published are barely comparable.[37]

The ADIs of nitrate and nitrite in the human body have been set by EFSA at 3.7[44] and 0.07 mg/kg of body weight (bw) per day,[23] respectively. Of studies reviewed, 12 reported nitrate and nitrite intake above the ADIs and 9 reported nitrate and nitrite intake below the ADIs. Table 1 lists these studies.[4,32,34–37,39,41,52–62] In particular, Roila et al[34] found in their study that the intake of nitrate from vegetables was above the ADI and the nitrite ingestion from processed meats was below the ADI.

### HEALTH-RELATED ISSUES CONCERNING DIETARY NITRATE AND NITRITE BY SOURCE OF INTAKE

Tables 2 and 3 summarize the risks[63–78] and benefits[38,65,79–90] of dietary nitrate and nitrite based on the epidemiological and clinical studies evaluated. Table 4 lists the studies that did not reveal an explicit relationship with potential health implications.[71,74,78,88,91–97]

### Fruits and vegetables

A weak inverse association between fruit consumption and cancers has been suggested; it was evident in current smokers with lung cancer, whereas high cereal-fiber consumption was linked to lower risk of CRC and other gastrointestinal cancers.[15] Fresh fruits and citrus fruits may help prevent diffuse and cardia GC,[14] but the adverse relationship between fruit and citrus intake and risk of GC appears to be limited to smokers and Northern European populations. Vegetable consumption is also linked to a lower incidence of CRC, which researchers partly attribute to the presence of endogenous nitrosation inhibitors.[65] Furthermore, in men, there appears to be an inverse relationship between consumption of green leafy vegetables and the risk of Barrett's esophagus.[85] The differential effects between vegetable and fruit ingestion and risk of Barrett's esophagus in both sexes were explained by differences in the incidence ratio (more frequent in men), esophageal pathophysiology, possible false-negative cases, and a lack of knowledge of *Helicobacter pylori* infection (which is inversely related to Barrett's esophagus).[85]

*Table 1* **Dietary intake of nitrite and nitrate relative to ADIs**

| Compound | Source of exposure | Reference | |
|---|---|---|---|
| | | Above the ADI | Below the ADI |
| Nitrite | Combined (fresh and processed food-stuffs, vegetables and fruit, drinking water, beverages) | van den Brand et al (2020)[4] Elias et al (2020)[55a] Chetty et al (2019)[56] Bahadoran et al (2019)[32c] Suomi et al (2016)[57d] Mancini et al (2015)[58e] Larsson et al (2011)[59f] Menard et al (2008)[37g] Thomson et al (2007)[60h] | Vlachou et al (2020)[61] Mancini et al (2015)[58b] Anyzewska et al (2014)[35] |
| | Vegetables | – | – |
| | Processed foodstuffs (processed meat products) | Adam et al (2017)[52i] Merino et al (2016)[53] Vin et al (2013)[54j] | Roila et al (2018)[34] |
| Nitrate | Combined (fresh and processed food-stuffs, vegetables, drinking water, beverages) | – – | Chetty et al (2019)[56] Anyzewska et al (2014)[35] |
| | Vegetables | Roila et al (2018)[34k] | Brkic et al (2017)[39] Mitek et al (2013)[62] Tamme et al (2006)[41] Temme et al (2011)[36] |
| | Processed foodstuffs (processed vegetables, cheeses, and meat products) | | |

[a]ADI for nitrite was >3.1% of children participating in the study.
[b]ADI levels for nitrate and nitrite are 3.7 and 0.06 mg/kg body weight, respectively.
[c]Nitrite consumption by more than every tenth child between the ages of 3 and 6 y exceeded the ADI for nitrite.
[d]When using the maximum permitted levels and upper-bound scenarios.
[e]When using measured concentrations for nitrite.
[f]When total nitrite consumption was evaluated, nitrite levels in nearly 12% of children aged 4 y were above the nitrite ADI.
[g]Individual nitrate consumption was greater than the ADI in 1.4%–1.5% of adults and 7.9%–8.4% of children.
[h]Approximately 10% of persons with an average rate of conversion and 50% of all persons with a high rate of conversion are expected to surpass the ADI.
[i]ADI exceeded in some adults and all children.
[j]ADI exceeded at tier 2 for children (except for those aged 4–18 y in the United Kingdom) and P97.5 for both adults and children.
[k]Nitrate was higher than the ADI for infants.
*Abbreviations*: –, no data; ADI, acceptable daily intake.

*Table 2* **Risks of dietary nitrate and nitrite in the human body in relation to source**

| Source | Reference | Case | Type of study |
|---|---|---|---|
| Animal (especially Red, processed, pan-fried meat) | Barry et al (2020)[63] | BLC | Case-control |
| | Jones et al (2019)[64] | CRC (colon) | Cohort |
| | Espejo-Herrera et al (2016)[65] | CRC (rectal) | Case-control |
| | Inoue-Choi et al (2015)[66] | OVC | Cohort |
| | Aschebrook-Kilfoy et al (2013)[67] | TC | Cohort |
| | Dellavalle et al (2013)[68] | RCC | Cohort |
| | Aschebrook-Kilfoy et al (2012)[69] | OVC | Cohort |
| | Aschebrook-Kilfoy et al (2011)[70] | PC | Cohort |
| | Cross et al (2011)[71] | ESCC | Cohort |
| | Ferrucci et al (2009)[72] | Colorectal adenoma | Cross-sectional |
| | Preston-Martin et al (1996)[73] | BT | Case-control |
| Drinking water | Barry et al (2020)[63] | BLC | Case-control |
| | Espejo-Herrera et al (2016)[65] | CRC (rectal) | Case-control |
| | Espejo-Herrera et al (2016)[74] | BC[a] | Case-control |
| Not defined (usually dietary and drinking water) | Dellavalle et al (2014)[75] | CRC | Cohort |
| | Loh et al (2011)[76] | CRC (rectal) | Cohort |
| | Ward et al (2010)[77] | TC | Cohort |
| | | Hypothyroidism | Cohort |
| | Knekt et al (1999)[78] | CRC[b] | Cohort |

[a]Among postmenopausal women with high red-meat consumption.
[b]Significant association of high intake of *N*-nitrosodimethylamine with smoked and salted fish and nonsignificant association with cured meat and sausages.
*Abbreviations*: BC, breast cancer; BLC, bladder cancer; BT, brain tumor; CRC, colorectal cancer; ESCC, esophageal squamous cell carcinoma; GI, gastrointestinal cancer; OVC, ovarian cancer; PC, pancreatic cancer; RCC, renal cell carcinoma; TC, thyroid cancer.

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**Table 3** Benefits of dietary nitrate and nitrite in the human body in relation to plant sources (fruits, vegetables, supplements)

| Reference | Case | Type of study |
|---|---|---|
| Gopinath et al (2020)[79] | Arteriolar and venular caliber | Cross-sectional |
| Cherukuri et al (2020)[80] | Endothelial function, BP | Cross-over |
| Kerley et al (2018)[81] | BP | Cross-over |
| Jonvik et al (2017)[38] | Energy | Cross-sectional |
| Asgary et al (2016)[82] | Endothelial function, HTN, systemic inflammation | Cross-over |
| Espejo-Herrera et al (2016)[65] | CRC (rectal cancer) | Case-control |
| Velmurugan, et al (2016)[83] | Vascular function[a] | Cross-over |
| Ashworth et al (2015)[84] | BP | Cross-over |
| Keszei et al (2014)[85] | BE | Cohort |
| Larsen et al (2011)[86] | Basal mitochondria function and whole-body oxygen consumption | Cross-over |
| Larsen et al (2010)[87] | Oxygen consumption | Cross-over |
| Larsen et al (2006)[88] | DBP[b] | Cross-over |
| Svetkey et al (1999)[89] | BP[c] | Cross-over |
| Appel et al (1997)[90] | BP[c] | Cross-over |

[a]In patients with hypercholesterolemia.
[b]In healthy, young study subjects.
[c]Intake from the Dietary Approaches to Stop Hypertension diet.
*Abbreviations*: BP, blood pressure; BC, breast cancer; BE, Barret's esophagus; CRC, colorectal cancer; CRC, colorectal cancer; DBP, diastolic blood pressure; HTN, hypertension.

**Table 4** No or weak association of dietary nitrate and nitrite in the human body in relation to source

| Source | Reference | Case | Type of study |
|---|---|---|---|
| Plant (fruits and vegetables) | Sundqvist et al (2020)[91] | BP | Cross-over |
| | Smeets et al (2020)[92] | Endothelial function | Cross-over |
| | Blekkenhorst et al (2018)[93] | BP | Cross-over |
| | Bondonno et al (2014)[94] | BP | Cross-over |
| | Dubrow et al (2010)[95] | Adult glioma | Cohort |
| | Larsen et al (2006)[88] | SBP, pulse rate[a] | Cross-over |
| Animal | Cross et al (2011)[71] | GC | Cohort |
| | Dubrow et al (2010)[95] | Adult glioma | Cohort |
| Not defined | Espejo-Herrera et al (2016)[74] | BC | Case-control |
| | Keszei et al (2013)[96] | ESCC[b] | Cohort |
| | | EAC | Cohort |
| | | GCA | Cohort |
| | | GNCA | Cohort |
| | Knekt et al (1999)[78] | Head/neck cancers[c] | Cohort |
| | | GC[c] | Cohort |
| | van Loon et al (1998)[97] | GI | Cohort |

[a]In healthy, young study subjects.
[b]An association with the risk of ESCC in men may be observed.
[c]Related to *N*-nitrosodimethylamine consumption.
*Abbreviations*: BP, blood pressure; BC, breast cancer; CRC, colorectal cancer; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; GC, gastric (stomach) cancer; GCA, gastric cardia adenocarcinoma; GI, gastrointestinal cancer; GNCA, gastric non-cardia adenocarcinoma; SBP, systolic blood pressure; TC, thyroid cancer.

Inorganic nitrate may have a key part in vegetables' cardiovascular health effects, possibly through enhancing NO bioavailability in the vascular system.[98] Endothelial function appears to robustly improve and reduce systolic blood pressure (BP) and diastolic BP in hypertensive people when plant bioequivalent inorganic nitrate and ascorbic acid are administered daily.[80] NO generated from the reduction of dietary nitrate may decrease BP and improve endothelial function through a variety of processes, including substrate use by the endothelial NO synthase, an increase in vasodilation, and inhibition of platelet aggregation and reactive oxygen species generation in mitochondria.[99] Dietary nitrate supplementation in the short run can enhance BP, endothelial function, and systemic inflammation[82] and could be used to prevent CVD in individuals with hypercholesterolemia and early vascular dysfunction.[83] It could also result in a decrease in diastolic BP and systolic BP,[80–82,84] although a study in healthy young people showed a decrease in diastolic BP but no effect on

systolic BD and pulse rate.[88] Generally, the Dietary Approaches to Stop Hypertension combination of a low-fat and cholesterol diet high in fruits and vegetables is indicated as a successful method for primary and secondary hypertension prevention in the general population and among those at high risk for CVD risk.[89,90] Dietary nitrate has been reported to have antihypertensive effects among people with uncontrolled hypertension,[81] and green leafy vegetables have been suggested to protect against the risk for CVD in normotensive women, because the intake of plants high in nitrate appears to significantly boost nitrate and nitrite levels in plasma and reduce BP.[84] Conversely, recently published data indicate that high nitrate intake from green leafy vegetables or nitrate supplementation over a 5-week period does not lower ambulatory systolic BP in individuals with hypertension, in comparison with a diet low in nitrate.[91] Previous studies indicated that, in comparison with low-nitrate vegetables, greater consumption of vegetables that are rich in nitrate did not reduce BP in people with untreated hypertension or those who were classified as prehypertensive,[84,93] and arterial stiffness was not reduced in normotensive individuals.[94] A randomized, double-blind, cross-over trial that enrolled men with abdominal obesity did not show an improvement in postprandial endothelial function after a single dose of nitrate-rich beetroot powder mixed with L-arginine.[92] In their systematic review, Blanch et al[100] agreed that higher amounts of potassium intake may help improve endothelial function; however, findings on beneficial effects of increasing fruit and vegetable intake are mixed. Specifically, diets containing fruit and vegetables consumed by individuals at high risk for cardiovascular disease may improve vascular endothelial function, whereas the effect on healthy persons is less obvious. The characteristics of the cross-over studies that have considered the correlation between dietary nitrate intake and BP are summarized in Table S5 in the Supporting Information online.

A positive association between nitrate ingestion and energy intake[38] and the possible protective effects of dietary nitrate[30] on body balance have been highlighted. Specifically, dietary nitrate lowers maximal oxygen consumption while maintaining work performance during maximal exercise[87] and might also have a significant impact on both basal human mitochondrial function and whole-body oxygen consumption during exercise.[86] The International Olympic Committee recently agreed that there is enough evidence on nitrate as a supplement to suggest that enhancement of performance is possible.[19] However, improvements in performance appear to be more difficult to obtain in highly trained athletes.[101]

Also, dietary nitrate has been linked with the alleviation of stomach ulcers[30] and, in older persons, with an improved retinal arterial and venous caliber.[79] Finally, adult glioma risk has not been proven to be reduced by eating fruits and vegetables with vitamin C or vitamin E.[95]

## Animal sources (with focus on red and processed meat)

Results between intake of nitrate and nitrite and the risk of cancers are controversial. A substantial positive correlation between N-nitrosodimethylamine intake and CRC incidence has been observed[78]; however, other studies do not support a clear or positive link between them (Table 4). No link was found between CRC and overall dietary nitrate or via vitamin C intake, although high dietary nitrate intake may increase CRC risk in subgroups in which higher endogenously produced NOCs is expected, particularly in those with a deficiency in vitamin C.[75] Dietary nitrate from animal sources has been linked to an elevated risk for rectal cancer,[65] and recent research has also linked a relatively high intake of red meat with a higher colon cancer risk, but this was not related to dietary nitrate and nitrite,[64] contributing to the ambiguous results of quantitative exposure-estimate studies. Moreover, colorectal adenomas have been linked with elevated consumption of red and fried meat, as well as to the 2-amino-3,4-dimethylimidazo [4,5-f]quinolone; exposures from other meat sources require more investigation.[72]

Higher amounts of processed meat intake were associated with an increased GC risk, but the link may be distorted or altered by dietary components such as vitamins C and E, which inhibit the synthesis of NOCs in the stomach.[102] In addition, an increased esophageal squamous cell cancer risk was found with red meat intake, and between 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline intake and gastric cardia adenocarcinoma.[71] NOCs may have an effect on the chance of esophageal squamous cell cancer development in men; no other apparent relationships were found with other esophageal and gastric subtypes.[96] Moreover, there is some evidence relating pancreatic cancer in men, but not women, with dietary nitrate and nitrite from processed meat.[70] In the United Kingdom, N-nitrosodimethylamine has been linked to an increased risk of rectal cancer, which could be influenced by plasma concentration of vitamin C.[76] Last, although of low evidence, higher nitrite intake from animal sources, mainly from processed meat, has been associated with thyroid cancer.[67]

In addition, a study based on the Iowa Women's Health Study showed that ovarian cancer was inversely

associated with dietary nitrate and positively with nitrite from processed meats.[66] The results also suggested that high nitrate levels could elevate the ovarian cancer risk in postmenopausal women. Women in the highest nitrate consumption quintile had a 31% increased risk of epithelial ovarian cancer, compared with women in the lowest quintile. Finally, although no link was found between overall dietary nitrite levels and ovarian cancer risk, women who consumed the most nitrite from animal sources had a 34% higher risk of ovarian cancer.[69]

In another study, risk for tumor occurrence increased with more frequent consumption of processed meats, as well as with mean daily cured meat or nitrite intake, but not with vegetables nitrate.[73] These effects were observed across social levels, age groups, and geographic regions, as well as for each of the 3 major histological types. Processed or red meat, as well as nitrite or nitrate consumption, have not been shown to increase the incidence of adult glioma.[95] There were also no significant links found between *N*-nitrosodimethylamine consumption and head and neck tumors combined.[78]

Findings also imply that nitrite derived from animal foods may increase the risk of renal cell carcinoma, predominantly clear-cell adenocarcinomas.[68] Also, nitrite, but not nitrate, increased risk for development of non-Hodgkin lymphoma risk.[103] Furthermore, an association was found between angioedema and anaphylaxis with processed meat intake containing nitrate or nitrite. Authors reporting that this association should be accounted for among individuals with food allergies.[104] Finally, dietary nitrite intakes at levels over the acceptable limit (0.07 mg/kg bw/d) may increase the risk for type 1 diabetes, although more research should be conducted to clarify this association.[105]

The International Agency for Research on Cancer (IARC) has categorized processed meat as a human carcinogen, on the basis of epidemiologic studies of the risk of CRC.[106] However, in a review, Govari and Pexara[24] reported a significant reduction in the nitrite content found in meat products over the past 20 years and concluded that the quantities of residual nitrite present in meat products did not jeopardize individuals' health. This raises the question of total nitrite in meat vs frequency of intake and portion size. Data on risk assessment in more populations are limited.

### Drinking water

Concerns about nitrate arose in the 1940s, when the first report of methemoglobinemia in infants, known as the *blue baby syndrome*, was related to high nitrate concentrations in well water.[31] The nitrate regulatory limit in sources of drinkable water was established to protect newborns from this syndrome. Other health problems, such as cancer and adverse reproductive effects, were not considered.[49]

Methemoglobinemia is the most commonly reported human adverse impact associated with nitrate exposure.[29,44] Methemoglobin is generated through the conversion of nitrate to nitrite via excreted saliva.[44] Specifically, nitrite can react with hemoglobin, leading to biochemical anemia because hemoglobin becomes unable to transporting oxygen to the body.[6] When the methemoglobin concentration approaches ≥10% of normal hemoglobin levels, methemoglobinemia results,[49] producing the blue effect known as cyanosis; at greater concentrations, the result is suffocation.[107] Methemoglobinemia, therefore, is a potentially fatal disease and led to the 0.07 mg/kg bw/d ADI derivation by EFSA. Infants, indeed, are particularly susceptible to methemoglobinemia, but adults may also be at risk if consumed vegetables are inappropriately stored and/or processed and/or contain elevated nitrite as a result of bacterial infectivity and increased activity of endogenous nitrate reductase.[108,109]

Long-term exposure to nitrate found in drinkable water at levels below the European legal levels has been associated with a higher risk of CRC, especially in people with additional risk factors.[65] Despite evidence linking nitrate intake from drinking water, even at concentrations lower than the regulatory limits, to CRC, thyroid disease, and neural tube abnormalities, there is insufficient well-designed research on individual health outcomes to draw solid conclusions.[49] Besides, overall high levels of dietary nitrate ingestion have been associated with hypothyroidism. But when intake from drinking water is also considered, high dietary levels of nitrate also have been linked with risk of thyroid cancer.[77] However, a later study found no link.[67] Breast cancer (BC) was solely linked to waterborne ingested nitrate, not dietary nitrate, among postmenopausal women who consumed a lot of red meat, irrespective of plant or animal source or menopausal status.[74] Drinking water and dietary nitrate were both identified as significant risk causes for bladder cancer (BLC).[63]

### DISCUSSION

The association of nitrate and nitrite in plant foods with good health benefits physiologically, nutritionally, and therapeutically justifies the inclusion of these components as nutrients.[29,110] An inverse association between their ingestion and the threat of coronary heart disease, stroke, CVD, total cancer, and all-cause mortality is supported.[13] Actually, many experts in the field have already issued statements indicating that dietary nitrate should be classified as an essential nutrient.[29,110–112]

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Athletes are using it as an effective performance-enhancing dietary component,[19] and consumers are increasingly accepting nitrate and nitrite as nutritional products when derived from plant sources.[16] Also, although the focus of this review is on human research, in support of the issued statements by the experts in this field, it is important to note that an experimental study using rodents fed nitrate- and nitrite-deficient diets developed hyperglycemia, excessive adiposity, and CVD.[113]

Epidemiological studies correlate dietary nitrite with GC or its subtypes, gastric cardia adenocarcinoma and gastric noncardia adenocarcinoma, but a link between with CRC or its subtypes has also been indicated.[23] No association has been found between dietary nitrate and nitrite and breast, bladder, colon, esophageal, renal cell, ovarian, pancreatic cancers, and non-Hodgkin lymphoma.[114,115] In a meta-analysis conducted in 2015, increased intake of nitrite and N-nitrodimethylamine appeared to be a risk factor for cancer, whereas high nitrate consumption was weakly connected to a lower risk of GC.[116] In addition, concerns have been raised about excessive nitrate levels in drinking water or dietary supplements, because of the possibility of isolated intake without the beneficial dietary components found in vegetables.[117]

The chemical, safety, and regulatory issues around the use of natural nitrate and nitrite for meat preservation have been addressed previously,[28] but finding the perfect alternatives is extremely challenging because of the multifunctional nature of meat processing.[6] Even if no confirmed evidence exists,[91] intakes should be monitored nevertheless, because adverse effects may arise when the ADIs are exceeded.[35] Because of the possible adverse effects and strong pressure from consumers for the manufacturing of meat products that are free of, or contain low levels of, nitrate and nitrite, the specific compounds should be restricted in the meat industry.[6]

On the basis of study findings discussed in this review, there seems to be a positive correlation between nitrate and nitrite present in fruits and vegetables and well-being, especially in terms of lowering BP and improvement of endothelial function, which are directly related to cardiovascular health. However, although the literature suggests a beneficial effect for lowering BP and hypertension has long been recognized as an essential public health objective for lowering CVD incidence, it is becoming increasingly obvious that numerous CVD risk factors must be addressed to appropriately treat this complex disease.[8] The biological functions and applications of dietary nitrate and nitrite need more investigation[30] so we have a better understanding of their long-term significance in preventing CVD

events.[8] No specific risks are reported in relation to the ingestion of nitrate and nitrite from plant sources.

Health risks seem to arise from the ingestion of processed meat or drinking water with an excess of nitrate and nitrite, particularly risks related to the emergence of cancers of the gastrointestinal tract (especially CRC). A clear or positive association between red and processed meat intake and GC was not supported by the epidemiological and clinical studies we reviewed, and caution should be used when interpreting study results, because weak but nonsignificant associations are often stated, although statistical findings are beyond the grey zone ($0.048 < P < 0.59$). These results should be interpreted as indicating no association. Also, the variables used to adjust for when in models differ in most cases, with age and sex being the only factors used by all models (Table S6 in the Supporting Information online). Many studies have not adjusted for total dietary intake or exposure to smoke or smoking status, nor have they all accounted for weight status. These factors are all associated with most health effects examined and linked to nitrate and nitrite exposure.

The IARC has classified nitrate or nitrite (after ingestion) in Group 2A, agents probably carcinogenic to humans.[1] With regard to animal studies, the IARC judged that there is substantial proof indicating nitrite's carcinogenicity when combined with amines or amides. With regard to human data, however, the IARC determined there is limited evidence for nitrite in food being carcinogenic; it has been linked to an increased incidence of GC in humans.[1] Furthermore, according to EFSA, the available information for nitrite does not show genotoxic potential in vivo,[23] and the available data for nitrate do not indicate a genotoxic potential.[44] Nevertheless, carcinogenicity research should focus on population subgroups with a higher risk of endogenous N-nitrosamine production (eg, smokers; persons consuming increased levels of nitrate or nitrite via the consumption of food supplements) if beneficial dietary components like polyphenols or vital nutrients are missing.[117] Research could eventually lead to assessments of broader groups of chemicals that cause endogenous nitrosation or circadian disruptions.[118]

It is impossible to distinguish nitrosamines created from nitrite added at permitted amounts from those found in the food per se.[23] Thus, it is important that a risk-benefit analysis be conducted for these specific substances when they are used as food additives.[25] Research should also address how nitrate via food can promote health in the context of overall nutritional quality, ensuring that consistent health messages are conveyed to the public.[25] Eventually, the risk-benefit equilibrium should be carefully considered before any

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new regulatory or public health recommendations for dietary nitrite and nitrate exposures.[119]

The importance of a concerted effort to develop standards to improve and harmonize the estimation of nitrate and nitrite ingestion should be emphasized.[51] The extent to which a food additive may endanger health is determined not only by its toxicity but also by its dietary intake.[120] According to the results of this review, the combined exposure estimates exceeded the ADI set for nitrite in several studies (Table 1). So, either the ADI established is unsuitable and has to be reconsidered, or those with a high rate of nitrate to nitrite conversion are at risk of nitrite-related deleterious effects.[60] Based on laboratory analyses and/or use levels provided to the EFSA, and using exposure data from 19 European countries (N = 66 025), the estimated nitrate exposure as a food additive did not surpass the ADI of the overall exposure to nitrate in food (it was <5%).[44] Exposure to nitrite salts as food additives did not result in the ADI for the general population being surpassed, with the exception of a minor excess in children at the highest percentile.[23] When all sources of dietary exposures were accounted for, the mean nitrate intake of the assessed population surpassed the ADI for all age categories, including children.[44] In the case of nitrite, the mean ADI was surpassed by infants, toddlers, and school-aged children only; adolescents and adults exceeded the ADI only at the maximum level of intake.[23] Therefore, research should be extended to all population groups—infants, children, adults older than 65 years, pregnant and lactating women, and people following special diets, such as vegetarians and vegans—because greater consumption of nitrate from plants may result in increased nitrite levels.[61] Moreover, the use of a national database on the nitrate and nitrite content of foods, together with valid food frequency questionnaires, could offer a reliable assessment of the targeted populations' dietary intakes.[32] For an assessment of nitrate levels in the body, an investigation of a vast variety of plant-based foods, meats, drinking water, and water-based beverages is required.[39] Methodologies for determining nitrate amounts in different foods, which would also take into consideration processing parameters that may affect the nitrate content, such as cooking and preservation procedures, are required.[50] In addition, more studies involving improved exposure assessment for populations connected to public water supplies, nitrate-reducing bacterium quantification, and evaluation of dietary and other variables that affect nitrosation are needed.[49]

Finally, changes in diet toward a more Western type, due to developing economies, known as nutrition transition, is associated with a decreased consumption of plant-based foods and a higher consumption of animal products.[121] This phenomenon may project a decrease in plant-based nitrate and nitrite intake,[24,51] with potentially adverse consequences. Therefore, it is critical to find ways to minimize this transition and promote a healthier, less-processed diet with emphasis on more nutrient-dense, nitrate-rich, plant-based foods.[121,122]

## CONCLUSION

Dietary nitrate and nitrite are plant nutrients, authorized food additives, and components of foodstuffs and drinking water and dietary supplements. There is no confirmation of a link between carcinogenicity and dietary exposure to nitrate and nitrite from fruit and vegetables. Research appears to support the health benefits of nitrate intake from vegetables, particularly because of the association with an improved endothelial function and a reduction in BP, and thus better vascular and metabolic health. However, recent clinical studies indicate that a high nitrate diet may be less beneficial in patients with hypertension. On the contrary, a considerable number of studies show a link between processed food nitrate and nitrite and an elevated risk of cancer, particularly in the gastrointestinal tract. Furthermore, red and/or processed meat intake increases cancer risk. The importance of drinking water as a risk factor for negative health outcomes has also been highlighted. The lack of coherence in exposure assessment among studies necessitates more prospective research to validate the link between dietary nitrate, nitrite, and nitrosamines with human health risks and benefits. Gaps seem to remain concerning the safety of the European population's exposure to nitrate and nitrite in food, particularly through processed meat consumption. More national data need to be gathered and the exposure assessment scenario refined for nitrate and nitrite intake to ensure that ADIs are not exceeded.

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publication and they do not necessarily represent the decisions, policy, or views of the Hellenic Food Authority.

## Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

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# SUPPORTING INFORMATION

*Table S1*: **Studies reviewed per type (Reference, Country [a])**

| Dietary exposure assessment studies N=21 | | | Epidemiological observational studies N=22 | | | Cross-over studies N=14 | Meta-analyses N=4 | Systematic Reviews N=4 | Reviews N=16 | Re-analyses N=1 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Combined dietary intake N=13** | **Intake from processed products N=5** | **Intake from vegetarian diet N=3** | **Cohort N=15** | **Case-control N=4** | **Cross-sectional N=3** | | | | | |
| van den Brand et al. (2020)[S1], Netherlands | Lee et al. (2018)[S2], US | Mitek et al. (2013)[S3], Poland | Jones et al. (2019)[S4], USA | Barry et al. (2020)[S5], USA | Gopinath et al. (2020)[S6], Australia | Sunqvist et al. (2020)[S7], Sweden | Yu et al. (2020)[S8] | Babateen et al. (2018)[S9] | Flores et al. (2020)[S10] | Gonzalez et al. (2012)[S11] |
| Elias et al. (2020)[S12], Estonia | Adam et al. (2017)[S13], Sudan | Brkic et al. (2017)[S14] Croatia, | Inoue-Choi et al. (2015)[S15], USA | Espejo-Herrera et al. (2016)[S16], Spain/Italy | Jonvik et al. (2017)[S17], Netherlands | Cherukuri et al. (2020)[S18], USA | Xie et al. (2016)[S19] | Jackson et al. (2018)[S20(b)] | Karwowska et al. (2020)[S21] | |
| Vlachou et al. (2020)[S22], Austria | Merino et al. (2016)[S23], Sweden | Tamme et al. (2006)[S24] Estonia | Dellavalle et al. (2014)[S25], China | Espejo-Herrera et al. (2016)[S26], Spain | Ferrucci et al. (2009)[S27], USA | Smeets et al. (2020)[S28], Netherlands | Song et al. (2015)[S29] | Aune et al. (2017)S[30(b)] | Bahadoran et al. (2019)[S31] | |
| Chetty et al. (2019)[S32], Fiji | Vin et al. (2013)[S33], UK/Ireland/ Italy/France | | Keszei et al. (2014)[S34], Netherlands | Preston-Martin et al. (1996)[S35], USA | | Blekkenhorst et al. (2018)[S36], Australia | Larsson et al. (2006)[S37] | Blanc et al. (2015)[S38] | Kalaycıoğlu et al. (2019)[S39] | |
| Bahadoran et al. (2019)[S31], Iran | Temme et al. (2011)[S40] Belgium | | Dellavalle et al. (2013)[S41], USA | | | Kerley et al. (2018)[S42], Ireland | | | Lundberg et al. (2018)[S43] | |

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| Dietary exposure assessment studies N=21 | | | Epidemiological observational studies N=22 | | | Cross-over studies N=14 | Meta-analyses N=4 | Systematic Reviews N=4 | Reviews N=16 | Re-analyses N=1 |
|---|---|---|---|---|---|---|---|---|---|---|
| Combined dietary intake N=13 | Intake from processed products N=5 | Intake from vegetarian diet N=3 | Cohort N=15 | Case-control N=4 | Cross-sectional N=3 | | | | | |
| Roila et al. (2018)[S44], Italy | | | Keszei et al. (2013)[S45], Netherlands | | | Asgary et al. (2016)[S46], Iran | | | Govari et al. (2018)[S47] | |
| Jackson et al. (2018)[S20], Australia | | | Aschebrook Kilfoy et al. (2013)[S48], China | | | Velmurugan et al. (2016)[S49], UK | | | Ma et al. (2018)[S50] | |
| Suomi et al. (2016)[S51], Finland | | | Aschebrook Kilfoy et al., (2012)[S52], USA | | | Ashworth et al. (2015)[S53], UK | | | Ward et al. (2018)[S54] | |
| Mancini et al. (2015)[S55], France | | | Loh et al. (2011)[S56], UK | | | Bondonno et al. (2014)[S57], Australia | | | Nuijic et al. (2017)[S58] | |
| Anyzewska et al. (2014)[S59], Poland | | | Cross et al. (2011)[S60], USA | | | Larsen et al. (2011)[S61], Sweden | | | Habermeyer et al. (2015)[S62] | |
| Larsson et al. (2011)[S63], Sweden | | | Aschebrook Kilfoy et al. (2011)[S64], US | | | Larsen et al. (2010)[S65], Sweden | | | Jones AM. (2014)[S66] | |

| Dietary exposure assessment studies N=21 | | | Epidemiological observational studies N=22 | | | Cross-over studies N=14 | Meta-analyses N=4 | Systematic Reviews N=4 | Reviews N=16 | Re-analyses N=1 |
|---|---|---|---|---|---|---|---|---|---|---|
| Combined dietary intake N=13 | Intake from processed products N=5 | Intake from vegetarian diet N=3 | Cohort N=15 | Case-control N=4 | Cross-sectional N=3 | | | | | |
| Menard et al. (2008)[S67], France | | | Ward et al. (2010)[S68], USA | | | Larsen et al. (2006)[S69], Sweden | | | Bradbury et al. (2014)[S70] | |
| Thomson et al. (2007)[S71], New Zealand | | | Dubrow et al. (2010)[S72], USA | | | Svetkey et al. (1999)[S73], USA | | | Weitzberg et al. (2013)[S74] | |
| | | | Knekt et al. (1999)[S75], Finland | | | Appel et al. (1997)[S76], USA | | | Hobbs et al. (2013)[S77] | |
| | | | van Loon et al. (1998)[S78], Netherlands | | | | | | Machha et al. (2012)[S79] | |
| | | | | | | | | | Cogliano et al. (2011)[S80] | |

[a] Country is stated only for the dietary exposure assessment, epidemiological and cross-over studies (not indicated for Reviews, Systematic Reviews, Meta-analyses and re-analyses)

[b] Systematic review and meta-analysis.

*Table S2*: **Characteristics of cohort studies**

| Country, Reference | Compound | Source | Outcome | Cases/Cohort, n | Age | Sex (M/F), n | Duration, y | Study population | Dietary assessment |
|---|---|---|---|---|---|---|---|---|---|
| USA, Jones et al. (2019)[S4] | Nitrate, Nitrite | Red meat | CRC | 624 colon & 158 rectal cancers/ 15910 | 55-69 | All F | 1 | Iowa Women's Health Study (IWHS) | 126-item validated FFQ |
| USA, Inoue-Choi et al. (2015)[S15] | Nitrate, Nitrite | Processed meat | OVC | 315/17216 | 55-69 | All F | 1 | Iowa Women's Health Study (IWHS) | 126-item validated FFQ |
| China, Dellavalle et al. (2014)[S25] | Nitrate, Nitrite | Diet | CRC | 619/73118 | 40-70 | All F | 11 | Shanghai Women's Health Study (SWHS) | 77-item FFQ |
| Netherlands, Keszei et al. (2014)[S34] | Nitrate | Vegetables, fruit, drinking water | BE | 433/3717 | 55-69 | 1833/1884 | 16,3 | Netherlands Cohort Study (NLCS) | 150-item FFQ |
| USA, Dellavalle et al. (2013)[S41] | Nitrate, Nitrite | Animal sources | RCC | 1816/491841 | 50-71 | 293248/198593 | 9 | NIH-AARP Diet and Health Study | 124-item validated FFQ |
| Netherlands, Keszei et al. (2013)[S45] | Nitrate, Nitrite, NDMA | Diet | ESCC EAC GCA GNCA | 110/4032151/40 32166/4032 497/4032 | 55-69 | 1947/2085 | 16,3 | Netherlands Cohort Study (NLCS) | 150-item FFQ |
| China, Aschebrook Kilfoy et al. (2013)[S48] | Nitrate, Nitrite | Animal sources, esp. processed meat | TC | 164/73317 | 40-70 | All F | 11 | Shanghai Women's Health Study (SWHS) | 77-item FFQ |
| USA, Aschebrook Kilfoy et al. (2012)[S52] | Nitrate, Nitrite | Diet | Epithelial OVC | 709/151316 | 50–71 | All F | 10 | NIH-AARP Diet and Health Study | 124-item validated FFQ |

| Country, Reference | Compound | Source | Outcome | Cases/Cohort, n | Age | Sex (M/F), n | Duration, y | Study population | Dietary assessment |
|---|---|---|---|---|---|---|---|---|---|
| UK, Loh et al. (2011)[S56] | NOCs, NDMA, Nitrite | Diet | Cancers | 3268/23363 | 40-79 | 10783/12580 | 11,4 | EPIC-Norfolk study | FFQ[(a)] |
| USA, Cross et al. (2011)[S60] | NOCs | Red meat | ESCC<br>EAC<br>GCA<br>GNCA | 215/494979<br>630/494979<br>454/494979<br>501/494979 | 50-71 | 295305/199674 | 10 | NIH-AARP Diet and Health study | 124-item validated FFQ |
| US, Aschebrook Kilfoy et al. (2011)[S64] | Nitrate, Nitrite, NOCs | Processed meat | PC | 1728/492226 | 50-71 | 293491/198735 | 10 | NIH-AARP Diet and Health Study | 124-item validated FFQ |
| USA, Ward et al. (2010)[S68] | Nitate | Diet, drinking water | TC | 45/20651 | 61 (mean) | All F | 19 | Iowa Women's Health Study (IWHS) | 126-item validated FFQ[1] |
| USA, Dubrow et al. (2010)[S72] | NOCs | Processed/red meat, nitrite, nitrate fruit/vegetable | Glioma | 585/545770 | 12-13, 50-71 | 322347/223423 | 1 | NIH-AARP Diet and Health Study | 124-item validated FFQ |
| Finland, Knekt et al. (1999)[S75] | Nitrate, Nitrite, NDMA | Diet | GI | 189/9985 | 15-99 | 5274/4711 | 24 | Finnish Mobile Clinic Health Examination Survey | 1-year dietary history interview |
| Netherlands, van Loon et al. (1998)[S78] | Nitrate, Nitrite | Diet | GC | 282/3123 | 55-69 | 1525/1598 | 6,3 | Netherlands Cohort Study (NLCS) | 150-item FFQ |

*Abbreviations:* ESCC-esophageal squamous cell carcinoma, GCA-gastric cardia adenocarcinoma, EAC-esophageal adenocarcinoma, GNCA-gastric noncardia adenocarcinoma, BE-Barret's oesophagus, NOCs-N-nitroso compounds, NDMA-N-nitrosodimethylamine, GC-Gastric Cancer, GI-Gastrointestinal Cancer, OVC-Ovarian Cancer, TC-Thyroid Cancer, EC-Esophageal cancer, PC-Pancreatic cancer, RCC-Renal Cell Carcinoma, BLC-Bladder cancer, BT-Brain Tumors, BP-Blood pressure, HTN-Hypertension, CRC-Colorectal Cancer, FFQ-Food Frequency Questionnaire

*Table S3:* **Characteristics of case-control studies**

| Country, Reference | Compound | Source | Outcome | Cases/Controls, n/n | Age | Sex (M/F), n/n | Duration, y | Study population | Dietary assessment |
|---|---|---|---|---|---|---|---|---|---|
| US (Northern New England), Barry et al. (2020)[S5] | Nitrate, Nitrite | Diet and drinking water | BLC | 987/1180 (drinking water) 1037/1225 (dietary) | 30-79 | 863/317 339/928 | 5 | New England Bladder Cancer Study (NEBCS) | 124-item dietay history questionnaire |
| Spain, Italy, Espejo-Herrera et al. (2016)[S26] | Nitrate | Diet and drinking water | CRC | 1869/3530 | 20-85 | 4190/3051 | 6 | Hospital-based incident BC cases and population-based controls | Validated 140-item FFQ |
| Spain, Espejo-Herrera (2016)[S16] | Nitrate | Diet and drinking water | BC | 1245/1520 | 58 (mean) | Al F | 6 | Hospital-based incident cases and population based (Spain) or hospital-based (Italy) controls | Validated 140-item FFQ[1] |
| US, Preston-Martin et al. (1996)[S35] | N-nitroso compounds | Maternall diet | BT | 540/801 | <20 | 298/242 | 7 | U.S. West Coast CBT study | Dietary recall and abstract food models |

*Abbreviations:* CRC-Colorectal Cancer, BLC-Bladder cancer, BT-Brain Tumors, BP-Blood pressure. FFQ-Food Frequency Questionnaire

*Table S4:* **Characteristics of cross-sectional studies**

| Country Reference | Health status | Outcome | Source | Sample, n | Age | Sex (M/F) | Study population | Dietary assessment |
|---|---|---|---|---|---|---|---|---|
| Australia, Gopinath et al. (2020)[S6] | Healthy | Retinal arteriolar and venular caliber | Diet (vegetables and non-vegetables) | 2813 | >49 | - | Blue Mountains Eye Study (BMES) | 145-item self-administered FFQ |
| Netherlands, Jonvik et al. (2017)[S17] | Healthy (Athletes) | Energy | Diet | 553 | NR | 226/237 | Dutch Sport Nutrition and Supplement Study (DSSS) (2012-2015) | 2-4 unannounced web-based (Compl-eatTM) 24-h dietary recalls and dietary supplement questionnaires |
| US, Ferrucci et al. (2009)[S27] | Not healthy Healthy | Colorectal adenoma | Meat | 158 CA 649 | 50-79 | All F | CONCeRN (COlorectal Neoplasia screening with Colonoscopy in asymptomatic women at Regional Navy/army medical centers) study | 124-item DHQ |

*Abbreviations:* FFQ-Food Frequency Questionnaire, NR-Not Reported, CA-Colorectal Adenoma, DHQ-Diet History Questionnaire

*Table S5:* **Characteristics of cross-over studies**

| Country Reference | Compound | Source | Outcome | Sample, n | Age | Sex (M/F) | Duration/Scheme/Phases |
|---|---|---|---|---|---|---|---|
| Sweden, Sundqvist et al. (2020)[S7] | Nitrate | Leafy green vegetable | BP | 231 (SBP 130-159mmHg) | 50-70 | - | 2w: nitrate restricted diet & 5 wk:1 of the following 3 interventions daily low nitrate vegetables + placebo pills, ow-nitrate vegetables + nitrate pills (300 mg nitrate), or leafy green vegetables containing 300 mg nitrate + placebo pills |
| USA, Cherukuri et al. (2020)[S18] | Nitrate | Beetroot extract | Endothelial function, BP | 67 (SBP and DBP>120 and 80 mmHg) | 40-75 | 26/41 | Daily dosing of 314 mM inorganic NO3 tablets or placebo. Duration of administration of study drug was 12 wks. Participants return at 2 wks and 12 wks to assess for any side effects. |
| Netherlands, Smeets et al. (2020)[S28] | Nitrate | Beetroot powder | Endothelial function | 18 (healthy abdominally obese) | 40-70 | 18 | 5d tests, each separated by a wash-out period of at least one week: blended meal with a control or nutritional supplement consisting of beetroot powder providing 200 mg nitrate, beetroot with 0.8 g of L-arginine, beetroot with 1.5 g of L-arginine, or 3.0 g of L-arginine. |
| Australia, Blekkenhorst et al. (2018)[S36] | Nitrate | Nitrate rich vegetables | BP | 30 (pre-hypertension or untreated grade 1 hypertension) | 55-70.5 (mean 63) | 20/10 | 4-week treatment periods separated by 4-week washout periods. Participants completed three treatments in random order: (1) increased intake (~200 g/d) of nitrate-rich vegetables (high nitrate, (HN), ~150 mg/d nitrate) (2) increased intake (~200 g/d) of nitrate-poor vegetables (low nitrate, |

| Country Reference | Compound | Source | Outcome | Sample, n | Age | Sex (M/F) | Duration/Scheme/Phases |
|---|---|---|---|---|---|---|---|
| | | | | | | | (LN), ~22 mg/d nitrate); and (3) no increase in vegetables (control, (C), ~6 mg/d nitrate). |
| Ireland, Kerley et al. (2018)[S42] | Nitrate | Beetroot juice | HTN uncontrolled | 20 | 62.5 (mean) | - | Daily nitrate compared with placebo in subjects. 7-d, double-blind, randomised, placebo (PL)-controlled, cross-over trial to assess the effect of dietary nitrate. Subjects were tested on three separate occasions – baseline (day 1), midpoint (day 8) and endpoint (day 15) – before and after each intervention period |
| UK, Velmurugan et al. (2016)[S49] | Nitrate | Beetroot juice | Vascular function | 69 (otherwise healthy hypercholesterolemic men and women with BMI (in kg/m2) from 18.5 to 40 | 18–80 | - | 6-wk once-daily intake of dietary nitrate compared with placebo intake (250mL nitrate-depleted beetroot juice) |
| Iran, Asgary et al. (2016)[S46] | Nitrate | Raw beetroot juice (RBJ) & Cooked beet (CB) | Endothelial function BP Systemic inflammation | 24 (SBP 130–139 mm Hg or DBP 85–89 mm Hg) | 25-68 | | 250 ml day− 1 of RBJ or 250 g day− 1 of CB each for a period of 2 weeks, followed by a 2-week washout period. |
| UK, Ashworth et al. (2015)[S53] | Nitrate | HNvegetable (green leafy vegetables) | BP | 19 | 20 | All F | HN vegetables (HN diet) or avoided HN vegetables (Control diet) for 1 week. |
| Australia Bondonno et al, (2014)[S57] | Nitrate | Green leafy vegetables | BP & arterial stiffness | 38 (High normal BP, | 30-70 | - | A 7 day high nitrate diet intervention (increased nitrate intake by at least 300 mg/day from |

| Country Reference | Compound | Source | Outcome | Sample, n | Age | Sex (M/F) | Duration/Scheme/Phases |
|---|---|---|---|---|---|---|---|
| | | | | SBP 120 to 139 mmHg | | | green leafy vegetables) were compared to a 7 day low nitrate diet intervention |
| Sweden, Larsen et al. (2011)[S61] | Nitrate | Dietary supplementation with sodium nitrate or an equivalent amount of sodium chloride (placebo) | Basal mitochondrial function & whole-body oxygen consumption | 14 healthy, nonsmoking subjects were included | $25 \pm 1$ years | M | 3 days prior to experiments, with a washout period of at least 6 days between tests |
| Sweden, Larsen et al. (2010)[S65] | Nitrate | Dietary supplementation with sodium nitrate or placebo (NaCl). | oxygen consumption | 9 healthy, nonsmoking volunteers | $30 \pm 2.3$ | | They received a dose corresponding to the amount found in 100–300g of a nitrate-rich vegetable such as spinach or beetroot 2 days before the test. |
| Sweden, Larsen et al. (2006)[S69] | Nitrate | Dietary supplementation (same as nitrate-rich vegetable such as spinach, beetroot, or lettuce) | BP | 17 (physically active, healthy volunteers, none of whom smoked) | 24 (mean) | 15/2 | 3-day dietary supplementation with either sodium nitrate (at a dose of 0.1 mmol per kilogram of body weight per day) or placebo (sodium chloride, at a dose of 0.1 mmol per kilogram per day) two different treatment periods during which the subjects received either nitrate or placebo; the treatment periods were separated by a washout period of at least 10 days |
| US, Svetkey, (1999)[S73] | Nitrate | DASH DIET[(b)] | BP (Untreated systolic BP <160mmHg | 459 | >22 | | Three phases (screening, run-in, and intervention). 3w: a control diet. Randomized to 8w of (1) control diet, (2) a diet rich in fruit |

| Country Reference | Compound | Source | Outcome | Sample, n | Age | Sex (M/F) | Duration/Scheme/Phases |
|---|---|---|---|---|---|---|---|
| | | | and diastolic BP 80-95mHg) | | | | and vegetables or 3) a combination diet rich in fruits, vegetables and low-fat dairy foods and reduced in saturated fat, total fat and cholesterol (the DASH combination diet). Weight and salt constant. |
| US, Appel (1197)[S76] | Nitrate | DASH DIET | BP SBP<160 mm Hg and BP 80- 95 mm Hg. | 17 | >22 | 15/2 | Three phases (screening, run-in, and intervention). 3w a control diet that was low in fruits, vegetables, and dairy products, with a fat content typical of the average diet in the United States. Randomly to 8w the control diet, a diet rich in fruits and vegetables, or a "combination" diet rich in fruits, vegetables, and low-fat dairy products and with reduced saturated and total fat. Sodium intake and body weight were maintained at constant levels. |

*Abbreviations:* SBP-Systolic Blood Pressure, DBP-Diastolic Blood Pressure, BP-Blood Pressure, HTN-Hypertension, HN-High Nitrate, DASH-Diet Approach to Stop Hypertension

*Table S6:* **Covariates included in statistical analyses in cohort studies[(a)]**

| Country, First author, y | Health Impact | Nitrates/Nitrites | | Source | Adjusted covariates |
|---|---|---|---|---|---|
| | | **Positive** | **Null /Negative** | | |
| USA, Jones et al. (2019)[S4] | CRC (Colon Cancer) | Red meat | Drinking water | Animal Sources | Age, heme iron, red meat, and mutually adjusted for total dietary nitrate or nitrite (processed meats: not mutually adjusted for nitrate from processed meat sources due to high correlation) |
| | | | | Drinking Water | Age, physical activity, smoking status, mutually adjusted for NO3-N or TTHM |
| USA, Inoue-Choi et al. (2015)[S15] | OVC | Processed meat, Drinking water | Plant Sources, Other animal sources than processed meat | Vegetables/Fruits, Animal Sources | BMI, family history of ovarian cancer, number of live births, age at menarche, age at menopause, age at first live birth, oral contraceptive use, estrogen use, and a history of unilateral oophorectomy, additionally adjusted for logarithmically transformed values of cruciferous vegetable and red meat intake |
| | | | | Drinking Water | BMI, family history of ovarian cancer, number of live births, age at menarche, age at menopause, age at first live birth, oral contraceptive use, estrogen use, and a history of unilateral oophorectomy, additionally mutually adjusted for logarithmically transformed values of NO3-N or TTHMs levels in public water |
| China, Dellavalle et al. (2014)[S25] | CRC | Preserved Foods (preserved vegetables-colon cancer) | Plant Sources, Animal Sources, Red meat | Vegetables/Fruits, Animal Sources | Age, energy intake, education, physical activity, dietary vitamin C intake, carotene and folate |
| Netherlands, Keszei et al. (2014)[S34] | BE | Vegetables, inverse association (beneficial) only among men (not | | Vegetables/Fruits | Age, smoking status, duration of cigarette smoking, number of cigarettes smoked per day, total energy intake, BMI, alcohol intake, levels of education, non- |

| Country, First author, y | Health Impact | Nitrates/Nitrites | | Source | Adjusted covariates |
|---|---|---|---|---|---|
| | | **Positive** | **Null /Negative** | | |
| | | from fruit consumption) | | | occupational physical activity and use of lower oesophageal sphincter-relaxing medications |
| USA, Dellavalle et al. (2013)[S41] | RCC | Animal sources | Plant sources | Vegetables/Fruits, Animal Sources | Age, sex, caloric intake, race, smoking status, family history of cancer, BMI, alcohol intake, education, history of hypertension, history of diabetes |
| China, Aschebrook Kilfoy et al. (2013)[S48] | TC | Animal sources (esp. processed meat) | Plant sources | Vegetables/Fruits, Animal Sources | Age, total energy intake, education, history of thyroid disease, vitamin C, carotene, and folate intake |
| USA, Aschebrook Kilfoy et al. (2012)[S52] | Epithelial OVC | Animal sources (no association with processed meats) | Plant sources | Vegetables/Fruits, Animal Sources | Age, cigarette smoking status, race, family history of cancer, BMI, menopausal status at baseline, parity, age at menarche, and total daily dietary vitamin C intake |
| USA, Cross et al. (2011)[S60] | ESCC EAC GCA GNCA | Red meat intake with ESCC, EAC | | Animal Sources | Age, sex, BMI, education, ethnicity, tobacco smoking, alcohol drinking, usual physical activity at work, vigorous physical activity, and the daily intake of fruit, vegetables, saturated fat, and calories |
| US, Aschebrook Kilfoy et al. (2011)[S64] | PC | | Plant sources, animal sources | Vegetables/Fruits, Animal Sources | Age, race, total energy intake, smoking status, family history of cancer, family history of diabetes, body mass index, and intakes of saturated fat, folate, and vitamin C |
| USA, Ward (2010)[S68] | TC | Public water supplies | | Drinking water | Age, vitamin C intake, and residence location |
| USA, Dubrow et al. (2010)[S72] | Adult Glioma | | Fruit and vegetable, processed meat, red meat | Vegetables/Fruits, Animal Sources | Sex, age, race, energy intake, education, height, and history of cancer at baseline |

| Country, First author, y | Health Impact | Nitrates/Nitrites | | Source | Adjusted covariates |
|---|---|---|---|---|---|
| | | Positive | Null /Negative | | |
| Finland, Knekt et al. (1999)[S75] | GI | Smoked and salted fish | Vegetables, fruits | Vegetables/Fruits | Data not shown |
| | | | | Animal Sources | Sex, age, municipality, smoking and energy intake |
| Netherlands, van Loon et al. (1998)[S78] | GC | | Drinking water, foods | Drinking water | Age, sex, smoking, highest level of education, coffee consumption, intake of vitamin C and beta-carotene, family history of stomach cancer, prevalence of stomach disorders, use of refrigerator and use of freezer |

[a] ESCC-esophageal squamous cell carcinoma, GCA-gastric cardia adenocarcinoma, EAC-esophageal adenocarcinoma, GNCA-gastric noncardia adenocarcinoma, BE-Barret's oesophagus, NOCs-N-nitroso compounds, FFQ-Food Frequency Questionnaire, NDMA-N-nitrosodimethylamine, GC-Gastric Cancer, GI-Gastrointestinal Cancer, OVC-Ovarian Cancer, TC-Thyroid Cancer, EC-Esophageal cancer, PC-Pancreatic cancer, RCC-Renal Cell Carcinoma, BLC-Bladder cancer, BT-Brain Tumors, BP-Blood pressure, HTN-Hypertension, CRC-Colorectal Cancer, BMI-Body Mass Index, TTHM- Total trihalomethanes

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# 3. THESIS OVERVIEW

## 3.1. RESEARCH GAPS

Further review of the evidence regarding the impact of nitrite and nitrate intake by source of origin was required (details in section 1.6).

Nitrite and nitrate intakes, mainly from processed meat products, have already been assessed in several countries [1–9] and at EU level [10,11]. However no such research has been conducted to date for Greek consumers, hence severe concerns remain about the relevant safety of Greek populace. The results of such a research could be of great value in order to guide consumer choices, set and prioritize dietary guidelines, and inform and support competent authorities with food reformulations to reduce risks [12].

Furthermore, the relationship of processed meat ingredients with key CVD risk factors, such as hypertension, remains unclear [13], although its understanding is crucial for public health. Consequently, this relationship needs to be further investigated.

## 3.2. SCOPE OF THE PhD THESIS

The current PhD thesis sought to identify gaps in the literature and research pertaining to nitrite and nitrate and subsequently, to evaluate the potential health effects associated with dietary nitrite and nitrate intake from processed meat in the Greek population.

## 3.3. RESEARCH DEVELOPMENT

The present study progressively established the following set of research inquiries and objectives to encompass multiple topics, including the scientific and regulatory framework, as well as the approach that ought to be adopted for conducting the risk assessment (RA) in light of factors such as data accessibility, time constraints, and resource restrictions.

### 3.3.1. Research Questions

1. What are the gaps in current research related to dietary nitrite and nitrate?

2. What are the data and tools available?

3. Is cumulative assessment possible?

4. What are other challenges pertaining to data for RA and tools employed?

5. What is the overall and age group specific risk of ingesting nitrite and nitrate among Greeks?

6. Are there any health associations that need to be further investigated?

7. How does our research contribute in bridging gaps indentified?

8. Is there an association between high intake of nitrite and nitrate (expressed as nitrite) with CVD risk factors?

9. Are there any confounding factors that need to be explored?

10. What are the major findings?

11. What are the differences or/and similarities with other studies on the subject?

12. What are the strengths and limitations of our studies?

13. Can we reach solid conclusions?

14. What are the key future challenges?

15. Could our findings be disseminated to inform public and authorities? How?

### 3.3.2. Objectives of PhD thesis

Following the findings of the extensive literature review and having explored data availability, considering also the challenging COVID-19 pandemy condition, we opted to conduct a health risk assessment of nitrite and nitrate used as additives in processed meat products. Our decision was dictated by the nutrition transition towards increased processed meat consumption and after taking into consideration that, despite its importance for public health, given that it has been linked with carcinogenicity in humans, such an evaluation had not been conducted before among Greeks. Consequetively, the second objective of this thesis was to assess the risk of processed meat and meat products nitrite and nitrate intake among Greeks.

Furthermore, although the outcomes of our literature review did not reveal a positive association between nitrite and nitrate from processed meat with CVD, the association

between processed meat consumption and CVD risk led us to investigate further this relationship, which remained a topic that had been inadequately studied internationally. Therefore, the third objective in this thesis was to investigate the association of dietary nitrite and nitrate from processed meat and meat products with BP.

### 3.3.3. THESIS FLOWCHART
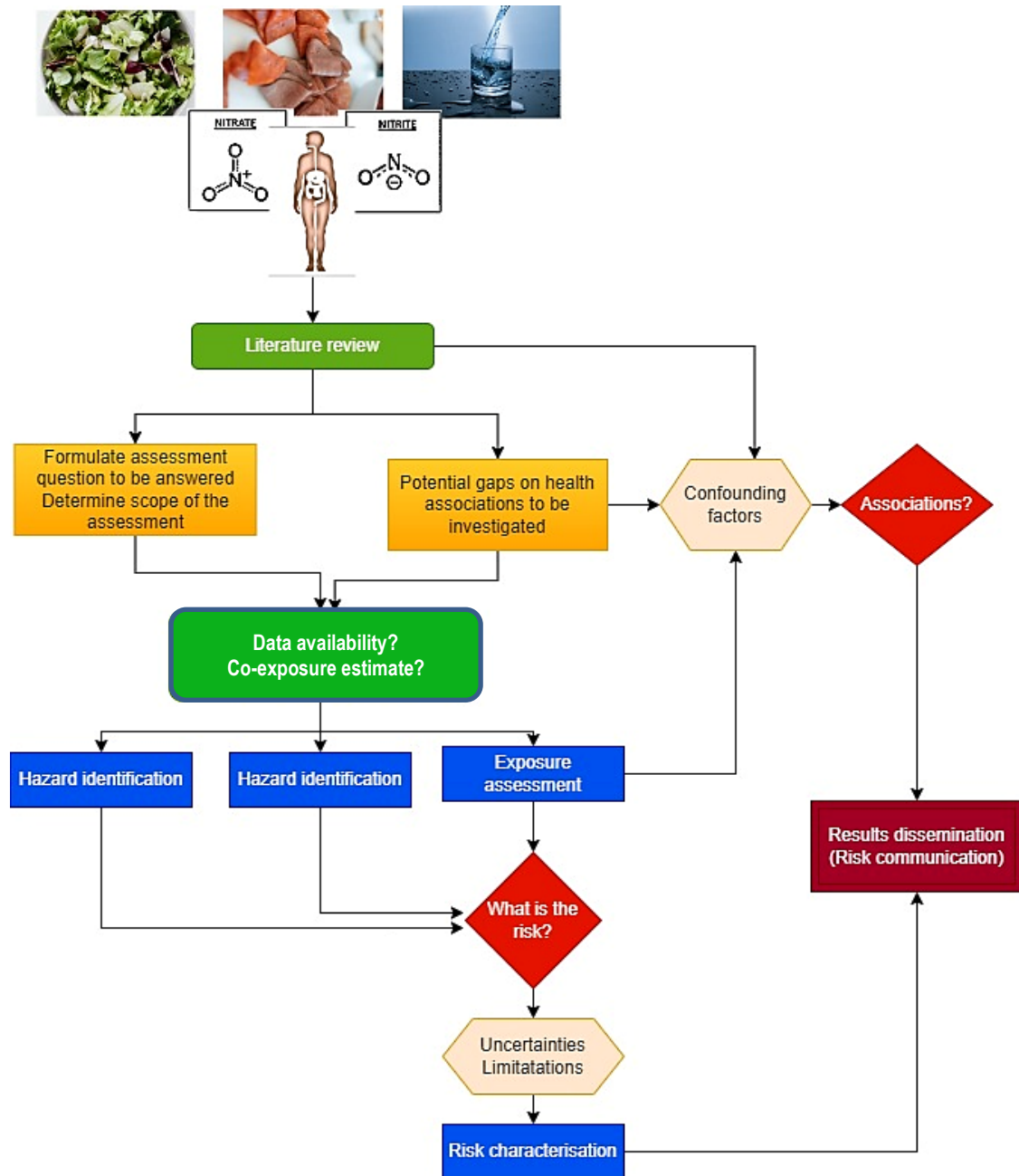
## 3.4. AIMS AND SPECIFIC OBJECTIVES BY PAPER IN SUPPORT TO PhD THESIS

**Paper 1:** The first aim of the current PhD thesis was to review the evidence on the health risks and benefits of dietary nitrate and nitrite, focusing on plant versus animal sources and water (as already set in section 1.7).

**Paper 2:** To (a) estimate the daily nitrite and nitrate intake from processed meat products consumption (b) assess the potential risk of exceeding the ADI and (c) identify the major contributors, all in total and by age group, using a nationally representative sample.

**Paper 3:** To examine the possible relation of dietary nitrite and nitrate intake from processed meat to BP, adjusting for major confounding variables in CVD causation, such as sodium intake.

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# B. PRESENTATION OF PAPERS II & III PERTAINING TO GAPS OBSERVED IN THE LITERATURE REVIEW

# PAPER II ▪ RISK ASSESSMENT OF NITRITE AND NITRATE INTAKE FROM PROCESSED MEAT PRODUCTS: RESULTS FROM THE HELLENIC NATIONAL NUTRITION AND HEALTH SURVEY (HNNHS)

## Graphical Abstract
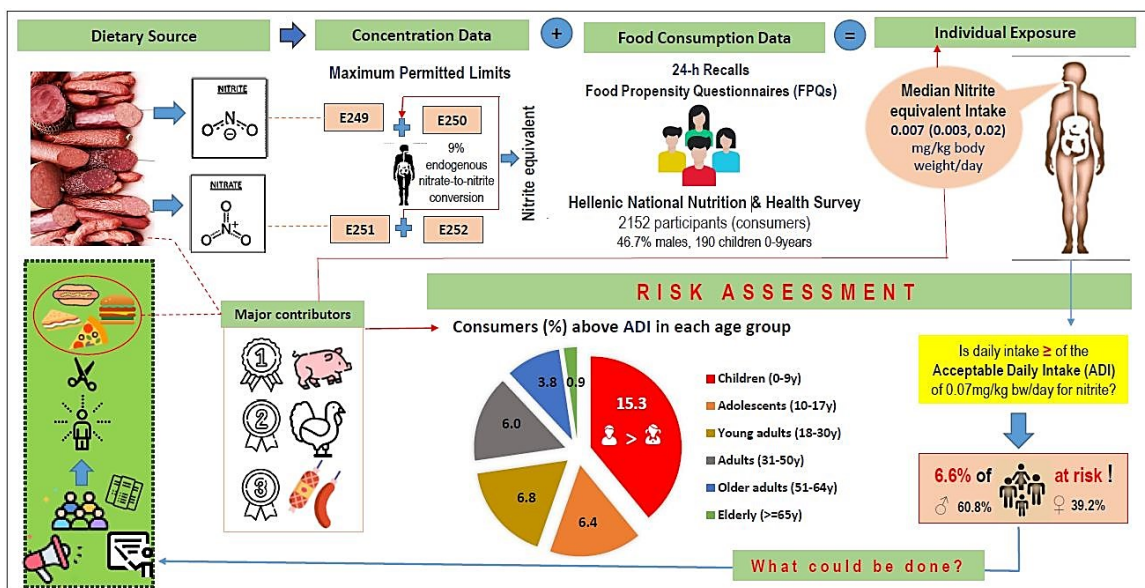
*Article*

# Risk Assessment of Nitrite and Nitrate Intake from Processed Meat Products: Results from the Hellenic National Nutrition and Health Survey (HNNHS)

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**Abstract:** Long-term exposure to a high nitrite and nitrate intake through processed meat is of concern, as it has been related to adverse health effects. Individual consumption data from 2152 participants (46.7% males) in the Hellenic National Nutrition and Health Survey (HNNHS) were linked with current Maximum Permitted Levels (MPLs) to calculate exposure to nitrite and nitrate from processed meat products (assessed as nitrite equivalent), evaluate potential risk and identify the major contributors. Processed meat intakes were determined by combining data from 24 h recalls and frequency of consumption reported in Food Propensity Questionnaires (FPQs). Median exposure was estimated to be within safe levels for all population groups. However, 6.6% ($n = 143$) of the consumers exceeded the Acceptable Daily Intake (ADI) of nitrite (0.07 mg/kg bw/day), of which 20.3% were children aged 0–9 years (N = 29) (15.3% of all children participants in the study, N = 190). In total, pork meat was the major contributor (41.5%), followed by turkey meat (32.7%) and sausages (23.8%), although contribution variations were found among age groups. The outcomes are of public health concern, especially exposure among children, and future research is warranted to evaluate possible associations with health effects, by using more refined occurrence data if available.

**Keywords:** nitric compounds; food additives; MPLs; dietary exposure; dietary intake; risk; meat consumption; processing; food group contribution; Greece

## 1. Introduction

Processed meat consumption has been linked with human carcinogenesis [1], based primarily on epidemiologic studies evaluating risk factors of colorectal cancer (CRC). Recommendations from the World Health Organization (WHO) [2], implemented also in Greece [3], suggest limiting or avoiding consumption, since no safe intake has been established. Specifically, processed meat is any meat that has been flavored or preserved by salting, curing, fermenting, smoking, or other techniques [4]. It may contain a high concentration of nitrite (potassium nitrite-E249 and sodium nitrite-E250) and nitrate (sodium nitrate-E251 and potassium nitrate-E252), both of which are legally and widely used food additives in the European Union [5], to fix color, limit microbial growth (particularly of Clostridium botulinum), or develop a distinguishable flavor [6]. However, controversies regarding the healthiness of nitrite and nitrate have been reported and their consumption should therefore be separately monitored from red meat. The hazard of dietary nitrite and nitrate for human health has previously been thoroughly reviewed [7] and an increased risk of cancers, especially of the gastrointestinal tract, when ingested from processed meat has been suggested. This is principally attributable to the production of genotoxic N-nitroso compounds (NOCs), nitrosamines, and nitrosamides under the proper conditions (pH, reactant concentration), as well as the lack of nitrosation inhibitors such as vitamin C [1]. Legislative Maximum Permitted Levels (MPLs) for the use of nitrite and nitrate in processed

meat products have been set [5] to ensure that the desired effect of the product is obtained without exceeding safe levels for human health.

Nutrition transition, linked with lifestyle changes seen in recent decades, has been characterized by a decrease in plant foods consumption and an increase in animal-based products consumption [8], as well as an increased consumption of processed and packaged foods [9]. This trend, therefore, has increased food additive consumption, including nitrite and nitrate, raising the risk of exceeding Acceptable Daily Intake levels (ADIs) [10]. ADI is a health-based reference value that characterizes the hazard and is used to represent the safe levels of the quantity of additives in food or drinking water that may be ingested every day during a lifetime, without posing significant harm to health [11]. The ADI for nitrite is 0.07 mg nitrite ion/kg bw/day [12] and for nitrate it is 3.7 mg nitrate ion/kg bw/day [13]. The toxicity of a food additive and its dietary intake determine the extent to which it may represent a health concern [9] and negative health impacts may arise when ADIs are surpassed [14]. Thus, intakes must be monitored by member states in order to facilitate risk assessment [5], through a scientifically based four-step procedure comprising of hazard identification, hazard characterization, exposure assessment and risk characterization, which is used to estimate the proportion of individuals and age-groups that exceed these limits, for public health monitoring [15].

Several studies have been published to assess dietary exposure to nitrite and nitrate from processed meat products in other countries [16–24]; however, to our knowledge, no relevant research has been conducted to date for Greek consumers. Moreover, Greeks seem to follow a dietary pattern that differs significantly from the Mediterranean diet, presenting a high intake of red meat and fast food [25] and the gradual adoption of harmful eating habits appears to have accelerated since the onset of the Greek debt crisis [26]. Consequently, the objectives of this study were as follows: (a) estimate the daily nitrite and nitrate intake from processed meat products consumption; (b) assess the potential risk of exceeding the ADI; and (c) identify the major contributors, all in total and by age group, using a nationally representative sample.

## 2. Materials and Methods

### 2.1. Study Design and Subjects

Participants from the Hellenic National Nutrition and Health Survey (HNNHS), a nationally representative health study of the general population conducted from September 2013 to May 2015, were included in the study; pregnant or nursing women, institutionalized individuals and those serving in the military forces were excluded. Multistage stratified sampling was based on data from the latest Hellenic Statistical Authority's geographical density criteria by area, age, and sex. Trained professionals interviewed all research participants using Computer Assisted Personal Interview (CAPI) software to collect data on socio-demographics parameters, dietary intake, and lifestyle. Details of the survey, as well as of the questionnaires used, have been published elsewhere [27,28] The Hellenic Data Protection Authority (HDPA) and the Ethics Committee of the Department of Food Science and Human Nutrition of the Agricultural University of Athens approved the activities after receiving individual permission and approval.

### 2.2. Exposure Assessment

2.2.1. Food Consumption Data

- Participants

Data of 2152 (47.5% of HNNHS participants, 46.7% males) individuals across all age groups consuming processed meat products for which the addition of nitrite and nitrate is permitted [5] (henceforth: consumers) were retrieved to be included in our risk assessment, out of a total of 4532 participants in the HNNHS (henceforth: general population). The population enrolled was primarily classified in age groups following the clustering suggested by EFSA [29]. For better power of statistical analysis, infants (<11 months, $n = 1$) and toddlers (<3 years, $n = 14$) were moved to the upper class of other children (3–9 years)

and were all grouped as children (0–9 years), and individuals ≥65 years old were not further divided as elderly (65–74 years) and very elderly (≥75 years). Even after further subgrouping of adults aged 18–64 years, the size of all age groups was large enough for the calculation of the 95th percentile of exposure (N ≥ 60) [29]. The age classification finally adopted included children: 0–9 years, adolescents: 10–17 years, young adults: 18–30 years, adults: 31–50 years, older adults: 51–64 years and elderly: ≥65 years. Misreporters, defined as individuals reporting energy intake <500 and >6000 kcal/day (N = 15, 3 under-reporters and 12 over-reporters) were also included, as recommended by EFSA to always use a conservative approach [30,31].

- Processed meat consumption frequency

For 1890 (87.8%) participants, the dietary intakes were collected by two non-sequential 24 h recalls. The 24 h recall techniques have been described in detail elsewhere [27]. The remaining 12.2% of participants (N = 262) took part in the dietary survey for only one day. Individuals that supply data for one dietary recall are typically excluded from chronic exposure assessments, since at least two survey days per subject are normally required [29]. To overcome this and adjust for the quantity of the processed meat consumed over time, the frequency of consumption of processed meat or meat containing dishes during a 24 h recall, as provided in validated Food Propensity Questionnaires (FPQs), was used. "Never", "less than once a month", "1–3 times per month", "once a week", "2–4 times a week", "5–6 times a week", "every day", "2–3 times a day", "4–5 times a day", "5–6 times a day" were among the options in the FPQs. Thus, all consumers were included and any processed meat consumption, whether whole or in mixed dishes/recipes, was evaluated. The frequency of consumption was converted to servings per day by dividing the mean of the stated frequencies by the overall number of days (for instance, for a frequency of 1–3 times per month, the mean was 2 times per month and was divided by 30 to result in 0.067 servings per day). To acquire a relative intake over time and reduce variability, this value was multiplied by all participants' individual processed meat-eating events (in grams). Individuals who replied "never" in the FPQs but had records of processed meat consumption were additionally assigned to appropriate consumption frequencies.

### 2.2.2. Occurrence Data

- Food groups classification

Processed meat products in which the use of nitrite and nitrate is authorized [5], as well as dishes/recipes containing those, were selected from HNNHS. Composite dishes/recipes were broken down to their components and then all products were classified in processed meat categories according to food classification and description system FoodEx2 [32], in order to consequently attribute the legislative MPLs and identify the major contributors.

Overall, 17 FoodEx2 food groups were considered in the present exposure assessment, based on their basic term codes. They were subsequently summed up into 4 broader food groups, including subcategories where needed, such as pork meat (bacon, ham, other), poultry meat (chicken, turkey), sausages and meat specialties. Pork and poultry meat together fall under the broader FoodEx category of preserved meat. The unique foods and composite dishes/recipes from HNNHS considered are listed in Table S1 of the Supplementary Materials per food group. Processed meats were more frequently consumed as part of mixed dishes and recipes (75.1%), mainly in toasts/sandwiches (44.2%) and pizzas (14.2%), as can be seen in Table S2.

The European Commission and EFSA suggest using a stepwise procedure to estimate additive intakes and among the recommended Tiers; the second one, known as the regulatory maximum level exposure assessment scenario (Tier 2), was used in this study since it links actual national food consumption data and MPLs [33,34].

- Maximum Permitted Limits (MPLs)

Nitrite and nitrate were assumed to be present in processed meat products at a concentration level equal to their legislative MPLs [5]. To attribute MPLs, FoodEx2 categories

were matched with food categories of Regulation (EC) 1333/2008, following the mapping conducted by EFSA [35]. The food groups with their corresponding MPLs used in our assessment are listed in Table S3 of the Supplementary Materials.

- Estimated nitrite and nitrate intake

Individual processed meat intakes per FoodEx2 category, as adjusted before with frequencies of consumption and after being converted in kg, were multiplied with corresponding MPLs (mg/kg), resulting in nitrite and/or nitrate intakes (in mg) per individual and eating event. The total nitrite and nitrate intake per participant were determined, averaged in case of two days of recall, and divided by individual body weight in order to acquire the quantified dietary exposure of nitrite and nitrate, separately, for each subject in the study in mg/kg bw/day [13].

Some aspects about dietary nitrate exposure from processed meat products were further considered. The nitrate intake when used as a food additive was estimated by EFSA, using the refined scenario, and it was found to be less than 5% of the overall dietary nitrate intake [13]. Generally, the nitrate intake is much higher from vegetables and vegetable products than from additive sources [36]; thus, the in vivo converted amount is hardly taken into consideration when dietary exposure to nitrite and nitrate through processed foods is studied [20]. Furthermore, the toxicity of nitrate is determined by its conversion to nitrite and potential endogenous nitrosation and only when nitrate is ingested at its ADI level of 3.7 mg/kg bw/day, the in vivo nitrate-to-nitrite conversion in the human body may result in an exposure of significantly above the ADI for nitrite [1]. Finally, the nitrate-to-nitrite conversion factor uncertainty has been discussed previously [37].

The highly conservative Tier 2 approach used in our study revealed a null median nitrate intake from processed meat products among Greeks (Table S4), both in the general population and in consumers. Even at the 95th percentile, the intake was 0.003 mg/kg bw/day for the general population and 0.012 mg/kg bw/day for consumers, which is well below the ADI of 3.7 mg/kg bw/day, suggesting that there is no risk for surpassing the ADI of nitrite from the in vivo conversion of nitrate-to-nitrite in the human body. Despite the low dietary nitrate exposure estimated, its conversion to nitrite (in mg) was further considered in order to account for the co-exposure of nitrite and nitrate intake, by exploring the following three different conversion factors: 1% and 9% suggested by EFSA as the lower and higher nitrate-to-nitrite conversion in the body [13] and 2.3% as a median conversion factor used in research before [37]. The nitrate-to-nitrite conversion (mg) was then added to the direct intake of nitrite and total nitrite intake was assessed for each subject by dividing by individual body weight.

As expected, differences between estimates were negligible (Table S4). Additionally, the highest conversion factor of 9% better serves the worst case scenario, already adopted with the maximum regulatory exposure assessment scenario used to assess exposure. Thus, the co-exposure of daily intake of nitrite and nitrate from processed meat products as determined by applying the 9% nitrate-to-nitrite conversion factor—hereafter also referred to as total nitrite intake—was further used to analyze, present, and discuss data in this study.

- Estimated food groups contribution to daily total nitrite intake

To calculate how much each food group contributes to the daily total nitrite exposure from processed meats, the following formula was used: (Total exposure per food group per day/Total exposure per day) × 100. This contribution was also estimated per age group in total (Total exposure per food group per day per age group/Total exposure per day) × 100 and among consumers of the same age group: (Total exposure per food group per day per age group/Total exposure per day in the reference age group) × 100.

### 2.3. Risk Characterization

The total nitrite intake exposure levels estimated were compared with the ADI value of nitrite (0.07 mg/kg bw/day), to identify the subgroups or extreme consumers that might be at risk of exceeding the ADI and which may have adverse impacts on their health. Dietary

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nitrite exposures were also reported as a percentage contribution to ADI [12], and if the percentage was less than 100% it was concluded that there was no risk from exposure to the additive.

### 2.4. Other Parameters

Trained health professionals interviewed participants to obtain sociodemographic and anthropometric data, gathering information on age, gender, and educational level. The educational level was divided into the following three categories: up to 6 years of schooling, 12 years of schooling and higher education (including colleges). Smoking patterns and levels of physical activity were also evaluated. Individuals were categorized as ex-smokers if they had been at least 30 days smoke free, smokers, or never-smokers. Physical activity (PA) was defined according to the International Physical Activity Questionnaire (IPAQ), as per the calculation guidelines [38]. Sedentary status was assigned to people who scored below the light activity level. Body Mass Index (BMI) was calculated using the measurements of weight (kg) and height (m), using the following formula: weight/height$^2$ (kg/m$^2$). Weight status was categorized as healthy weight $\leq 25$ kg/m$^2$, $25 \leq$ overweight $< 30$ kg/m$^2$, and obese $\geq 30$ kg/m$^2$. Children and adolescents were classified using the extended International Obesity Task Force (IOTF) tables [39]. Total fat, trans fatty acid (TFA) and saturated fat acid (SFA) intakes had been estimated before, as % of total energy intakes [40]. Sodium intake was categorized per approximately 800 mg intake as <1500, $\geq 1500$ and <2300 and $\geq 2300$. Adherence to the Mediterranean diet was evaluated using the MedDiet score, ranging from 0 to 55 [41]. The variable was dichotomized to two final MedDiet categories, <23 and $\geq 23$ for low and high adherence, respectively, based on the median value of the population, since it has been shown that for every 11-unit rise in Med Diet score, there was a 37% odds decrease in acute coronary event [42].

### 2.5. Statistical Analysis

Baseline variables were stratified based on participants' level of total nitrite intake, of below or above the ADI of 0.07 mg/kg bw/day of nitrite, to assess significant differences. Continuous variables were presented as mean (standard deviation-sd) when normally distributed and as median (Interquartile Range-IQR: 25th percentile, 75th percentile) for skewed distributions. Categorical variables were presented as frequencies. The nonparametric Kruskall–Wallis rank sum and ANOVA test were employed to test group differences for skewed and continuous variables, respectively. For categorical variables, chi square testing was conducted. A *p*-value < 0.05 was considered statistically significant. Where significant differences were identified, the variables were entered into a logistic regression model to account for potential confounding. Variables considered in the logistic regression were age group, sex, weight, employment status, total energy intake, sodium intake category and MedDiet category. Missing age, weight and frequency of consumption data were imputed. All data statistical analyses were carried out using the STATA 13.0 (StataCorp, College Station, TX, USA) statistical software.

### 3. Results

Median total nitrite intake from processed meat products was found to be 0.007 (0.003, 0.02) mg/kg bw/day, accounting for 10% (4.3%, 28.6%) of the ADI of 0.07 mg/kg bw/day for nitrite. Table S5 presents the distribution of daily nitrite intake in mg/kg bw/day and as a percentage of the ADI, in total and per age group and sex for consumers only, which ranged from 0.003 (0.001, 0.007) mg/kg bw/day in the elderly ($\geq 6$ ears) to 0.02 (0.008, 0.042) mg/kg bw/day in children (0–9 years). The results are summarized in Table 1.

**Table 1.** Summary of dietary exposure to nitrite from processed meat products (in mg/kg bw/day and as % of ADI).

| Age Group | Dietary Exposure to Nitrite | | | | | |
| | Median | | Mean | | 95th Percentile | |
| | mg/kg bw/day | % ADI [1] | mg/kg bw/day | % ADI | mg/kg bw/day | % ADI |
|---|---|---|---|---|---|---|
| Minors (<18 years) [2] | 0.014 | 14.3 | 0.030 | 42.9 | 0.126 | 180.0 |
| Children 0–9 years | 0.02 | 28.6 | 0.038 | 54.3 | 0.173 | 247.1 |
| Adolescents 10–17 years | 0.01 | 14.3 | 0.022 | 31.4 | 0.076 | 108.6 |
| Adults (≥18 years) [3] | 0.007 | 10.0 | 0.021 | 30.0 | 0.078 | 111.4 |
| Young adults 18–30 years | 0.008 | 11.4 | 0.025 | 35.7 | 0.087 | 124.3 |
| Adults 31–50 years | 0.007 | 10 | 0.020 | 28.6 | 0.079 | 112.9 |
| Older adults 51–64 years | 0.004 | 5.7 | 0.014 | 20.0 | 0.056 | 80 |
| The elderly ≥65 years | 0.003 | 4.3 | 0.008 | 11.4 | 0.048 | 68.6 |
| Total | 0.007 | 10 | 0.022 | 31.4 | 0.173 | 118.6 |

[1] ADI: Acceptable Daily Intake.

Figure 1 shows that ADI was surpassed in most of the age groups and in total (118.6% of the ADI), when daily intake was estimated at the 95th percentile. Specifically, all females up to 30 years and all males up to 64 years old surpassed it. Additionally, nitrite exposure estimated for children 0–9 years exceeded the ADI already at the 90th percentile for both sexes (115.7% of the ADI for girls and 164.3% of the ADI for boys).
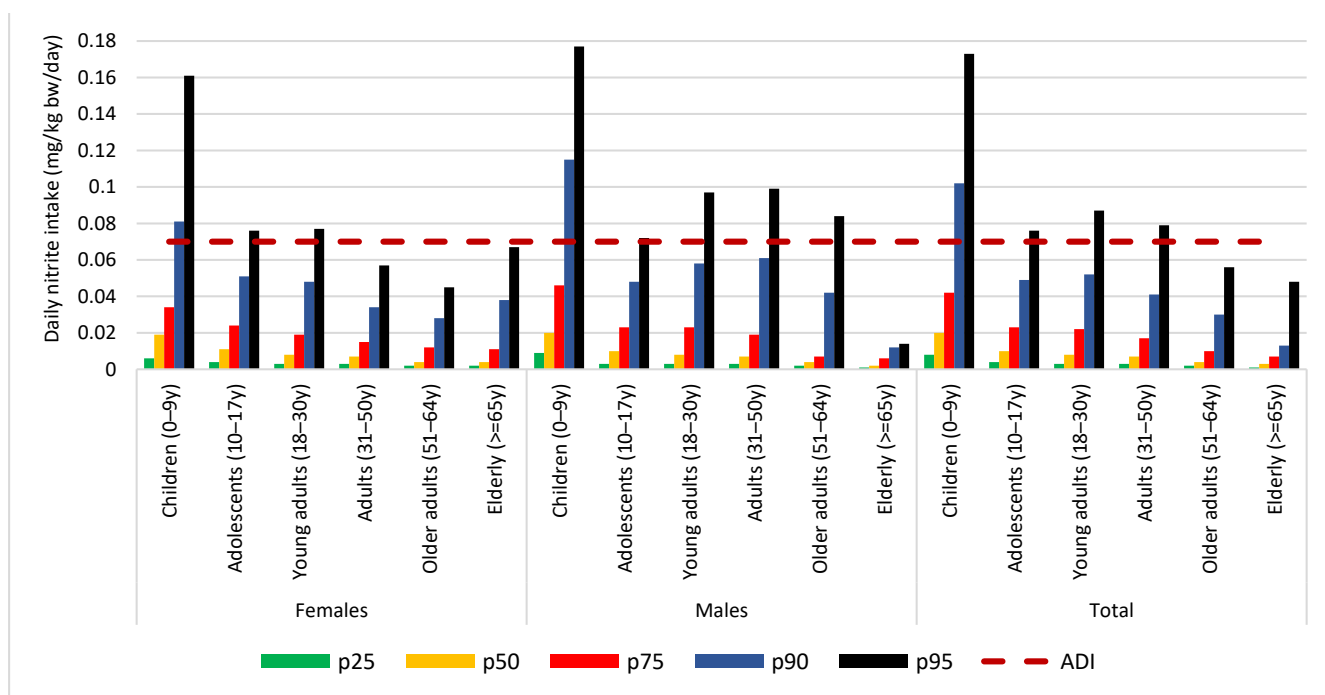


**Figure 1.** Distribution of daily total nitrite intake (mg/kg bw/day) by sex and age group, in comparison to ADI of 0.07 mg/kg bw/day for nitrite.

The main characteristics and baseline variables differences stratified by level of intake (above and below the ADI of 0.07 mg/kg bw/day) of the population studied are summarized in Table 2. A total of 6.6% (N = 143) of consumers exceeded the ADI of nitrite from the ingestion of processed meat products only (3.2% of the general population). When over consumers (total energy > 6000 kcal/day, N = 12) were excluded, the proportion of those exceeding the ADI was estimated to be 6.5% (N = 138), hence the results did not differ. Among those exceeding the ADI, young adults aged 18–30 years had the highest

proportion (37.1%), followed by adults aged 31–50 years (28%) and children aged 0–9 years (20.3%). Overall, more males exceeded the ADI (60.8%) than females (39.2%). Significant differences were found in age and age groups, sex, weight, employment status, total energy intake, total sodium intake (in both mg and category) and in MedDiet score and category. The area of residence, marital status, education level, smoking status, physical activity level and total fat, TFA and SFA intakes, and BMI/weight status of children only did not significantly differ. The BMI of adults significantly differed by ADI levels of intake, but this was not significant by weight status.

**Table 2.** Baseline variables of the participants by level of intake (above and below ADI of 0.07 mg/kg bw/day).

| Variable [1] | Total N = 2152 | Below ADI N = 2009 (93.4%) | Above ADI N = 143 (6.6%) | *p*-Value (by Intake Level) [2] |
|---|---|---|---|---|
| Age(years), median (IQR) | 29 (21, 42) | 29 (21, 42) | 26 (15, 36) | <0.001 |
| Age group (years), *n* (%) | | | | <0.001 |
| Children (0–9) | 190 (8.8) | 161 (8.0) | 29 (20.3) | |
| Adolescents (10–17) | 188 (8.7) | 176 (8.8) | 12 (8.4) | |
| Young adults (18–30) | 782 (36.4) | 729 (36.3) | 53 (37.0) | |
| Adults (31–50) | 664 (30.9) | 624 (31.0) | 40 (28.0) | |
| Older adults (51–64) | 211 (9.8) | 203 (10.1) | 8 (5.6) | |
| Elderly (>=65) | 117 (5.4) | 116 (5.8) | 1 (0.7) | |
| Sex, *n* (%) | | | | <0.001 |
| Females | 1144 (53.3) | 1088 (54.4) | 56 (39.2) | |
| Males | 1001 (46.7) | 914 (45.6) | 87 (60.8) | |
| Weight (kg), mean (sd) | 67.9 (20.7) | 68.2 (20.4) | 62.9 (24.2) | <0.05 |
| Area of residence, *n* (%) | | | | 0.215 |
| Attiki and Thessaloniki | 1429 (67.0) | 1335 (67.1) | 94 (66.7) | |
| Islands (including Crete) | 219 (10.3) | 199 (10.0) | 20 (14.2) | |
| Mainland | 483 (22.7) | 456 (22.9) | 27 (19.1) | |
| Marital status [3], *n* (%) | | | | 0.165 |
| Divorced/Separated/Widowed/Single | 1044 (62.0) | 980 (61.6) | 64 (68.8) | |
| Married/Cohabiting | 639 (38.0) | 610 (38.4) | 29 (31.2) | |
| Education level [3], *n* (%) | | | | 0.146 |
| Up to 6 years of school | 88 (5.1) | 87 (5.3) | 1 (1.1) | |
| 12 years of school | 661 (38.4) | 626 (38.5) | 35 (36.8) | |
| Higher education (including colleges) | 974 (56.5) | 915 (56.2) | 59 (62.1) | |
| Employment status [3], *n* (%) | | | | 0.034 |
| Unemployed | 535 (31.0) | 507 (31.1) | 28 (29.2) | |
| Employed | 1009 (58.5) | 944 (58.0) | 65 (67.7) | |
| Pension | 180 (10.5) | 177 (10.9) | 3 (3.1) | |
| Smoking status [3], *n* (%) | | | | 0.119 |
| Never smoker | 847 (48.1) | 801 (48.3) | 46 (45.1) | |
| Current smoker | 656 (37.3) | 622 (37.5) | 34 (33.3) | |
| Ex-smoker | 257 (14.6) | 235 (14.2) | 22 (21.6) | |
| Physical activity status [3,4], *n* (%) | | | | 0.930 |
| Low | 255 (15.5) | 239 (15.3) | 16 (17.2) | |
| Moderate | 624 (37.8) | 588 (37.8) | 36 (38.7) | |
| Sedentary | 101 (6.1) | 95 (6.1) | 6 (6.5) | |
| Very | 670 (40.6) | 635 (40.8) | 35 (37.6) | |
| Total energy intake (kcal/day), median (IQR) | 1917.8 (1443.1, 2518.5) | 1894.4 (1433.7, 2493.3) | 2249.9 (1639.6, 3357.4) | <0.001 |
| Total fat intake (%energy) [3], mean (sd) | 38.1 (9.5) | 38.0 (9.5) | 39.6 (9.5) | 0.1246 |
| Total TFA intake (%energy) [3], median (IQR) | 0.6 (0.4, 0.8) | 0.5 (0.4, 0.8) | 0.6 (0.4, 0.8) | 0.3107 |
| Total SFA intake (%energy) [3], mean (sd) | 13.4 (4.0) | 13.4 (4.0) | 13.9 (4.0) | 0.2235 |
| Total sodium intake (mg), mean (sd) | 2303 (690.1) | 2276.3 (666.2) | 2770.3 (906.1) | <0.001 |

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**Table 2.** *Cont.*

| Variable [1] | Total N = 2152 | Below ADI N = 2009 (93.4%) | Above ADI N = 143 (6.6%) | *p*-Value (by Intake Level) [2] |
|---|---|---|---|---|
| Sodium intake [3], *n* (%) | | | | <0.001 |
| <1500 | 104 (6.4) | 103 (6.7) | 1 (1.1) | |
| >=1500 and <2300 | 851 (52.3) | 816 (53.1) | 35 (38.5) | |
| >=2300 | 673 (41.3) | 618 (40.2) | 55 (60.4) | |
| BMI adults(kg/m$^2$) [3,5] mean (sd) | 24.0 (5.2) | 24.0 (5.2) | 23.0 (5.1) | 0.020 |
| BMI adults categories [3,5], *n* (%) | | | | 0.488 |
| Healthy weight | 977 (56.6) | 921 (56.6) | 56 (57.1) | |
| Overweight | 507 (29.4) | 475 (29.2) | 32 (32.7) | |
| Obese | 241 (14.0) | 231 (14.2) | 10 (10.2) | |
| BMI children [6], mean (sd) | 18.8 (4.2) | 18.9 (4.3) | 18.1 (3.8) | 0.2680 |
| BMI children categories [5,6], *n* (%) | | | | 0.602 |
| Healthy weight | 332 (92.2) | 293 (91.8) | 39 (95.1) | |
| Overweight | 21 (5.8) | 19 (6.0) | 2 (4.9) | |
| Obese | 7 (2.0) | 7 (2.2) | 0 (0) | |
| MedDiet score [3], mean (sd) | 27.0 (6.4) | 27.1 (6.3) | 25.0 (6.5) | <0.05 |
| MedDiet category [3], *n* (%) | | | | <0.05 |
| MD < 23 | 373 (22.9) | 341 (22.2) | 32 (35.2) | |
| MD >= 23 | 1255 (77.1) | 1196 (77.8) | 59 (64.8) | |

[1] Continuous variables were presented as mean and standard deviation (sd) when normally distributed and median and interquartile range (IQR: 25th percentile, 75th percentile) when skewed. Categorical variables were presented as frequencies. [2] Group differences were tested using chi square test for proportions and Kruskall–Wallis rank sum or ANOVA test depending on data distribution. Level of significance was set at alpha = 5%. [3] Adults only. [4] Physical activity (PA) was defined according to the International Physical Activity Questionnaire (IPAQ). [5] Body Mass Index (BMI) was calculated from measurements of weight (kg) and height (m): weight/height$^2$ (kg/m$^2$). Weight status was categorized as healthy weight $\leq$25 kg/m, $25 \leq$ overweight < 30 kg/m$^2$, and obese $\geq$ 30 kg/m$^2$. Children and adolescents were classified using the extended International Obesity Task Force (IOTF) tables [39]. [6] Children only.

A logistic regression model was used to assess the likelihood of consuming total nitrite from processed meats above the ADI (Table S6). The results revealed that high adherence to Mediterranean diet (MedDiet score $\geq$ 23) significantly decreased the odds of exceeding the ADI of nitrite from the consumption of processed meat products (OR: 0.6, 95% CI: 0.36–0.90). Among the consumers of processed meat products in our study, 54.3% reported a frequency of consumption of once a week and 22.9% reported a frequency of 1 to 3 times per month. Among those with an intake of total nitrite exceeding the ADI, however, 39.2% consumed processed meat products 2 to 4 times a week, 19.6% consumed them every day, 16.8% from 5 to 6 times a week and a 12.6% from 2 to 3 times a day (Table S7).

Figure 2 depicts individuals (%) that exceeded the ADI per sex and age group over total number of exceeders (N = 143) as well as over the total number of participants of the same age group (the data are presented in detail in Table S8). Specifically, 15.3% (N = 29) of children aged 0–9 years that participated in the study (N = 190) were found to exceed the ADI, with a higher proportion of males over females (18.1% and 11.8%, respectively).
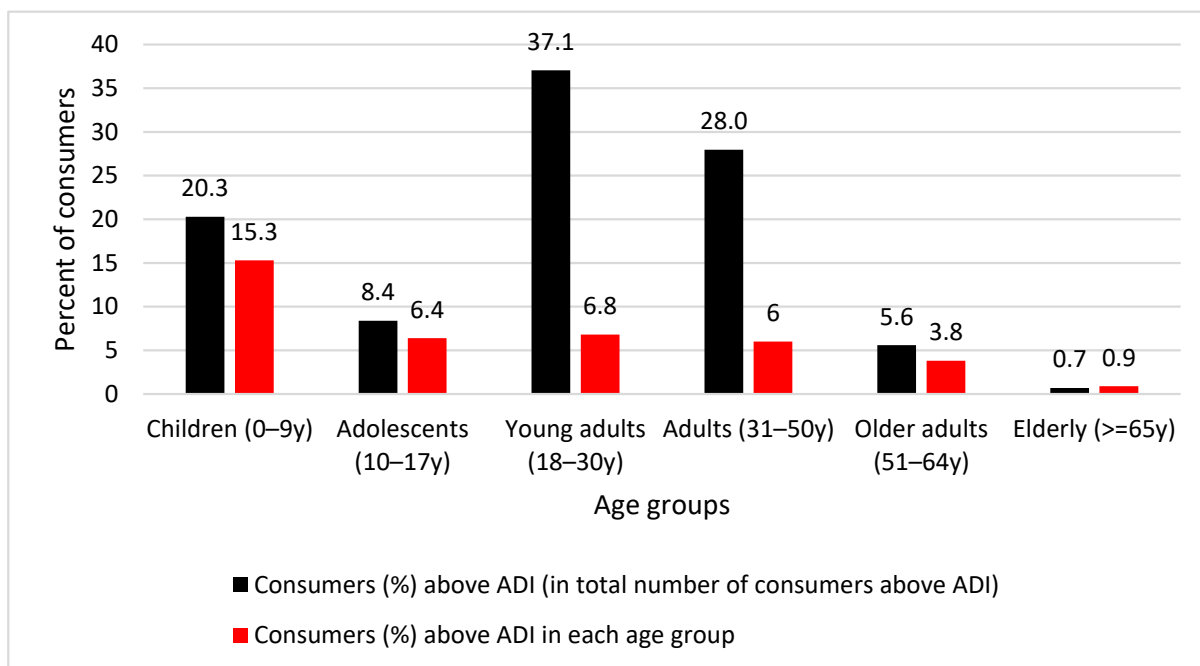
**Figure 2.** Proportion of the population per age group that exceeds the ADI of 0.07 mg/kg bw/day for nitrite in total exceeders and within the same age group.

The major contributors to total nitrite exposure, when based on FoodEx2 groups classification, was by far A023T-Cooked Turkey Meat (26.83%), followed by A0EYP-Preserved or partly preserved sausages (18.23%), A023H-Cooked cured (or seasoned) pork meat (17.56%) and A022T-Ham pork (13.04%) (Figure S1 in the Supplementary Materials). When FoodEx2 groups were divided into broader food groups, as already presented in Table S1, products from pork meat in general, including ham, bacon and other pork meats, contributed the most to nitrite exposure (41.53%), in relation to poultry meat products (34.39%), sausages (23.82%) and meat specialties (0.26%) (Table S9). Nitrite exposure from poultry meat was primarily due to turkey meat (32.7% of the total 34.4%), whereas exposure from pork meat was primarily due to non-specific products (20.4%) and ham (17.7%). Figure 3 depicts the contribution (%) of different food groups to the total nitrite intake among consumers of the same age group. As it shows, processed pork meat was the major contributor for all age groups, with the exception of young adults aged 18–30 years, where poultry meat slightly outperformed (39.1% vs. 38.2% for pork meat), and the elderly (65+ years), where pork meat and sausages were found to contribute equally to total nitrite exposure, at 39.6% and 39.4%, respectively. Moreover, pork meat products were by far the major contributors in children aged 0–9 years (51.9%), with unclassified pork meat contributing the most (25.7%), followed by ham (21.8%). Poultry meat was the second source of nitrite intake in children, with turkey meat accounting for 25.9% of total nitrite intake among children. For adolescents aged 10–17 years, the processed meat that contributed the most to nitrite exposure was pork (39.5%), with ham accounting for 18.1%, but sausages contributed to 30.9% of consumption and poultry meat, primarily turkey (29.5%) also contributed significantly and by about the same level. Young adults aged 18–30 years ingested more nitrite from poultry meat (39.1%, with turkey contributing 37.4%) and pork meat (38.2%), with ham contributing 17.2%, while pork meat was by far the major contributor in adults aged 31–50 years old (40.5%) and in older adults aged 51–64 years (46.8%), followed by poultry meat (35.28%) and sausages (30.94%), respectively.

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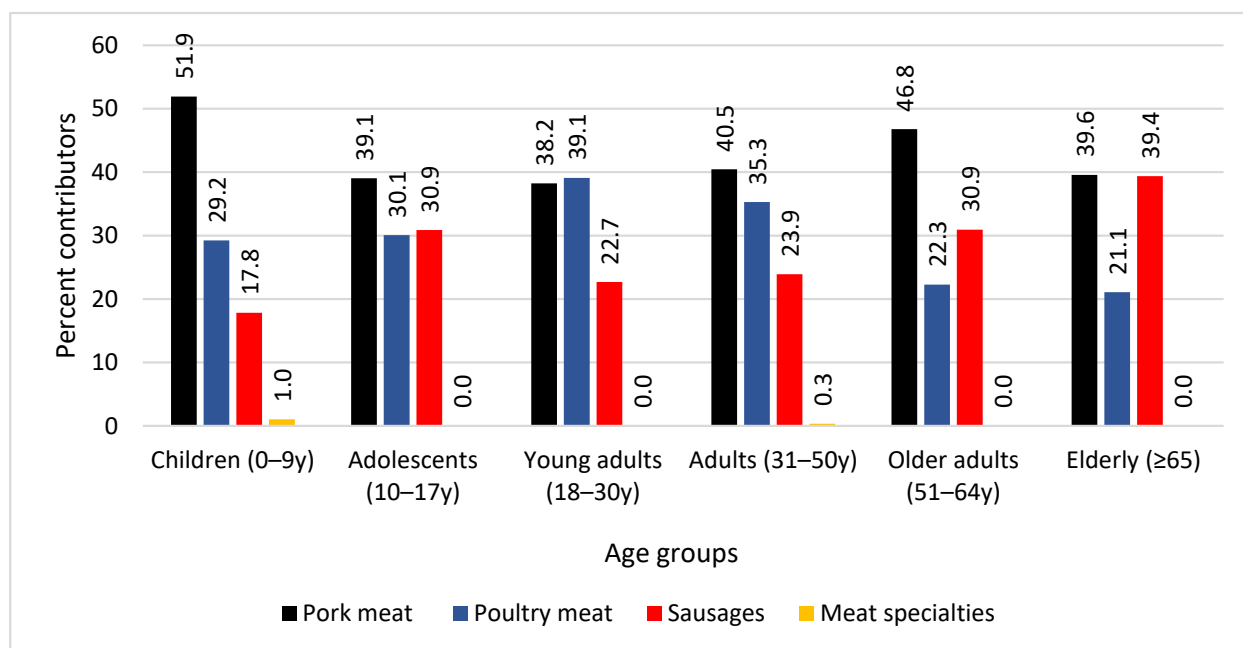
**Figure 3.** Main processed meat food groups contributing to total nitrite intake among consumers of the same age group.

## 4. Discussion

The findings of this study indicate that a considerable proportion of Greek consumers (6.6%) may be at risk, as their consumption of nitrite and nitrate (assessed as nitrite equivalent) already exceeded the ADI via ingestion of processed meats only. The risk seems to be higher among children aged 0–9 years, since 15.3% of participants exceeded the ADI, compared to 6.4% in adolescents and 5.7% in adults, a proportion that corresponds to 160.625 children based on the latest data provided by the Hellenic Statistical Authority [43]. The major food groups contributing to total nitrite intake were processed products from meat of pork and turkey, most frequently consumed as part of mixed dishes (mainly in toasts, sandwiches and pizzas).

Due to differences in the design of the studies estimating the nitrate and nitrite intakes from processed meat products that have been conducted at EU level [12] and in other countries [16–24] (such as additive evaluations, sources of intake, occurrence data, age groupings, dietary assessment methodologies, conversion factors utilized, and more), the results are quite difficult to compare. Children aged 3 to 9 years in the EU were found to have a mean dietary exposure to nitrites from their use solely as food additives ranging from 0.03 to 0.027 mg/kg bw/day [12]. For this estimate, a sample of 838 children from the Greek island of Crete was included, whose nitrite intake in Tier2 ranged from 0.03 to 0.15, similar to the 0.038 (0.055) mg/kg bw/day found in the present study. The proportion of children exceeding the ADI among children 0–9 years in this study (15.3%) was higher than that of Serbian children < 9 years old (9.33%) [23], Estonian children 1–6 years (3.1%) [24] and Swedish children aged 4, 8–9 and 11–12 years old (0.1%) [44]. Based on the aforementioned studies, mean intakes as % of ADI were 40.0% for Serbian children, 21.9% and 22.9% among Estonian children 12–35 months and 3–10 years, respectively, and 10% for Swedish children, versus the higher 54.3% found for Greek children aged 0–9 years. In comparison to the median of 0.02 mg/kg bw/day and mean of 0.038 mg/kg bw/day for children aged 0–9 years in our study, median nitrite intakes of 0.016, 0.040, and 0.033 mg/kg bw/day were found for children aged 1, 3, and 6 years old in Finland [20] and mean intakes of 0.110–0.521 mg/kg bw/day for children in Sudan [21]. The daily intake of nitrite for adults was estimated to be in the range of 0.027–0.130 mg/kg bw/day in Sudan [21] and 0.038–0.063 mg/kg bw/day for adults and older children aged 4–18 years in the UK [18], in

comparison to 0.02 and 0.03 mg/kg bw/day for adults and minors (<18 years) in our study. Finally, the average daily intake of nitrite was 6% of ADI in a Belgian population ≥15 years old (source: processed meat products) [17] and 75.9% for an Austrian adult population (source: meat products and drinking water) [45], compared with 28.6% of Greek adult participants in our study (source: processed meat products). Additionally, preserved meat was found to contribute considerably more to nitrite ingestion in children (81.1%) in our study than in EFSA's estimate based on the participants from the Greek island of Crete (48.7%), with sausages contributing 17.8% against the 51.3% projected by EFSA. Those differences may be due to the fact that the sample used by EFSA was not representative of the Greek child population. Additionally, the children were aged 3–9 years old, whereas infants and toddlers (0–3 years, N = 15) were also included in the present study. Therefore, the outcomes cannot be compared with precision. Finally, major contributors were minced cooked sausages and canned meat for Serbian children [23] and whole muscle meat cured in brine (54%) and cured, cooked sausages (45%) for the U.S. population ≥2 years [22].

Our study had strengths as well as limitations. As far as we know, this is the first study to use a nationally representative sample to evaluate the nitrite and nitrate intake from processed meat products. The survey-specific FPQs were utilized to adjust for the quantity of processed meat ingested over time. The possible effect of the endogenous conversion of nitrate to nitrite was investigated and the higher 9% conversion factor suggested by EFSA was used [12] in order to securely assess the maximum possible risk for consumers, although the in vivo converted amount is rarely taken into consideration when dietary exposure to nitrite and nitrate through processed foods is studied [20]. As already indicated by experts, in order to achieve consistency and thus more reliable nitrite exposure estimates, the conversion factor has to be defined more accurately [46,47].

Uncertainties in the exposure assessment of nitrite and nitrate intake from processed meat products have been summarized in Table S10, according to the relevant guidance provided by EFSA [48]. The lack of national data on the use and occurrence of nitrite and nitrate salts in processed meat products posed a limitation to this study. The regulatory scenario used is considered the worst case scenario, as it tends to systematically overestimate current intakes [48]; overall, our results could be an overestimation of nitrite ingested from processed meat, and therefore, a more refined assessment may be required. However, the European Commission that suggests using a stepwise procedure to estimate the additive intakes [33], with Tier 3 (based on individual food consumption data and measured data on additives occurrence) to be performed only when Tier 2 is exceeded [49]. Given the difficulty in obtaining accurate concentration data, it has also been suggested to combine the actual national food consumption data with the MPL when the additive is listed in the label of the food product (Tier 2a) [50]. Additionally, EFSA identifies the fact that the compounds could also be used at levels higher than permitted in the legislation as an extrapolation uncertainty affecting the estimation of additives [48]. Furthermore, the reported levels of nitrite and nitrate usage in meat products, as provided by European industry [12,13] were equal to the MPLs, with the exception of E250 when used solely as a flavor enhancer [13]; a detail that could not be taken into account in this study as such detailed information was not provided.

Some other methodological limitations include the challenge in FoodEx2 codification and mapping with the categories outlined in the legislation [5], as thorough meat product classification may have not been permitted by the information available in the survey. This challenge is also indicated by EFSA, as restrictions/exceptions in the regulation could not be taken under consideration in similar projects [12,35]. Finally, residual levels might be affected by the processing time and temperature, the primary additive dosage, pH, the addition of ascorbate and/or other antioxidant components, and the existence of microorganisms, resulting in a continuous reduction during storage [51]. These factors could not be considered in this risk assessment, as no relevant data were available.

EFSA concluded that that the ADI would be surpassed at the EU level if all dietary nitrite sources were taken into account at the mean in infants, toddlers and children and

at the highest exposure for all age groups [12]. Nitrate intake was also suggested to be exceeded for all age groups at the mean and highest percentiles if all sources of dietary intake (food additives, natural presence and contaminants) were assessed [13]. Other national studies have already evaluated the contribution from the conversion of dietary nitrate to nitrite in various foods, especially vegetables and fruits [19,45], although a direct link between nitrate and nitrite intake from vegetables and fruits and adverse health effects has not been identified [7]. Further research may be restricted by the lack of national data on the concentration of nitrite and nitrate in a number of food categories where these compounds are present as naturally occurring species (vegetable/fruits) or contaminants (drinking water). Surveillance data could allow this uncertainty of over/underestimation to be quantified; therefore, regulatory authorities could plan and maintain a system to monitor the content of nitrite and nitrate in products in which these compounds may occur naturally, as contaminants or additives, within a risk-based approach with appropriate frequency.

Finally, our outcomes are in accordance with the Scientific Committee on Food (SCF) of the European Union that specifically recommended that the subpopulation of children be given special attention, because of their higher ingestion levels relative to their body weight [33]. Increasing public knowledge of the potentially harmful consequences of nitrite and nitrate in processed meats could result in a change in dietary preferences and habits among Greeks, particularly in children and their caretakers. Moreover, the actual nitrite consumption from processed meat products might depend on the type of meat, since chicken products have been suggested to have higher residual nitrite levels than pork and beef products [44]. This could result in an elevated nitrite exposure in consumers switching from consuming processed red meat to white meat products [19] given the public's perception of poultry meat as being healthier, and could be also considered in future studies and dietary guidelines. If diet habits keep changing, deviating from the Mediterranean diet, and processed meat consumption keeps rising, the intake may increase beyond even higher levels; thus, the idea of the food industry shifting toward using healthier, "greener" preservation technologies is generally supported.

## 5. Conclusions

The median nitrite and nitrate intake from processed meat products, estimated as nitrite equivalent, revealed that a significant proportion of Greek consumers were at risk of exceeding the ADI for nitrite from the consumption of processed meat alone, mainly that of processed products from pork and turkey meat consumed as part of mixed dishes (more frequently on toast, sandwiches, and pizza). Special attention should be given to children aged 0–9 years, who had by far the highest proportion of exceeders among them. Considering the cumulative impact of chronic exposure to additional dietary sources of nitrite and nitrate, these results are alarmingly high and could indicate the need for competent authorities to establish relevant educational campaigns aiming to raise public awareness of the potential adverse health effects of nitrite and nitrate found in processed meat. Public awareness creates indirect pressure on the food industry and could effectively lead to the decrease in or even elimination of these compounds. Other techniques that will adequately support food safety may be developed to replace these additives. Furthermore, competent authorities should develop and maintain a monitoring plan for the nitrite and nitrate content of various products in the Greek market. Future research could assess the nitrite and nitrate dietary intakes using refined occurrence data. Finally, potential associations of dietary intakes of nitrite and nitrate with adverse health effects other than cancer, in total but especially for those at risk, could be explored if relevant data are made available.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph191912800/s1, Table S1: Food groups and items (including composite disks and recipes) involved in the exposure assessment; Table S2: Frequency of processed meat products and mixed dishes/recipes containing processed meat products in HNNHS; Table S3:

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Concentrations levels of nitrite (E 249–250) and nitrate (E 251–252) used in the regulatory maximum level scenario (mg/kg); Table S4: Dietary daily intakes of nitrate, nitrite and combined nitrite and nitrate via processed meat products using different nitrate-to-nitrite conversion factors (a) for general population and (b) for consumers only; Table S5: Distribution of daily nitrite intake (in mg/kg bw/day and as % of ADI), estimated in total and per sex and age group for consumers only; Table S6: Likelihood of exceeding ADI of nitrite from total nitrite intake from processed meat products; Table S7: Proportion of population per frequency of consumption of processed meat products, as reported in HNNHS, among (a) consumers and (b) consumers with total nitrite intake exceeding the ADI of 0.07 mg/kw bw/day; Table S8: Consumers with total nitrite intake from processed meat products above the ADI of nitrite (0.07 mg/kg bw/day) per sex and age group in number of individuals ($n$), as a percentage of individuals of the same age group [a = ($n$/N) × 100] and as a percentage of total number of individuals exceeding ADI ($n$ = 143) [b = ($n$/143)*100]; Figure S1: Main FoodEx2 group contribution to total nitrite intake among processed meat products; Table S9: Contribution (%) of meat type and FoodEx2 food groups to total nitrite exposure per age group; Table S10. Qualitative evaluation of influence of uncertainties on the dietary exposure estimated.

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**Table S1.** Food groups and items (including composite disks and recipes) involved in the exposure assessment.

| |
|---|
| **FOODEX Category** |
| **Food group (if different than FOODEX Category)** |
| **Food subgroup (if any)** |
| **x.  FoodEx2 Code & Term** |
|     HNNHS Foods |

**PRESERVED MEAT**

**Pork Meat**

**Pork meat, Bacon**

1. **A022X Bacon**
   Caesar salad
   Cheese soufflé
   Processed meat products, bacon
   Crepes
   Hamburger
   Lasagna soufflé
   Omelet
   Pasta au gratin
   Pasta carbonara
   Peinirli ("pizza boat")
   Pizza
   Potatoes au gratin
   Sandwich
   Soup
   Tart
   Toast

**Pork meat, Ham**

2. **A022T Ham, pork**
   Caesar salad
   Cheese soufflé
   Chef salad
   Chicken with ham
   Processed meat products, ham
   Crepes
   Croissant
   Lasagna soufflé
   Omelet
   Pasta au gratin
   Peinirli ("pizza boat")
   Pie
   Pizza
   Sandwich
   Toast
   Tortilla sandwich

3. **A023K Cooked pork ham**
   Caesar salad

**FOODEX Category**
**Food group (if different than FOODEX Category)**
**Food subgroup (if any)**
x.  **FoodEx2 Code & Term**
    HNNHS Foods

Chef salad

Processed meat products, ham

Crepes

Lasagna soufflé

Peinirli ("pizza boat")

Pie

Pizza

Sandwich

**Pork meat, other**

4.  **A022S Cured seasoned pork meat**

Caesar salad

Chef salad

Crepes

Pasta au gratin

Pie

Pizza

Sandwich

Processed meat products

Processed meat products, prosciutto

Toast

Meat preserved with salt, "apaki"

Meat preserved with salt, pork

5.  **A023H Cooked cured (or seasoned) pork meat**

Processed meat products

Toast

Tortilla sandwich

Pizza

Sandwich

Processed meat products, prosciutto

Crepes

**Poultry meat**

**Poultry meat, Chicken**

6.  **A023S Cooked cured (or seasoned) poultry meat**

Processed meat products, chicken

Sandwich

Toast

7.  **A023X Cooked other poultry meat**

Processed meat products, chicken

**Poultry meat, Turkey**

8.  **A023E Cured seasoned poultry meat**

Processed meat products, turkey

Pasta carbonara

**FOODEX Category**
**Food group (if different than FOODEX Category)**
**Food subgroup (if any)**
x.  **FoodEx2 Code & Term**
    HNNHS Foods

Sandwich

Toast

9.  **A023T Cooked turkey meat**

Processed meat products, turkey

Crepes

Croissant

Omelet

Pasta carbonara

Pizza

Salad

Sandwich

Toast

Tomato sauce

Tortilla sandwich

**SAUSAGES**

10. **A0EYP Preserved or partly preserved sausages**

Beans

Peinirli ("pizza boat")

Pie

Piroshky

Pizza

Sandwich

Sausage

Sausage, breakfast type

"Spetsofai" (Sausages with peppers)

Tomato sauce

Sausage, soutzouki

Sausage, turkey

Sausage, tzoumagias-type

Sausage, village type

Toast

Sausage, cocktail-type

11. **A025C Chorizo and similar**

Paella

12. **A026B Frankfurt-type sausage**

Hot dog

Sandwich

Sausage, Frankfurt

Tomato sauce

13. **A025Q Mortadella-type sausage**

Processed meat products, mortadella

Sandwich

Toast

**FOODEX Category**
**Food group (if different than FOODEX Category)**
**Food subgroup (if any)**
x. **FoodEx2 Code & Term**
   HNNHS Foods

14. **A025B Pepperoni/paprika-type sausage**

   Pizza

15. **A024X Salami-type sausage**

   Processed meat products, air-dried salami

   Processed meat products, Hellenic-type salami

   Processed meat products, salami

   Processed meat products, salami Hungarian type

   Omelet

   Pizza

   Sandwich

   Sausage, beer salami

   Sausage, salami

   Souvlaki

   Toast

**MEAT SPECIALTIES**

16. **A026M Liver based spreadable-textured specialties**

   Pate and meat pastes, pate, duck liver

   Pate and meat pastes, pate, other poultry liver

17. **A026Q Pate, chicken liver**

   Pate and meat pastes, pate, chicken liver

**Table S2.** Frequency of processed meat products and mixed dishes/recipes containing processed meat products in HNNHS.

| Foods/Mixed Dishes/Recipes | Frequency in HNNHS (%) |
|---|---|
| **Foods** | **24.91%** |
| Processed meat products | 1.85% |
| Processed meat products, turkey | 10.92% |
| Processed meat products, air-dried salami | 0.87% |
| Processed meat products, bacon | 0.34% |
| Processed meat products, chicken | 1.08% |
| Processed meat products, ham | 4.35% |
| Processed meat products, Hellenic salami | 0.08% |
| Processed meat products, loin | 0.44% |
| Processed meat products, mortadella | 0.36% |
| Processed meat products, prosciutto | 0.20% |
| Processed meat products, salami | 0.03% |
| Processed meat products, salami Hungarian type | 0.08% |
| Pate and meat pastes, pate, chicken liver | 0.08% |
| Pate and meat pastes, pate, duck liver | 0.03% |
| Pate and meat pastes, pate, other poultry liver | 0.06% |
| Meat preserved with salt, "apaki" | 0.11% |
| Meat preserved with salt, pork | 0.06% |
| Sausages | 1.65% |
| Sausage, beer salami | 0.06% |
| Sausage, breakfast type | 0.03% |
| Sausage, cocktail | 0.08% |
| Sausage, Frankfurt | 0.08% |
| Sausage, salami | 0.08% |
| Sausage, soutzouki | 0.14% |
| Sausage, turkey | 0.14% |
| Sausage, tzoumagias-type | 0.59% |
| Sausage, village type | 1.12% |
| **Mixed dishes/Recipes** | **75.09%** |
| Beans | 0.03% |
| Caesar salad | 0.47% |
| Cheese soufflé | 0.06% |
| Chef salad | 0.28% |
| Chicken with ham | 0.56% |
| Crepes | 1.43% |
| Croissant | 0.64% |
| Hamburger | 0.59% |
| Hot dog | 0.73% |
| Lasagna soufflé | 1.42% |
| Omelet | 0.36% |
| Paella | 0.42% |
| Pasta au gratin | 1.59% |
| Pasta carbonara | 1.71% |

| Foods/Mixed Dishes/Recipes | Frequency in HNNHS (%) |
| --- | --- |
| Peinirli ("pizza boat") | 1.01% |
| Pie | 4.13% |
| Piroshky | 0.45% |
| Pizza | 14.24% |
| Potatoes au gratin | 0.17% |
| Salad | 0.03% |
| Sandwich | 7.58% |
| Soup | 0.03% |
| "Spetsofai" (Sausages with peppers) | 0.36% |
| Tart | 0.03% |
| Toast | 36.61% |
| Tomato sauce | 0.12% |
| Tortilla sandwich | 0.03% |
| Souvlaki | 0.03% |
| **Total** | **100.00%** |

**Table S3.** Concentration levels of nitrite (E 249-250) and nitrate (E 251-252) used in the regulatory maximum level scenario (mg/kg).

| Food category number | Food category name | FOODEX category | FOODEX Code | FOODEX Name | Frequency in HNNHS % | NITRITE MPL (mgkg) | NITRATE MPL (mgkg) |
|---|---|---|---|---|---|---|---|
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A022S | Cured seasoned pork meat | 1.08 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A022X | Bacon | 9.02 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A023E | Cured seasoned poultry meat | 3.26 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A024X | Salami-type sausage | 1.77 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A025B | Pepperoni/paprika-type sausage | 0.63 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A025C | Chorizo and similar | 0.41 | 150 | 150 |
| **08.3.1** | **Total** | | | | **16.17** | | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A022T | Ham, pork | 23.97 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023H | Cooked cured (or seasoned) pork meat | 21.13 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023K | Cooked pork ham | 2.43 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023S | Cooked cured (or seasoned) poultry meat | 1.38 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023T | Cooked turkey meat | 25.71 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023X | Cooked other poultry meat | 0.08 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A025Q | Mortadella-type sausage | 0.63 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A026B | Frankfurt-type sausage | 0.86 | 150 | |
| 08.3.2 | Heat–treated meat products | Meat specialties | A026Q | Pate, chicken liver | 0.08 | 150 | |
| 08.3.2 | Heat–treated meat products | Meat specialties | A026M | Liver based spreadable-textured specialties | 0.08 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A0EYP | Preserved or partly preserved sausages | 7.48 | 150 | |
| **08.3.2** | **Total** | | | | **83.83** | | |
| **TOTAL** | | | | | **100.00** | | |

**Table S4.** Dietary daily intakes of nitrate, nitrite and combined nitrite and nitrate via processed meat products using different nitrate-to-nitrite conversion factors (a) for general population and (b) for consumers only.

| Population | Intakes | Median (IQR) | p90 | p95 |
|---|---|---|---|---|
| **General population (N=4532)** | Nitrate (mg/kg bw/day) | 0 (0, 0) | 0 | 0.003 |
| | Nitrite (mg/kg bw/day) | 0 (0, 0.007) | 0.023 | 0.048 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 1% (mg/kg bw/day) | 0 (0, 0.007)) | 0.023 | 0.048 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 2.3% (mg/kg bw/day) | 0 (0, 0.007)) | 0.024 | 0.048 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 9% (mg/kg bw/day) | 0 (0, 0.007) | 0.023 | 0.049 |
| | Nitrite and nitrate intake[1] (% ADI[2]) | 0 (0, 10.0) | 32.9 | 70.0 |
| **Consumers only (N=2152, 47.5% of the general population)** | Nitrate (mg/kg bw/day) | 0 (0, 0) | 0.003 | 0.012 |
| | Nitrite (mg/kg bw/day) | 0.007 (0.003, 0.020) | 0.050 | 0.082 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 1% (mg/kg bw/day) | 0.007 (0.003, 0.020) | 0.050 | 0.082 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 2.3% (mg/kg bw/day) | 0.007 (0.003, 0.020) | 0.050 | 0.083 |
| | Nitrite and nitrate, with nitrate-to-nitrite conversion factor of 9% (mg/kg bw/day) | 0.007 (0.003, 0.020) | 0.051 | 0.083 |
| | Nitrite and nitrate intake[1] (% ADI[2]) | 10.0 (4.3, 28.6) | 72.9 | 118.6 |

[1.] Using nitrate-to-nitrite conversion factor of 9%
[2.] ADI of 0.07mg/kg bw/day for nitrite

**Tabel S5.** Distribution of daily nitrite intake (in mg/kg bw/day and as %of ADI), estimated in total and per sex and age group for consumers only.

| Age group (years) | N | % | Daily nitrite intake (mg/kg bw/day | | | | | | | | Contribution to ADI (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | p25 | p50 | p75 | p90 | p95 | p99[1] | Mean | SD | p25 | p50 | p75 | p90 | p95 | p99[1] | Mean | SD |
| **FEMALES** | **1144** | **53.3** | **0.003** | **0.007** | **0.018** | **0.044** | **0.067** | **0.309** | **0.022** | **0.064** | **4.3** | **10.0** | **25.7** | **62.9** | **95.7** | **441.4** | **31.4** | **91.4** |
| **Minors (<18)** | **170** | **14.8** | **0.005** | **0.014** | **0.032** | **0.062** | **0.134** | 0.359 | **0.032** | **0.065** | **7.1** | **20.0** | **45.7** | **88.6** | **191.4** | 512.9 | **45.7** | **92.9** |
| Children (0-9) | 85 | 7.4 | 0.006 | 0.019 | 0.034 | 0.081 | 0.161 | 0.359 | 0.037 | 0.059 | 8.6 | 27.1 | 48.6 | 115.7 | 230.0 | 512.9 | 52.9 | 84.3 |
| Adolescents (10-17) | 85 | 7.4 | 0.004 | 0.011 | 0.024 | 0.051 | 0.076 | 0.608 | 0.027 | 0.070 | 5.7 | 15.7 | 34.3 | 72.9 | 108.6 | 868.6 | 38.6 | 100.0 |
| **Adults (≥18)** | **974** | **85.2** | **0.003** | **0.007** | **0.015** | **0.038** | **0.064** | **0.309** | **0.020** | **0.064** | **4.3** | **10.0** | **21.4** | **54.3** | **91.4** | **441.4** | **28.6** | **91.4** |
| Young adults (18-30) | 442 | 38.7 | 0.003 | 0.008 | 0.019 | 0.048 | 0.077 | 0.332 | 0.025 | 0.081 | 4.3 | 11.4 | 27.1 | 68.6 | 110.0 | 474.3 | 35.7 | 115.7 |
| Adults (31-50) | 348 | 30.4 | 0.003 | 0.007 | 0.015 | 0.034 | 0.057 | 0.181 | 0.017 | 0.042 | 4.3 | 10.0 | 21.4 | 48.6 | 81.4 | 258.6 | 24.3 | 60.0 |
| Older adults (51-64) | 129 | 11.3 | 0.002 | 0.004 | 0.012 | 0.028 | 0.045 | 0.076 | 0.014 | 0.054 | 2.9 | 5.7 | 17.1 | 40.0 | 64.3 | 108.6 | 20.0 | 77.1 |
| Elderly (≥65) | 55 | 4.8 | 0.002 | 0.004 | 0.011 | 0.038 | 0.067 | 0.073 | 0.011 | 0.018 | 2.9 | 5.7 | 15.7 | 54.3 | 95.7 | 104.3 | 15.7 | 25.7 |
| **MALES** | **1001** | **46.7** | **0.003** | **0.007** | **0.022** | **0.058** | **0.098** | **0.220** | **0.023** | **0.049** | **4.3** | **10.0** | **31.4** | **82.9** | **140.0** | **314.3** | **32.9** | **70.0** |
| **Minors (<18)** | **206** | **20.6** | **0.006** | **0.014** | **0.030** | **0.075** | **0.126** | 0.195 | **0.029** | **0.042** | **8.6** | **20.0** | **42.9** | **107.1** | **180.0** | 278.6 | **41.4** | **60.0** |
| Children (0-9) | 105 | 10.5 | 0.009 | 0.020 | 0.046 | 0.115 | 0.177 | 0.195 | 0.040 | 0.051 | 12.9 | 28.6 | 65.7 | 164.3 | 252.9 | 278.6 | 57.1 | 72.9 |
| Adolescents (10-17) | 101 | 10.1 | 0.003 | 0.010 | 0.023 | 0.048 | 0.072 | 0.106 | 0.019 | 0.026 | 4.3 | 14.3 | 32.9 | 68.6 | 102.9 | 151.4 | 27.1 | 37.1 |
| **Adults (≥18)** | **795** | **79.4** | **0.003** | **0.006** | **0.019** | **0.054** | **0.090** | **0.296** | **0.022** | **0.051** | **4.3** | **8.6** | **27.1** | **77.1** | **128.6** | **422.9** | **31.4** | **72.9** |
| Young adults (18-30) | 338 | 33.8 | 0.003 | 0.008 | 0.023 | 0.058 | 0.097 | 0.319 | 0.025 | 0.056 | 4.3 | 11.4 | 32.9 | 82.9 | 138.6 | 455.7 | 35.7 | 80.0 |
| Adults (31-50) | 313 | 31.3 | 0.003 | 0.007 | 0.019 | 0.061 | 0.099 | 0.220 | 0.023 | 0.054 | 4.3 | 10.0 | 27.1 | 87.1 | 141.4 | 314.3 | 32.9 | 77.1 |
| Older adults (51-64) | 82 | 8.2 | 0.002 | 0.004 | 0.007 | 0.042 | 0.084 | 0.140 | 0.013 | 0.027 | 2.9 | 5.7 | 10.0 | 60.0 | 120.0 | 200.0 | 18.6 | 38.6 |
| Elderly (>=65) | 62 | 6.1 | 0.001 | 0.002 | 0.006 | 0.012 | 0.014 | 0.049 | 0.005 | 0.009 | 1.4 | 2.9 | 8.6 | 17.1 | 20.0 | 70.0 | 7.1 | 12.9 |
| **TOTAL** | **2152** | **100** | **0.003** | **0.007** | **0.020** | **0.051** | **0.083** | **0.266** | **0.022** | **0.058** | **4.3** | **10.0** | **28.6** | **72.9** | **118.6** | **380.0** | **31.4** | **82.9** |
| **Minors (<18)** | **378** | **17.5** | **0.005** | **0.014** | **0.030** | **0.072** | **0.126** | **0.241** | **0.030** | **0.053** | **7.1** | **20.0** | **42.9** | **102.9** | **180.0** | **344.3** | **42.9** | **75.7** |
| Children (0-9) | 190 | 8.8 | 0.008 | 0.02 | 0.042 | 0.102 | 0.173 | 0.269 | 0.038 | 0.055 | 11.4 | 28.6 | 60.0 | 145.7 | 247.1 | 384.3 | 54.3 | 78.6 |
| Adolescents (10-17) | 188 | 8.7 | 0.004 | 0.01 | 0.023 | 0.049 | 0.076 | 0.19 | 0.022 | 0.051 | 5.7 | 14.3 | 32.9 | 70.0 | 108.6 | 271.4 | 31.4 | 72.9 |
| **Adults (≥18)** | **1774** | **82.5** | **0.003** | **0.007** | **0.017** | **0.046** | **0.078** | **0.296** | **0.021** | **0.058** | **4.3** | **10.0** | **24.3** | **65.7** | **111.4** | **422.9** | **30.0** | **82.9** |
| Young adults (18-30) | 782 | 36.3 | 0.003 | 0.008 | 0.022 | 0.052 | 0.087 | 0.332 | 0.025 | 0.071 | 4.3 | 11.4 | 31.4 | 74.3 | 124.3 | 474.3 | 35.7 | 101.4 |
| Adults (31-50) | 664 | 30.9 | 0.003 | 0.007 | 0.017 | 0.041 | 0.079 | 0.22 | 0.02 | 0.048 | 4.3 | 10.0 | 24.3 | 58.6 | 112.9 | 314.3 | 28.6 | 68.6 |
| Older adults (51-64) | 211 | 9.8 | 0.002 | 0.004 | 0.01 | 0.03 | 0.056 | 0.113 | 0.014 | 0.045 | 2.9 | 5.7 | 14.3 | 42.9 | 80.0 | 161.4 | 20.0 | 64.3 |
| Elderly (>=65) | 117 | 5.5 | 0.001 | 0.003 | 0.007 | 0.013 | 0.048 | 0.067 | 0.008 | 0.014 | 1.4 | 4.3 | 10.0 | 18.6 | 68.6 | 95.7 | 11.4 | 20.0 |

1. Calculations at the 99th percentiles when the number of subjects is lower than 300 (children, adolescents, older adults and elderly) have been indicated with grey colour, as those results may not be statistically robust and should be interpreted cautiously [1]

**Table S6.** Likelihood of exceeding ADI of nitrite from total nitrite intake from processed meat products.

| Level of nitrite intake | Odds Ratio | Std. Err. | z | P>z | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| Age group | | | | | | |
| 31-50 | 0.777991 | 0.194701 | -1 | 0.316 | 0.476379 | 1.270566 |
| 51-64 | 0.6998 | 0.317109 | -0.79 | 0.431 | 0.287911 | 1.700942 |
| 65+ | 0.238563 | 0.285731 | -1.2 | 0.231 | 0.022809 | 2.495178 |
| Sex | 1.285699 | 0.370155 | 0.87 | 0.383 | 0.731268 | 2.260485 |
| Weight | 0.999971 | 0.008643 | 0 | 0.997 | 0.983173 | 1.017056 |
| Employment status | | | | | | |
| Pension | 0.725481 | 0.553252 | -0.42 | 0.674 | 0.162742 | 3.234091 |
| Unemployed | 0.894391 | 0.231454 | -0.43 | 0.666 | 0.538579 | 1.485268 |
| Total energy intake | 1.000468 | 0.000113 | 4.14 | 0 | 1.000246 | 1.000689 |
| Sodium intake category | | | | | | |
| >=1500 & <2300 | 2.672544 | 2.743197 | 0.96 | 0.338 | 0.357454 | 19.98156 |
| >=2300 | 3.117198 | 3.242189 | 1.09 | 0.274 | 0.405912 | 23.93849 |
| Med diet category | | | | | | |
| MD>=23 | 0.567615 | 0.134508 | -2.39 | 0.017 | 0.356734 | 0.903157 |

**Table S7.** Proportion of population per frequency of consumption of processed meat products, as reported in HNNHS, among (a) consumers and (b) consumers with total nitrite intake exceeding the ADI of 0.07mg/kw bw/day.

| Frequency of consumption of processed meat products | (a) Consumers (%) | (b) Consumers with total nitrite intake ≥ADI (%) |
|---|---|---|
| Less than once a month | 3.0 | 0 |
| 1 to 3 times per month | 22.95 | 0 |
| once a week | 54.32 | 6.29 |
| 2-4 times a week | 17.34 | 39.16 |
| 5-6 times a week | 1.65 | 16.78 |
| every day | 0.48 | 19.58 |
| 2 to 3 times a day | 0.20 | 12.59 |
| 4 to 5 times a day | 0.05 | 4.90 |
| 5 to 6 times a day | 0.01 | 0.70 |
| Total | 100.00 | 100.00 |

**Table S8.** Consumers with total nitrite intake from processed meat products above the ADI of nitrite (0.07mg/kg bw/day) per sex and age group in number of individuals (n), as a percentage of individuals of the same age group [a=(n/N) × 100] and as a percentage of total number of individuals exceeding ADI (n=143) [b=(n/143) ) × 100].

| Age group | N | Consumers with nitrite intake above ADI | | |
|---|---|---|---|---|
| | | n | (a) % of consumers within the same sex/age group | (b) % of total consumers exceeding ADI |
| **Females** | **1144** | **56** | **4.9** | **39.2** |
| Children (0-9y) | 85 | 10 | 11.8 | 7.0 |
| Adolescents (10-17y) | 85 | 5 | 5.9 | 3.5 |
| Young adults (18-30y) | 442 | 26 | 5.9 | 18.2 |
| Adults (31-50y) | 348 | 12 | 3.4 | 8.4 |
| Older adults (51-64y) | 129 | 2 | 1.6 | 1.4 |
| Elderly (>=65y) | 55 | 1 | 1.8 | 0.7 |
| **Males** | **1001** | **87** | **8.7** | **60.8** |
| Children (0-9y) | 105 | 19 | 18.1 | 13.3 |
| Adolescents (10-17y) | 101 | 7 | 6.9 | 4.9 |
| Young adults (18-30y) | 338 | 27 | 8.0 | 18.9 |
| Adults (31-50y) | 313 | 28 | 8.9 | 19.6 |
| Older adults (51-64y) | 82 | 6 | 7.3 | 4.2 |
| Elderly (>=65y) | 62 | 0 | 0.0 | 0.0 |
| **Total** | **2152** | **143** | **6.6** | **100.0** |
| **<18 years** | **378** | **41** | **10.9** | **28.7** |
| Children (0-9y) | 190 | 29 | **15.3** | 20.3 |
| Adolescents (10-17y) | 188 | 12 | 6.4 | 8.4 |
| **>=18 years** | **1774** | **102** | **5.7** | **71.3** |
| Young adults (18-30y) | 782 | 53 | 6.8 | 37.1 |
| Adults (31-50y) | 664 | 40 | 6.0 | 28.0 |
| Older adults (51-64y) | 211 | 8 | 3.8 | 5.6 |
| Elderly (>=65y) | 117 | 1 | 0.9 | 0.7 |
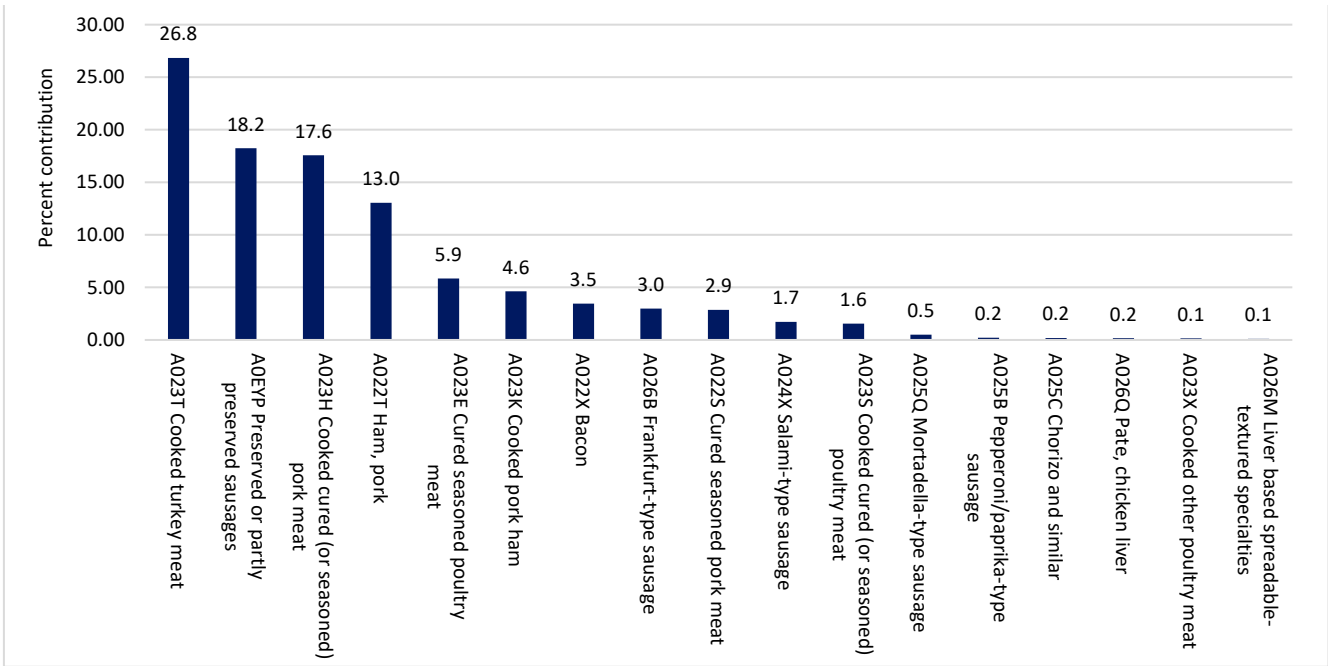
**Figure S1**. Main FoodEx2 group contribution (%) to total nitrite intakeamong processed meat products.

**Table S9.** Contribution (%) of meat type and FoodEx2 food groups to total nitrite exposure per age group.

| FOODEX CATEGORY<br>Food Group (if different than FOODEX Category)<br>Food Subgroup (if any)<br>FoodEx2 Category | Age group (years) | | | | | | TOTAL |
|---|---|---|---|---|---|---|---|
| | **0-9** | **10-17** | **18-30** | **31-50** | **51-64** | **65+** | |
| **PRESERVED MEAT** | | | | | | | |
| **Pork meat** | **51.91** | **39.05** | **38.23** | **40.45** | **46.77** | **39.57** | **41.53** |
| **Pork meat, ham** | **21.84** | **18.01** | **17.19** | **14.82** | **21.47** | **21.43** | **17.66** |
| A022T Ham, pork | 17.98 | 14.72 | 11.05 | 12.26 | 12.53 | 20.90 | 13.04 |
| A023K Cooked pork ham | 3.86 | 3.29 | 6.14 | 2.55 | 8.94 | 0.53 | 4.62 |
| **Pork meat, bacon** | **4.40** | **3.43** | **3.32** | **3.60** | **2.34** | **0.63** | **3.46** |
| A022X Bacon | 4.40 | 3.43 | 3.32 | 3.60 | 2.34 | 0.63 | 3.46 |
| **Pork meat, other** | **25.67** | **17.61** | **17.72** | **22.04** | **22.95** | **17.51** | **20.41** |
| A023H Cooked cured (or seasoned) pork meat | 23.65 | 15.91 | 14.37 | 19.25 | 18.71 | 16.72 | 17.56 |
| A022S Cured seasoned pork meat | 2.02 | 1.70 | 3.35 | 2.79 | 4.24 | 0.79 | 2.85 |
| **Poultry meat** | **29.23** | **30.07** | **39.08** | **35.28** | **22.27** | **21.07** | **34.39** |
| **Poultry meat, turkey** | **25.87** | **29.54** | **37.35** | **34.24** | **19.99** | **20.01** | **32.68** |
| A023T Cooked turkey meat | 24.99 | 27.88 | 28.21 | 29.23 | 12.70 | 17.80 | 26.83 |
| A023E Cured seasoned poultry meat | 0.88 | 1.66 | 9.14 | 5.01 | 7.29 | 2.21 | 5.85 |
| **Poultry meat, other (chicken)** | **3.36** | **0.53** | **1.73** | **1.04** | **2.27** | **1.06** | **1.71** |
| A023S Cooked cured (or seasoned) poultry meat | 2.55 | 0.53 | 1.70 | 0.99 | 2.27 | 1.06 | 1.56 |
| A023X Cooked other poultry meat | 0.82 | 0.00 | 0.03 | 0.05 | 0.00 | 0.00 | 0.15 |
| **SAUSAGES** | **17.82** | **30.88** | **22.68** | **23.92** | **30.94** | **39.36** | **23.82** |
| A024X Salami-type sausage | 3.96 | 0.39 | 0.95 | 2.11 | 1.95 | 0.16 | 1.72 |
| A025B Pepperoni/paprika-type sausage | 0.71 | 0.00 | 0.12 | 0.14 | 0.31 | 0.17 | 0.22 |
| A025C Chorizo and similar | 0.00 | 0.32 | 0.35 | 0.01 | 0.03 | 0.02 | 0.18 |
| A025Q Mortadella-type sausage | 0.00 | 0.00 | 0.36 | 0.26 | 4.01 | 1.96 | 0.50 |
| A026B Frankfurt-type sausage | 1.88 | 0.82 | 4.12 | 3.42 | 0.18 | 0.05 | 2.98 |
| A0EYP Preserved or partly preserved sausages | 11.26 | 29.35 | 16.78 | 17.99 | 24.46 | 37.00 | 18.23 |
| **MEAT SPECIALTIES** | **1.04** | **0.00** | **0.01** | **0.35** | **0.02** | **0.00** | **0.26** |
| A026M Liver based spreadable-textured specialties | 0.00 | 0.00 | 0.01 | 0.35 | 0.02 | 0.00 | 0.10 |
| A026Q Pate, chicken liver | 1.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.16 |

**Table S10.** Qualitative evaluation of influence of uncertainties on the dietary exposure estimated.

| Sources of uncertainties | | Direction |
|---|---|---|
| 1. | Regulatory maximum level exposure assessment scenario (food categories authorized at MPL according to Annex II to Regulation (EC) No 1333/2008 | + |
| 2. | Extrapolation from recipes to their ingredients. | ± |
| 3. | Uncertainty of nitrate-to-nitrite conversion factor | ± |
| 4. | Misclassification of meat products; incorrect FoodEx coding and mismatch with food categories of Regulation (EC) 1333/2008 | ± |
| 5. | No use of procession factors | ± |

+, uncertainty with potential to cause overestimation of exposure

-, uncertainty with potential to cause underestimation of exposure

**Reference**

1.  EFSA Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. *EFSA J.* **2011**, *9*, doi:10.2903/j.efsa.2011.2097.

# PAPER III ▪ NITRITE AND NITRATE INTAKE FROM PROCESSED MEAT IS ASSOCIATED WITH ELEVEATED DIASTOLIC BLOOD PRESSURE (DBP)

Original article

# Nitrite and nitrate intake from processed meat is associated with elevated diastolic blood pressure (DBP)

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## ARTICLE INFO

## SUMMARY

Background and aims: Processed meat consumption has been linked to high blood pressure (BP), a key risk factor for cardiovascular disease (CVD), but gaps remain with regards to the ingredients which contribute to this association. This study, therefore, aimed to examine the association between nitrite and nitrate intake from processed meat with diastolic (DBP) and systolic (SBP) blood pressure, while accounting for sodium intake.
Methods: Dietary nitrite and nitrate intake from processed meat, assessed as total nitrite equivalent, was estimated for 1774 adult, processed meat consumers (≥18 years, 55.1% females) who had enrolled in the Hellenic National Nutrition and Health Survey (HNNHS). To avoid selection and reverse causality bias, associations with measured DBP and SBP were considered instead of self-reported data of hypertension presence. Participants were divided by tertile of dietary nitrite intake and by level of dietary guideline adherence for sodium (<1500; 1500−2300; ≥2300). Multiple regression models were used to examine associations with SBP and DBP, including an interaction term of nitrite with dietary sodium intake, for potential synergy.
Results: Overall, DBP increased by 3.05 mmHg (95% CI: 0, 6.06), per tertile increase in nitrite intake and 4.41 mmHg (95% CI: 0.17, 8.64) per level increase in sodium intake, when the interaction effect between nitrite and total sodium intakes was accounted for. By considering the significant synergistic effect of the two factors, DBP finally increased by 0.94 mmHg overall and 2.24 mmHg for subjects in the third tertile compared to those in the first. Also, a rise in total sodium intake of approximately 800 mg, above 1500 mg, caused a 2.30 mmHg increase in DBP. No significant correlations were found with SBP.
Conclusions: Higher nitrite and nitrate intake from processed meats contributed to the increase of DBP, but the interaction effect with total sodium intake levels should be accounted for to properly interpret the findings.

## 1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality globally [1], with the majority of events being attributed to specific health behaviors and factors [2]. Hypertension, or elevated blood pressure (BP), is one such known health risk factor for CVD [3,4], and therefore can be considered a notable public health problem that needs to be mitigated. Hypertensive individuals aged 30−79 years old have doubled worldwide,

although detection, treatment and control rates vary greatly across countries [5]. The proportion of individuals that are aware, treated, and/or have controlled blood pressure levels remains low [6]. In fact, 46% of hypertensive adults worldwide (41% of women and 51% of men) are not aware of their condition, more than half of hypertensive individuals (53% of women and 62% of men) are not properly treated and only 1 in 4 women and 1 in 5 men with hypertension have their blood pressure effectively controlled with medication [5].

Lifestyle habits have also been associated with CVD, including related health behaviors such as smoking, level of physical activity, and diet [2], with the latter having attracted much interest [7,8]. Healthy dietary patterns, high in fruits, vegetables, seeds, whole grains, legumes, fish, and low in sweets, alcohol and non-processed

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dairy & meat products, may reduce BP or help to avoid/delay the occurrence of hypertension and thus lower the risk of CVD [9,10]. Ultra-processed foods consumption has been linked with an elevated risk of CVD [11] and overall mortality [12], with higher processed meat intake specifically being one of the main contributors to this finding [13−15]. The role of processed meat on CVD therefore requires further investigation.

Processed meat is defined as a product that is cured, salted, fermented or smoked, in order to improve its durability, color and/or taste [16]. It may contribute to elevated CVD risk due to the presence of preservatives [17], not naturally found in their non-processed alternatives, such as sodium which has been suggested as the underlying mechanism for the somewhat different association observed between unprocessed and processed red meat and CVD [18], and additives, potentially nitrite [19]. Nitrite (potassium nitrite-E249 and sodium nitrite-E250) and nitrate (sodium nitrate-E251 and potassium nitrate-E252) [20] are the most commonly used additives to improve the visual appeal, taste, safety, and quality in processed meat products [21]. The importance of nitrate in assessing the pathways related to health risks has been already indicated by *Etemadi* et al., who reported that 72% of CVD deaths were accounted for by nitrate intake from processed red meat consumption [22]. However, the authors neither studied the effect specifically on BP nor considered the effect of sodium (or salt) in their analysis. Although, nitrite and nitrate, as well as sodium, have been suggested as major contributors to Coronary Heart Disease (CHD) risk [23], the link between CVD and nitrite/nitrate intake from processed meat products in relation to sodium remains understudied. Additionally, the relationship of ingredients found in processed meat with major risk factors for CVD, such as hypertension, remains unclear [14], although its understanding is crucial for public health to guide consumer choices, set and prioritize dietary guidelines, and inform and support competent authorities with food reformulations to reduce risks [17]. As a result, the aim of this study was to investigate the association between dietary nitrite and nitrate intake from processed meat and BP, accounting for important confounding factors that may play a role in CVD causation [24], such as sodium intake.

## 2. Methods

### 2.1. Study design

The study included participants from the Hellenic National Nutrition and Health Survey (HNNHS), a population-based, nationally representative health study carried out in Greece from September 2013 to May 2015. The sampling was based on age, sex, and geographical density as determined by the Hellenic Statistical Authority. Pregnant and lactating women, institutionalized individuals, and military servants were excluded from the Survey. For this study, a total of 1774 adult consumers of processed meat (≥18 years, 45% males) were included (46.6% of the adult population participating in HNNHS, n = 3803).

Trained professionals, utilizing Computer Assisted Personal Interview (CAPI) software, interviewed the participants who had provided informed consent and recorded sociodemographic and lifestyle data, as well as use of medication. Detailed information on the Survey and the used questionnaires has been published elsewhere [25,26]. All activities were in accordance with the Declaration of Helsinki and were approved by the Hellenic Data Protection Authority (HDPA) and the Ethics Committee of the Department of Food Science and Human Nutrition of the Agricultural University of Athens.

### 2.2. Hypertension definition and measurements

According to World Health Organization (WHO) and European Society of Cardiology (ESC)/European Society of Hypertension (ESH), hypertension, commonly referred to as high or raised BP for epidemiological studies, is defined as a persistent elevation in SBP ≥ 140 mmHg and/or DBP≥90 mmHg [9,27], stating that in most patients a BP of at least 130/80 mmHg is suggested but not less than 120/70 mmgHg (often referred to as prehypertensive). The latter is in line with the new and stricter definition for hypertension used by the American College of Cardiology (ACC)/American Heart Association (AHA); SBP ≥ 130 mmHg and/or DBP ≥ 80 mmHg [28,29].

BP measurements were performed by trained medical assistants following guidelines recommended by the ESH and the ESC [30]. In detail, BP was measured using validated and calibrated auscultatory devices (Omron Hem 907, Vernon Hills, IL, USA) and appropriately sized cuffs (small, medium, and large cuff sizes). Participants were asked to sit with their backs upright and their arms well-supported at a 45-degree angle from the trunk at the heart level. Individuals' BP was assessed based on three consecutive measures given on the same day. To characterize and report the study population's mean SBP and DBP values, the average values of these measurements were employed. Hypertension was defined as an average BP of at least 140/90 mmHg or on antihypertensive treatment [31]. BP measurements (addressed below) were performed for 29.3% of participants (n = 519, 41% males), who had provided informed consent. The remaining population reported their blood pressure status using the CAPI method. Specifically, individuals were asked to report whether they have been diagnosed with hypertension by a physician in the past. They were also asked to report if they were on any medication for high blood pressure. Individuals' response was cross validated with the list of medications that the individuals were asked to name during the study (general question: do you take any medications? If yes, which one? Do you take any other medications?). Non-measured individuals were classified as hypertensive if they reported as having been diagnosed or if they were on any anti-hypertensive treatment.

### 2.2.1. Definitions for prehypertensives, individuals with abnormal BP, and individuals unaware of abnormal BP

For the purpose of this study and based on measurements of BP, prehypertensives were defined as those whose BP was at least 130/80 mmHg and less than 140/90 mmHg; individuals with abnormal BP as those with a BP of at least 130/80 mmHg (including prehypertensives and hypertensives); and individuals unaware of abnormal BP as those who reported negatively as having hypertension but were found to have an abnormal BP.

### 2.3. Dietary and processed meat nitrite and nitrate intake assessment

The present regulatory maximum level exposure assessment considered 16 FoodEx2 food categories to which the corresponding MPLs were assigned [20,32] (Table S1). The methodology followed for the exposure assessment has been described in detail elsewhere [33]. In short, two 24-h recalls were obtained using the Automated Multiple Pass Method, one at the initial face-to-face interview and the second during a telephone interview following 8−20 days on a different non-consecutive day. Specific, validated food atlases and standardized household measurements were employed as portion anchors for the appropriate processed meat intake assessment. The frequency of processed meat consumption, using a validated Frequency Propensity Questionnaires (FPQs) [26], was used to quantify processed meat intake over time, including processed meat consumed in whole meals or in mixed dishes (added in recipes).

The highest nitrate-to-nitrite endogenous conversion factor of 9% was used, as recommended by EFSA [34]. The total nitrite equivalent (including nitrate) was divided with individual body weight (kg) to determine chronic dietary exposure to nitrite from processed meat consumption (in mg/kg bw/day).

Information on other nutrients associated with BP were also derived. Total fat, trans fatty acid (TFA) and saturated fat acid (SFA) intakes, as % of total energy intakes, were estimated based on mean intake from foods in grams per day, amount that was converted to kilocalories/day (kcal/day) and was finally adjusted by mean total energy intake ([gr/day $\times$ 9 kcal per gram]/mean total energy intake in kcal) $\times$ 100. Details have been published elsewhere [35]. Sodium intake from food (salt added during cooking or at the table not included) was also obtained in milligrams per day (mg/day). This was further categorized based on recommended intakes as <1500, $\geq$1500 & <2300 and $\geq$2300 mg/day, as per recommendations by the American Heart Association (AHA: 1500 mg, strict guidelines) and Institute of Medicine of the National Academies (IOM) as well as US Department of Agriculture (USDA) (tolerable upper intake level <2300 mg/day) [36—38]. Adherence to the Mediterranean diet was estimated to account for total dietary pattern followed. This was evaluated using the MedDiet score, which ranges from 0 to 55 [39]. The variable was dichotomized to two final MedDiet categories, <23 and $\geq$23, based on the median value of the population, since research has shown that for every 11 units rise in MedDiet score, the odds of having an acute coronary event decreased by 37% [40].

### 2.4. Other parameters

Information on educational level and lifestyle were also collected. Three categories of education levels were established: up to six years of formal education, twelve years of formal education, and higher education (including colleges). Employment status was divided in the following categories: employed, pension and unemployed. Marital status was dichotomized to two categories: divorced, separated, widowed, single and married, consented individuals. Additionally assessed were smoking habits and levels of physical activity. Individuals were classified as never smokers, smokers, or ex-smokers if they had abstained from smoking for at least 30 days when included in the descriptive tables, and as smokers or non-smokers, when included in the models. Physical activity (PA) was defined using the International Physical Activity Questionnaire (IPAQ) [41]. People who scored below the high activity level were given the label of sedentary. Based on weight (kg) and height (m) measurements, Body Mass Index (BMI) was used [weight/height$^2$ (kg/m$^2$)] to define the individuals weight status; (i) Healthy weight: BMI $\leq$ 25 kg/m$^2$ since no individual was found to have a BM < 17.0 kg/m$^2$, (ii) overweight: 25 $\geq$ BMI < 30 kg/m$^2$, and (iii) obese: BMI $\geq$ 30 kg/m$^2$. Dyslipidemia was defined if total triglycerides>150 mg/dl and/or total cholesterol > 200 mg/dl or on lipid-lowering medication and diabetes as fasting blood glucose 125 mg/dl or if on diabetic medication [31].

### 2.5. Statistical analysis

All data statistical analyses were carried out using the STATA 13.0 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP) statistical software. Missing data for age, weight, and consumption frequency were imputed with Multiple Imputation technique. Normality was determined using P−P and Kernel density plots for all continuous data. If the continuous variables were normally distributed, they were presented as means (SD) and if skewed, they were presented as median (interquartile range, IQR). The nonparametric Kruskall−Wallis rank sum and

ANOVA tests were employed to test group differences for skewed and continuous variables, respectively. Categorical variables were presented as relative frequencies (n, %) and Chi square test was used to assess between group distribution differences.

Nitrite intake (main exposure) was categorized in tertiles of consumption, and the associations (*p*-value and *p*-trend) with the following parameters were examined: age, age categories, sex, total processed meat intake, total energy intake, total fat intake, total SFA intake, BMI and BMI category (weight status), area of residence, educational level, employment status, marital status, MedDiet score and categories, physical activity status, total sodium intake and sodium intake categories, smoking status (ex-smoker, current smoker, never smoker), hypertension prevalence, SBD and DBD measured mean values, dyslipidemia, CVD as well as Type 2 Diabetes (T2D) prevalence.

Further analysis assessed associations with mean values of SBP and DBP (outcome/independent variable) by using linear regression models adjusted for a-priori known factors. Nitrite intake tertiles and total sodium intake levels were the main dependent variables introduced in the model. An interaction term between nitrite intake tertiles and total sodium intake levels was examined in a separate model, to assess their potential additive effects on BP (the main outcome). All models were further adjusted with more parameters known to be associated with BP, to limit confounding effects. Specifically, the following confounders were included: (a) age, total SFA (energy adjusted), and mean total energy (to account for residual confounding of energy variation), all introduced as continuous variables, and (b) sex, educational level, BMI category (weight status), marital status, MedDiet categories, smoking status and physical activity level, all introduced as categorical variables. Statistical significance was defined as a *p*-value $\leq$ 0.05 and *p*-for trend was tested post hoc.

### 2.5.1. Sensitivity analyses

Additional statistical analyses were performed, using multiple logistic regression models to investigate odds of being hypertensive, based on self-reporting as well as being prehypertensive, with abnormal BP or unaware of abnormal BP, as defined in Section 2.2.1 of the present study, versus normotensive individuals. These analyses were performed taking into consideration all factors accounted for in linear regression models conducted as well as the interaction between nitrite intake tertiles and total dietary sodium levels.

## 3. Results

In Table 1, data on anthropometric, dietary, demographic, and other personal characteristics, are depicted in total and by tertile of total nitrite intake. Median nitrite intake from processed meat was 0.007 (0.003, 0.017) mg/kg bw/day, ranging from 0.002 mg/kg bw/day in the first tertile to 0.026 mg/kg bw/day in the third tertile. Mean DBP was 72.1 ($\pm$10.7) mmHg and mean SBP was 118 ($\pm$14.5) mmHg.

Information regarding hypertensive status was missing for 4.4% (n = 78) of the study population. Out of the remaining (n = 1696), a proportion of 89.9% (n = 1524) reported being normotensive and 10.1% (n = 172) being hypertensive. In addition, hypertensive individuals accounted for 7.2% (n = 36) of those having their BP measured (n = 519). Prehypertensives, individuals with abnormal BP, and those unaware of having abnormal BP, as defined earlier in this study, accounted for 27.6% (n = 143), 28.5% (n = 148) and 23.7% (n = 123) of those having their BP measured (n = 519), respectively.

Hypertensive individuals were found to consume significantly lower nitrite from processed meat (*p* < 0.001, *p*-for trend<0.001), although no significant differences were found in measured DBPs

**Table 1**
Main anthropometric, dietary, demographic, and other personal characteristics in total and by tertile of total total nitrite equivalent intake.

| Variables | Population N = 1774; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.007 (0.003, 0.017)) | Total nitrite & nitrate intake tertiles of adult consumers | | | *p*-Value | *p*-trend |
|---|---|---|---|---|---|---|
| | | 1st Tertile N = 595; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.002 (0.001, 0.003) | 2nd Tertile N = 588; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.007 (0.005, 0.008) | 3rd Tertile N = 591; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.026 (0.017, 0.054) | | |
| Age (years), median (25th, 75th percentile) | 33.0 (25.0, 45.0) | 37.0 (27.0, 52.0) | 32.0 (24.0, 43.0) | 30.0 (24.0, 39.0) | <0.001 | <0.001 |
| Age categories, n (%) | | | | | <0.001 | <0.001 |
| 18−30 years | 782 (44.1) | 212 (35.6)[a] | 263 (44.7) | 307 (51.9)[a] | | |
| 31−50 years | 664 (37.4) | 213 (35.8) | 233 (39.6) | 218 (36.9) | | |
| 51−64 years | 211 (11.9) | 106 (17.8)[a] | 57 (9.7) | 48 (8.1)[a] | | |
| ≥65 years | 117 (6.6) | 64 (10.8)[a] | 35 (6.0) | 18 (3.1)[a] | | |
| Sex, n (%) | | | | | 0.917 | 0.898 |
| Females | 974 (55.1) | 324 (54.5) | 326 (55.7) | 324 (54.9) | | |
| Males | 795 (44.9) | 270 (45.5) | 259 (44.3) | 266 (45.1) | | |
| BMI (kg/m²), mean (sd) | 25.1 (4.7) | 25.7 (4.9) | 24.9 (4.7) | 24.5 (4.4) | <0.001 | <0.001 |
| BMI Category (weight status), n (%) | | | | | <0.001 | <0.001 |
| Healthy weight | 977 (56.6) | 284 (49.1)[a] | 336 (58.7) | 357 (62.1)[a] | | |
| Overweight | 507 (29.4) | 191 (33.1.) | 159 (27.8) | 157 (27.3) | | |
| Obese | 241 (14.0) | 103 (17.8)[a] | 77 (13.5) | 61 (10.6) | | |
| Hypertension, n (%) | | | | | <0.001 | <0.001 |
| Yes | 172 (10.1) | 90 (15.8)[a] | 41 (7.3) | 41 (7.2) | | |
| No | 1524 (89.9) | 479 (84.2)[a] | 520 (92.7) | 525 (92.8) | | |
| Systolic BP in mmHg, mean (sd) | 118.0 (14.5) | 118 (16.0) | 118.1 (14.5) | 117.8 (12.7) | 0.971 | 0.589 |
| Diastolic BP in mmHg, mean (sd) | 72.1 (10.7) | 72.4 (11.0) | 72.5 (10.1) | 71.5 (11.2) | 0.688 | 0.919 |
| Dyslipidemia, n (%) | | | | | 0.114 | <0.05 |
| Yes | 405 (25.4) | 149 (27.8) | 138 (26.1) | 118 (22.4) | | |
| No | 1188 (74.6) | 387 (72.2) | 391 (73.9) | 410 (77.6) | | |
| CVD, n (%) | | | | | <0.05 | <0.05 |
| Yes | 42 (2.5) | 21 (3.8) | 15 (2.7) | 6 (1.1) | | |
| No | 1621 (97.5) | 538 (96.2) | 536 (97.3) | 547 (98.9) | | |
| Diabetes mellitus, n (%) | | | | | <0.05 | <0.05 |
| Yes | 47 (2.8) | 25 (4.4) | 10 (1.8) | 12 (2.2) | | |
| No | 1626 (97.2) | 539 (95.6) | 543 (98.2) | 544 (97.8) | | |
| Total sodium (mg), mean (sd) | 2303.0 (690.1) | 2120.9 (570.0) | 2292.2 (629.2) | 2495.4 (799.2) | <0.001 | <0.001 |
| Sodium intake, n (%) | | | | | <0.001 | <0.001 |
| ≤1500 mg/d | 104 (6.4) | 57 (10.5)[a] | 31 (5.7) | 16 (2.9)[a] | | |
| ≥1500 & <2300 mg/d | 851 (52.3) | 322 (59.0)[a] | 288 (53.4) | 241 (44.4)[a] | | |
| ≥2300 mg/d | 673 (41.3) | 166 (30.5)[a] | 221 (40.9) | 286 (52.7)[a] | | |
| Total processed meat intake (g), median (25th, 75th percentile) | 2.9 (1.3, 8.1) | 0.9 (0.4, 1.3) | 2.9 (2.2, 4.0) | 12.2 (8.1, 24.3) | <0.001 | <0.001 |
| Total fat intake, % energy, mean (sd) | 38.12 (9.46) | 37.58 (9.26) | 37.95 (9.66) | 38.82 (9.44) | 0.079 | <0.05 |
| Total TFA intake, % energy, median (25th, 75th percentile) | 0.55 (0.38, 0.80) | 0.50 (0.35, 0.76) | 0.57 (0.40, 0.80) | 0.58 (0.41, 0.81) | <0.05 | <0.05 |
| Total SFA intake, % energy, mean (sd) | 13.41 (4.02) | 13.05 (3.87) | 13.41 (4.16) | 13.78 (3.98) | <0.05 | <0.001 |
| Total energy intake (kcal/day), median (25th, 75th percentile) | 19,445 (1475, 2585) | 1812 (1366, 2411) | 1902 (1440, 2480) | 2163 (1614, 2892) | <0.001 | <0.001 |
| MedDiet Score, mean (sd) | 27.0 (6.4) | 27.7 (6.3) | 27.0 (6.5) | 26.3 (6.2) | <0.001 | <0.001 |
| MedDiet Category n (%) | | | | | 0.062 | <0.05 |

*(continued on next page)*

**Table 1** (*continued*)

| Variables | Population N = 1774; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.007 (0.003, 0.017)) | Total nitrite & nitrate intake tertiles of adult consumers | | | p-Value | p-trend |
|---|---|---|---|---|---|---|
| | | 1st Tertile N = 595; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.002 (0.001, 0.003) | 2nd Tertile N = 588; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.007 (0.005, 0.008) | 3rd Tertile N = 591; Total nitrite and nitrate intake, mg/kg bw/day, median (p25, p75): 0.026 (0.017, 0.054) | | |
| MD < 23 | 373 (22.9) | 106 (19.4) | 134 (24.8) | 133 (24.5) | | |
| MD ≥ 23 | 1255 (77.1) | 439 (80.6) | 406 (75.2) | 410 (75.5) | | |
| Physical activity status, n (%) | | | | | 0.060 | 0.056 |
| Sedentary | 101 (6.1) | 33 (5.9) | 35 (6.4) | 33 (6.0) | | |
| Low | 255 (15.5) | 105 (18.8) | 87 (16.0) | 63 (11.5) | | |
| Moderate | 624 (37.8) | 202 (36.1) | 19 (36.6) | 223 (40.8) | | |
| Very | 670 (40.6) | 219 (39.2) | 223 (41.0) | 228 (41.7) | | |
| Smoking status, n (%) | | | | | 0.446 | 0.816 |
| Ex-smoker | 257 (14.6) | 95 (16.1) | 88 (15.2) | 74 (12.6) | | |
| Current smoker | 656 (37.3) | 214 (36.1) | 222 (38.3) | 220 (37.4) | | |
| Never smoker | 847 (48.1) | 283 (47.8) | 270 (46.5) | 294 (50.0) | | |
| Area of residence, n (%) | | | | | <0.001 | 0.112 |
| Attiki & Thessaloniki | 1238 (70.5) | 391 (66.4) | 440 (75.7)[a] | 407 (69.4) | | |
| Islands (including Crete) | 165 (9.4) | 52 (8.8) | 43 (7.4) | 70 (12.0) | | |
| Mainland | 353 (20.1) | 146 (24.8)[a] | 98 (16.9) | 109 (18.6) | | |
| Educational level, n (%) | | | | | <0.001 | 0.464 |
| Up to 6 years of school | 88 (5.1) | 52 (9.0)[a] | 21 (3.7) | 15 (2.6)[a] | | |
| 12 years of school | 661 (38.4) | 223 (38.5) | 229 (39.9) | 209 (36.7) | | |
| Higher education, including colleges | 974 (56.5) | 304 (52.5) | 324 (56.4) | 346 (60.7) | | |
| Employment status, n (%) | | | | | <0.001 | 0.672 |
| Employed | 1009 (58.5) | 324 (56.0) | 33 (58.3) | 351 (61.4) | | |
| Pension | 180 (10.5) | 98 (16.9)[a] | 50 (8.7) | 32 (5.6)[a] | | |
| Unemployed | 535 (31.0) | 157 (27.1) | 189 (33.0) | 189 (33.0) | | |
| Marital Status, n (%) | | | | | <0.001 | <0.001 |
| Divorced/ separated/ widowed/ single | 1044 (62.0) | 301 (53.0)[a] | 354 (63.3) | 389 (70.0)[a] | | |
| Married/ consented | 639 (38.0) | 267 (47.0)[a] | 205 (36.7) | 167 (30.0)[a] | | |

[a] Statistical significant at p ≤ 0.05. Continuous variables were presented as mean and standard deviation (sd) when normally distributed and median and interquartile range (IQR: 25th percentile, 75th percentile) when skewed. Categorical variables were presented as frequencies. Group differences were tested using chi square test for proportions and Kruskall–Wallis rank sum or ANOVA test depending on data distribution. Level of significance was set at alpha = 5%. Physical activity (PA) was defined according to the International Physical Activity Questionnaire (IPAQ). Body Mass Index (BMI) was calculated from measurements of weight (kg) and height (m): weight/height$^2$ (kg/m$^2$). BMI category (weight status) was categorized as healthy weight ≤25 kg/m$^2$, 25 ≤ overweight <30 kg/m$^2$, and obese ≥30 kg/m$^2$.

and SBPs. Mean sodium intake increased by tertile of nitrite intake (*p*-for trend<0.001), with a larger proportion of individuals in the 3rd tertile having significant higher sodium intake as well. A larger proportion of overweight and obese individuals, as well as those with CVD, T2D and higher adherence to Mediterranean Diet (MD ≥ 23), were categorized on the lowest tertile compared to their counterparts, with significant trends observed. Overall, significant differences were found with total energy, TFA and SFA intakes, both expressed as % of total energy intake, age and age categories (more than half of the individuals in the age category 18–30 years consume higher nitrite intake), educational level, employment status and marital status.

Table 2 presents the main results from the two linear regression models for mean DBP and SBP; the 1st model was adjusted for sodium intake categories, and the 2nd model included the interaction effect of sodium and nitric compounds. Detailed results for

all confounding variables the models were adjusted for can be found in Table S2 of the Supplementary Material.

In the 1st model no significant effect was found between nitrite intake in tertiles and DBP levels, nor with sodium. However, in the 2nd model, DBP was significantly associated independently with nitrite intake tertiles and total sodium intake levels. The interaction term was also significant and showed an attenuating effect by −2.11 mmHg (95%CI: −4.13, −0.09), meaning that DBP actually increased by 0.94 mmHg overall; the increase specifically for subjects in the third tertile of nitrite intake was 2.24 mmHg compared to those in the first (Table S3 of the Supplementary Material). The actual in-crease per total sodium intake level was 2.30 mmHg. No significant relationship was found between SBP and nitrite intake tertiles in any of the models examined and no differences were found when over-consumers were identified (n = 1) and excluded as per sensitivity analysis.

**Table 2**
Linear Regression models of DBP and SBP (mmHg) using 2 models adjusted for: (a) nitrite intake tertiles and sodium intake levels (Model 1) (b) also including the interaction effect of sodium and nitric compounds (Model 2), n = 494.

| | Model 1 | | | Model 2 | | |
|---|---|---|---|---|---|---|
| | Coef. | [95% conf. interval] | | Coef. | [95% conf. interval] | |
| DBP, mmHg | | | | | | |
| Nitrite intake tertile | 0.18 | −1.02 | 1.38 | 3.05[a,b] | 0.05 | 6.06 |
| Sodium intake level | 0.53 | −1.51 | 2.57 | 4.41[a,c] | 0.17 | 8.64 |
| Interaction of nitrite intake tertile & sodium intake level | | | | −2.11[a] | −4.13 | −0.09 |
| SBP, mmHg | | | | | | |
| Nitrite intake tertile | 0.13 | −1.38 | 1.63 | 2.68 | −1.09 | 6.44 |
| Sodium intake level | 1.68 | −0.87 | 4.23 | 5.12 | −0.19 | 10.42 |
| Interaction of nitrite intake tertile & sodium intake level | | | | −1.87 | −4.41 | 0.66 |

[a] Statistical significant at p ≤ 0.05. Nitrite intake and sodium intake were assessed as continuous variables by tertile and level respectively. Model 2 included nitrite intake tertile##total sodium intake level. Models were adjusted for (a) continuous variables: age (years), total energy intake (kcal/day), total SFA intake (% energy) and (b) categorical variables (baseline levels): sex (males versus females), BMI category/weight status (overweight and obese versus healthy weight), educational level (higher education and 12 years of school versus up to 6years of school versus), employment status (unemployed and pension versus employed), marital status (Married-consented versus divorced-separated-widowed-single), MedDiet category (MD ≥ 23 versus MD < 23), smoking status (smokers versus nonsmokers), physical activity level (low, moderate, very versus sedentary).
[b] Final additive effect by nitrite intake tertile: 0.94 mmHg (3.05 mmHg-2.11 mmHg).
[c] Final additive effect by sodium intake level: 2.30 mmHg (4.41 mmHg−2.11 mmHg).

Figure 1 presents the results of the sensitivity analyses performed, revealing the likelihood of being hypertensive, pre-hypertensive, with abnormal BP and unaware of abnormal BP by nitrite intake tertile and total sodium intake level. When hypertensives were considered, no significant associations were identified with nitrite intake tertile, total sodium intake level or their interaction effect. On the other hand, the likelihood of being pre-hypertensive or with abnormal BP, whether aware of it or not, increased significantly with the nitrite intake tertile and total sodium intake level. The interaction term was found significant in both cases as well, and thus the odds presented in Fig. 1 are the ones attenuated by the interaction effect.



**Fig. 1.** Likelihood of being hypertensive, prehypertensive, with abnormal BP and unaware of having abnormal BP by tertile intake of nitrite from processed meat products and total sodium intake level (sensitivity analysis). *Statistical significant at p ≤ 0.05. Hypertensive: an average BP of at least 140/90 mmHg or on antihypertensive treatment, n = 172 (10.1% of consumers of processed meat products, n = 1774); pre-hypertensive: an average measured BP of at least 130/80 mmHg but less than 140/ 90 mmHg, n = 143 (27.6% of those having their BP measured, n = 519); With abnormal BP: an average measured BP of at least 130/80 mmHg, n = 148 (28.5% of those having their BP measured, n = 519); Unaware of abnormal BP: an average measured BP of at least 130/80 mmHg, but reported being no hypertension or on treatment, n = 123 (23.7% of those having their BP measured, n = 519). Results based on multiple logistic regression models for (a) hypertensive (Nirite intake tertile, 95% CI: 0.46, 1.50; Sodium intake level, 95% CI: 0.23, 1.21) (b) prehypertensive (Nirite intake tertile, 95% CI: 1.20, 6.18; Sodium intake level, 95% CI: 0.1.82, 16.14) (c) with abnormal BP, including pre-hypertensives and hypertensives (Nirite intake tertile, 95% CI: 1.10, 5.49; Sodium intake level, 95% CI: 1.75, 14.89) (d) unaware of abnormal BP (Nirite intake tertile, 95% CI: 1, 5.73; Sodium intake level, 95% CI: 1.21, 13.15), compared to normotensives. Models included nitrite intake tertile##total sodium intake level and were adjusted for (a) continuous variables: age (years), total energy intake (kcal/day), total SFA intake (% energy) and (b) categorical variables (baseline levels): sex (males versus females), BMI category/weight status (overweight and obese versus healthy weight), educational level (higher education and 12 years of school versus up to 6years of school versus), employment status (unemployed and pension versus employed), marital status (Married-consented versus divorced-separated-widowed-single), MedDiet category (MD ≥ 23 versus MD < 23), smoking status (smokers versus non smokers), physical activity level (low, moderate, very versus sedentary).
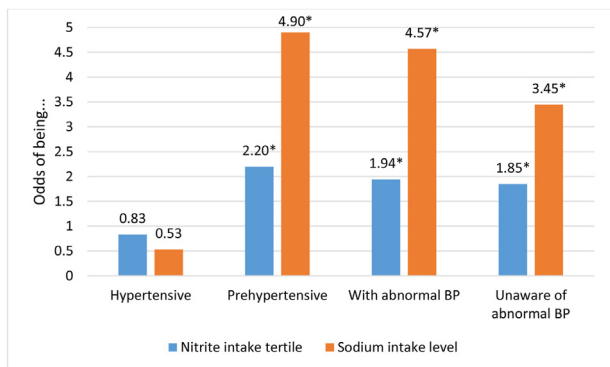
## 4. Discussion

### 4.1. Major findings

The major finding of this study was that higher nitrite intake levels from processed meat contributed to the increased DBP only when its intake is joint with sodium; meaning that the effect of nitrite equivalent on DBP from processed meat, is dependent on sodium intake. The likelihood of having higher than normal DBP among those reporting normotensives (unaware) increased with higher intakes of nitrite and sodium by 1.85% and 3.45% respectively. If we were to extrapolate these findings to the Greek adult consumer population, it is estimated that about 5155−9613 adults [42] could be affected.

### 4.2. Rational of findings

This paper addressed effects of nitrite and nitrate from processed meat, since this food is the main contributor of these additives and is consumed by a large proportion of the population, despite guidelines to limit. Processed meat has been identified as carcinogenic to humans [43] and individuals of all ages are advised to avoid it [44], as no level of intake can confidently be associated with a lack of risk [45]. Processed meat has been studied with respect to hypertension and CVD risk [14,18,46−48] with highly contradicting results pertaining on the effect of its constituents, other to saturated fats, on CVD risk [23], although dietary guidelines suggest to emphasize on avoiding preservatives [18]. To date, research has related the nitrite/nitrate intake through processed meat mainly with cancer, due to the development of carcinogenic N-nitrosocompounds, and has.

Nitrite/nitrate intake and health is complex since the main food vector that contributes to their intake can be natural or processed in origin. To date only the positive effect on CVD nitrate from plant based natural food sources has been highlighted, due to their beneficial effect on endothelial function and reduction of BP [49]. The association of nitrite/nitrate intake from processed meat intake

with CVD has also been studied with controversial results [19,22], but the relationship with BP, a significant risk factor for CVD, has not been specifically studied. It is worth mentioning that in a study of van den Brandt [19] both processed meat and nitrite intakes were found to be independently related to CVD mortality, but when adjusted for nitrite intake, the association with processed meat intake was attenuated and became non-significant, meaning that the effect was mainly attributed to the nitrite compounds. However, the effect on BP was not specifically studied, and total dietary sodium intake was not included in the analysis, despite the fact that processed meat contains a high concentration of sodium as well. To our knowledge, our study is the first to focus on the association of dietary nitrite/nitrate from processed meat with BP, accounting also for total dietary sodium intake, and our findings are consistent with those of the only other study investigating the effect of nitrite as an additive on the risk of hypertension [50].

Sodium from processed meat, as salt and compound in other preservatives, has been identified as the second major contributor to dietary salt intake [51] and excessive salt consumption may heighten risk for hypertension, increasing likelihood of CVD [52]. Mean total sodium intake was found significantly higher among individuals categorized in the third nitrite tertile, as expected since sodium intake increases due to processing. Specifically, the effect of higher sodium intake above 2300 mg set as the highest norm, also increased BP levels, although sodium from food alone. It should also be noted that although the interaction between sodium and nitrite intake mitigated nitrite effects on DBP, this remained significant. Effects of sodium were also mitigated, showing that the two variables have a joint action on DPB, and the effect of nitrite/nitrate intake from processed meat is dependent on higher sodium intake. And yet, although the precise pathogenic mechanism of hypertension is still not fully understood, the potential contribution of nitrite/nitrate intake from processed meat products to the etiology of hypertension has not been specifically studied. Generally, incomplete accounting for potential confounders is a frequent issue in epidemiological research [14].

### 4.3. Strengths and limitations

Study strengths include the use of a nationally representative sample of adults, survey-specific FPQs, the 9% endogenous nitrate-to-nitrite conversion factor, and direct BP measurements to avoid potential masking from various treatments and thus deal with possible selection and reverse causality biases. Adults with confirmed hypertension are typically advised to adhere to a thorough plan of care and treatment [30] that includes reduced salt ingestion (less than 5 g daily), increased consumption of fruits and vegetables, limited consumption of saturated fat-rich meals and trans-fat intake, reduced alcohol intake, regular exercise, control of weight status, and cessation of smoking [9,53]. Therefore, including only individuals already aware of having elevated BP in such studies could enhance masked, incorrect results. This said, based on the sensitivity analyses performed, no significant effects were found on DBP by nitrite tertile intake or total sodium intake level for hypertensives versus normotensives. On the contrary, DBP significantly increased by tertile of intake in prehypertensives, probably due to the inclusion of hypertensives with uncontrolled BP, as well as in those with abnormal BP, whether aware of it or not. This can be explained by the fact that those unaware have not altered their lifestyle and dietary habits, therefore reverse causality effect is avoided, strengthening the results of this study. When all individuals measured were considered in the analysis, including those that were aware of their status as well, the results remained significant, since these individuals were not controlled, hence were probably not adhering to the proposed medical regime.

This study has some limitations as well. These include the lack of national occurrence data and use of legislative MPLs, which could have led to an overestimation of intakes, challenges in FoodEx2 codification, no use of processing factors, and the small sample of individuals identified as hypertensive per ESC/ESH guidelines (DBP/SBP$\geq$140/90 mmHg, n = 29) which did not allow a further sensitivity analysis. Extra salt added during cooking and/or at the table, which may have further increased the effect, could not be included since urine for analysis was not available. Furthermore, a large proportion of the individuals were not measured, although this was counteracted with specific methodologically valid for epidemiological studies questionnaires [54].

### 4.4. Final remarks

Our results demonstrate that nitrite and nitrate intake from processed meat is associated with elevated DBP and highlight the significance of further consideration of underlying mechanisms and policy implications. Specifically, they add more evidence to the debate on current regulations regarding the use of nitrite as a food additive. The French parliament recently committed to gradually reducing nitrite in cured meats based on a relative formal opinion of the French Agency for Food, Environment, and Occupational Health and Safety (ANSES) [55]. Given that nearly a quarter of those in our study were unaware of being hypertensive according to American standard guidelines (130/80 mmHg), such a measure could be very important for public health. In addition, adults need to be further informed that higher processed meat intake contributes to higher salt and nitrite/nitrate consumption, both of which are positively associated with increased BP. Finally, future research should take into account the interaction effect of nitrite/nitrate and sodium intake levels in order to thoroughly investigate the association between dietary nitrite and nitrate from processed meat and cardiometabolic disorder risk and elucidate underlying mechanisms that could explain findings.

### 5. Conclusions

Our study revealed that DBP, but not SBP, increased with higher processed meat consumption due to nitric compound intake when the mediating effect of total sodium intake was accounted for. This finding adds support to public health recommendations to minimize processed meat intake and indicates the need for awareness campaigns by the competent authorities. It also provides additional arguments in the ongoing debate over updating regulations for nitrite and nitrate used as food additives. Future research, required to further advance knowledge on potential mechanisms underlying the link between the key ingredients of processed meat and hypertension, should not overlook the interaction effect of dietary nitric compounds and total sodium intakes in order to properly interpret their individual associations with BP levels.

### Author contribution

### Funding statement

(Ministry of Health) under the Operational Program "Human Resources Development 2007−2013".

## Conflicts of interest

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.03.015.

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**Table S1.** Concentration levels (mg/kg) of nitrite (E 249-250) and nitrate (E 251-252) used in the exposure assessment.

| Food category number | Food category name | FOODEX category | FOODEX Code | FOODEX Name | Frequency in HNNHS % | NITRITE MPL (mgkg) | NITRATE MPL (mgkg) |
|---|---|---|---|---|---|---|---|
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A022S | Cured seasoned pork meat | 1.9 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A022X | Bacon | 7.0 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Preserved meat | A023E | Cured seasoned poultry meat | 3.2 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A024X | Salami-type sausage | 1.8 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A025B | Pepperoni/paprika-type sausage | 0.6 | 150 | 150 |
| 08.3.1 | Non-heat-treated meat products | Sausages | A025C | Chorizo and similar | 0.5 | 150 | 150 |
| **08.3.1** | **Total** | | | | **15.0** | | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A022T | Ham, pork | 23.0 | 150 | |

| Food category number | Food category name | FOODEX category | FOODEX Code | FOODEX Name | Frequency in HNNHS % | NITRITE MPL (mgkg) | NITRATE MPL (mgkg) |
|---|---|---|---|---|---|---|---|
| 08.3.2 | Heat–treated meat products | Preserved meat | A023H | Cooked cured (or seasoned) pork meat | 21.1 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023K | Cooked pork ham | 1.8 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023S | Cooked cured (or seasoned) poultry meat | 1.7 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023T | Cooked turkey meat | 27.6 | 150 | |
| 08.3.2 | Heat–treated meat products | Preserved meat | A023X | Cooked other poultry meat | 0.1 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A025Q | Mortadella-type sausage | 1.0 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A026B | Frankfurt-type sausage | 1.0 | 150 | |
| 08.3.2 | Heat–treated meat products | Meat specialties | A026M | Liver based spreadable-textured specialties | 0.1 | 150 | |
| 08.3.2 | Heat–treated meat products | Sausages | A0EYP | Preserved or partly preserved sausages | 7.6 | 150 | |

| Food category number | Food category name | FOODEX category | FOODEX Code | FOODEX Name | Frequency in HNNHS % | NITRITE MPL (mgkg) | NITRATE MPL (mgkg) |
|---|---|---|---|---|---|---|---|
| 08.3.2 | Total | | | | 85.0 | | |
| TOTAL | | | | | 100.00 | | |

**Table S2.** Detailed results of linear Regression models of DBP and SBP (mmHg) using 2 models adjusted for: (a) nitrite intake tertiles and sodium intake levels (Model 1) (b) also including the interaction effect of sodium and nitric compounds (Model 2), n=494.

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Coef. | P>\|t\| | [95% conf. | interval] | Coef. | P>\|t\| | [95% conf. | interval] |
| **DBP, mmHg** | | | | | | | | |
| Nitrite intake tertile | 0.18 | 0.770 | -1.02 | 1.38 | 3.05*[1] | **0.046** | 0.05 | 6.06 |
| Sodium intake level | 0.53 | 0.610 | -1.51 | 2.57 | 4.41*[2] | **0.041** | 0.17 | 8.64 |
| Interaction of nitrite intake tertile & sodium intake level | | | | | -2.11* | **0.041** | -4.13 | -0.09 |
| Age | 0.16* | **0.006** | 0.05 | 0.27 | 0.17* | **0.004** | 0.05 | 0.28 |
| Sex | 1.38 | 0.187 | -0.67 | 3.44 | 1.59 | 0.130 | -0.47 | 3.65 |
| Total energy intake | 0.00 | 0.666 | 0.00 | 0.00 | 0.00 | 0.532 | 0.00 | 0.00 |
| Total Saturated Fat Acid (SFA) intake | 0.11 | 0.384 | -0.14 | 0.35 | 0.13 | 0.305 | -0.12 | 0.37 |
| BMI Category (weight status) | | | | | | | | |

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Coef. | P>\|t\| | [95% conf. interval] | | Coef. | P>\|t\| | [95% conf. interval] | |
| Overweight | 5.38* | **0.000** | 3.13 | 7.62 | 5.37* | **0.000** | 3.13 | 7.61 |
| Obese | 7.05* | **0.000** | 4.19 | 9.92 | 7.20* | **0.000** | 4.35 | 10.06 |
| Educational level | | | | | | | | |
| 12 years of school | 2.02 | 0.551 | -4.62 | 8.65 | 2.27 | 0.502 | -4.35 | 8.88 |
| Higher education, including colleges | 0.89 | 0.793 | -5.76 | 7.53 | 1.05 | 0.756 | -5.58 | 7.67 |
| Employment status | | | | | | | | |
| Pension | -2.08 | 0.322 | -6.21 | 2.05 | -1.97 | 0.347 | -6.09 | 2.15 |
| Unemployed | -0.67 | 0.544 | -2.82 | 1.49 | -0.50 | 0.651 | -2.65 | 1.66 |
| Marital status | | | | | | | | |
| Married/consented | 0.41 | 0.733 | -1.94 | 2.76 | 0.34 | 0.776 | -2.01 | 2.69 |
| MedDiet Category | | | | | | | | |
| MD>=23 | -0.46 | 0.706 | -2.83 | 1.92 | -0.27 | 0.826 | -2.64 | 2.11 |

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | **Coef.** | **P>\|t\|** | **[95% conf.** | **interval]** | **Coef.** | **P>\|t\|** | **[95% conf.** | **interval]** |
| Smoking status | | | | | | | | |
| Smokers | 0.07 | 0.945 | -1.84 | 1.98 | 0.02 | 0.987 | -1.89 | 1.92 |
| Physical activity status | | | | | | | | |
| Low | 3.68 | 0.108 | -0.81 | 8.17 | 3.22 | 0.160 | -1.27 | 7.72 |
| Moderate | 3.90 | 0.067 | 0.28 | 8.08 | 3.72 | 0.080 | -0.45 | 7.89 |
| Very | 2.47 | 0.249 | -1.73 | 6.67 | 2.28 | 0.286 | -1.91 | 6.47 |
| **SBP, mmHg** | | | | | | | | |
| Nitrite intake tertile | 0.13 | 0.870 | -1.38 | 1.63 | 2.68 | 0.163 | -1.09 | 6.44 |
| Sodium intake level | 1.68 | 0.197 | -0.87 | 4.23 | 5.12 | 0.059 | -0.19 | 10.42 |
| Interaction of nitrite intake tertile & sodium intake level | | | | | -1.87 | 0.147 | -4.41 | 0.66 |
| Age | 0.28* | **0.000** | 0.14 | 0.43 | 0.29* | **0.000** | 0.15 | 0.43 |
| Sex | 10.64* | **0.000** | 8.06 | 13.21 | 10.82* | **0.000** | 8.24 | 13.40 |

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Coef. | P>\|t\| | [95% conf. interval] | | Coef. | P>\|t\| | [95% conf. interval] | |
| Total energy intake | 0.00 | 0.682 | 0.00 | 0.00 | 0.00 | 0.586 | 0.00 | 0.00 |
| Total Saturated Fat Acid (SFA) intake | 0.23 | 0.141 | -0.08 | 0.53 | 0.24 | 0.114 | -0.06 | 0.55 |
| BMI Category (weight status) | | | | | | | | |
| Overweight | 4.17* | **0.004** | 1.36 | 6.97 | 4.16* | **0.004** | 1.35 | 6.96 |
| Obese | 5.12* | **0.005** | 1.54 | 8.69 | 5.25* | **0.004** | 1.67 | 8.82 |
| Educational level | | | | | | | | |
| 12 years of school | -3.73 | 0.377 | 12.03 | 4.56 | -3.51 | 0.406 | -11.81 | 4.78 |
| Higher education, including colleges | -5.48 | 0.196 | 13.79 | 2.83 | -5.34 | 0.207 | -13.64 | 2.97 |
| Employment status | | | | | | | | |
| Pension | -1.32 | 0.615 | -6.49 | 3.84 | -1.22 | 0.641 | -6.38 | 3.93 |
| Unemployed | 1.13 | 0.409 | -1.56 | 3.83 | 1.28 | 0.351 | -1.42 | 3.98 |
| Marital status | | | | | | | | |

|  | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
|  | Coef. | P>|t| | [95% conf. interval] | | Coef. | P>|t| | [95% conf. interval] | |
| Married/consented | -2.09 | 0.164 | -5.03 | 0.86 | -2.15 | 0.152 | -5.09 | 0.79 |
| MedDiet Category | | | | | | | | |
| MD>=23 | -0.76 | 0.616 | -3.73 | 2.21 | -0.59 | 0.697 | -3.57 | 2.39 |
| Smoking status | | | | | | | | |
| Smokers | -2.33 | 0.056 | -4.72 | 0.06 | -2.38 | 0.051 | -4.76 | 0.01 |
| Physical activity status | | | | | | | | |
| Low | 3.48 | 0.225 | -2.14 | 9.09 | 3.07 | 0.285 | -2.56 | 8.71 |
| Moderate | 1.08 | 0.686 | -4.15 | 6.31 | 0.91 | 0.732 | -4.32 | 6.14 |
| Very | 1.67 | 0.533 | -3.59 | 6.92 | 1.50 | 0.575 | -3.75 | 6.75 |

*Statistical significant at p≤0.05. Nitrite intake and sodium intake were assessed as continuous variables by tertile and level respectively. Models were adjusted for (a) continuous variables: age (years), total energy intake (kcal/day), total SFA intake (% energy) and (b) categorical variables (baseline levels): sex (males versus females), BMI category/weight status (overweight and obese versus healthy weight), educational level (higher education and 12 years of school versus up to 6years of school versus), employment status

(unemployed and pension versus employed), marital status (Married-consented versus divorced-separated-widowed-single), MedDiet category (MD≥23 versus MD<23), smoking status (smokers versus non smokers), physical activity level (low, moderate, and very versus sedentary).

[1] Final additive effect by nitrite intake tertile: 0.94mmHg (3.05 mmHg-2.11mmHg).

[2.] Final additive effect by sodium intake level: 2.30 mmHg (4.41mmHg-2.11mmHg).

**Table S3.** Linear Regression model of mean DBP (mmHg) adjusted for the interaction between nitrite intake tertiles and sodium intake levels, when nitrite intake tertiles are introduced as categorical paremeters.

| Mean DBP, mmHg | Coef. | P>\|t\| | [95% conf. interval] | |
|---|---|---|---|---|
| Nitrite intake tertile | | | | |
| 2nd tertile of nitrite intake | 0.82 | 0.763 | -4.52 | 6.16 |
| 3rd tertile of nitrite intake[1] | **6.70\*** | 0.035 | 0.49 | 12.90 |
| Sodium intake level | 1.69 | 0.266 | -1.30 | 4.68 |
| Interaction of nitrite intake tertile & sodium intake level | | | | |
| 2nd tertile of nitrite intake | -0.50 | 0.799 | -4.32 | 3.33 |
| 3rd tertile of nitrite intake[1] | **-4.46\*[1]** | 0.035 | -8.60 | -0.31 |

*Statistical significant at p≤0.05. Nitrite intake tertile was assessed as categorical variable (1st tertile the reference level) and sodium intake as continuous variable by level. Models were adjusted for (a) continuous variables: age (years), total energy intake (kcal/day), total SFA intake (% energy) and (b) categorical variables (baseline levels): sex (males versus females), BMI category/weight status (overweight and obese versus healthy weight), educational level (higher education and 12 years of school versus up to 6years of school versus), employment status (unemployed and pension versus employed), marital status (Married-consented versus divorced-separated-widowed-single), MedDiet category (MD ≥23 versus MD<23), smoking status (smokers versus non smokers), physical activity level (low, moderate, and very versus sedentary).

[1] Final additive effect for subjects in the 3rd nitrite intake tertile: 2.24 mmHg (6.70 mmHg-4.46 mmHg).

# C. GENERAL DISCUSSION-CONCLUSIONS

# 1. GENERAL DISCUSSION

## 1.1. STATEMENT OF MAIN RESULTS

To the best of our knowledge, this thesis represents the first attempt to assess the nitrite and nitrate consumption from processed meat products within the Greek population, adopting a risk assessment approach. Moreover, the extensive literature review conducted on health implications by source of origin along with the investigation of the correlation between nitrite and nitrate intake from processed meat products and BP, while controlling for potential confounding variables, complement the limited number of studies on these subjects worldwide. Overall, the present study investigated the risk as well as the health impact of nitrite and nitrate consumption from processed meat, given that this food source represents the primary contributor of these additives and is widely consumed by a significant proportion of the populace, notwithstanding recommendations to restrict intake.

The findings of the extensive literature review carried out indicated that the consumption of nitrite and nitrate derived from plants has been proposed to confer advantageous outcomes on metabolic and vascular health. In contrast, an elevated consumption of the stated compounds derived from processed animal products has been associated with higher risk of cancer, particularly in the gastrointestinal tract. No clear links between nitrite and nitrate from plant sources and cancer as well as nitrite and nitrate from animal sources and cardiovascular disease (CVD) were identified. The presence of nitrite and nitrate in drinking water is also an issue of concern, mainly due to associations with methemoglobinemia and carcinogenesis.

Subsequent to the aforementioned comprehensive analysis of existing literature, we opted to conduct a health risk assessment regarding the nitrite and nitrate ingestion from processed meat and meat-based products. The decision to exclusively assess intakes from processed meat was influenced by several factors. Firstly, the ingestion of nitrate and nitrite from vegetable sources, despite being consumed in large quantities, was deemed improbable to present any health hazards owing to their advantageous properties. Secondly, the ongoing nutrition transition has resulted in an increased consumption of processed meat products, which are known to contain these two additives, the most commonly used in the processed meat industry. Thirdly, the COVID-19 pandemic has

presented challenges in the production and availability of extra occurrence data, other than the MPLs. Finally, despite its significance for public health, and the relevant risk assessments of nitrite and nitrate intakes carried out by EFSA at EU level [1,2], such an evaluation had not been conducted among Greeks before.

The results of our risk assessment study suggest that a significant segment of Greek processed-meat consumers may be susceptible to potential health hazards, as their intake of nitrite and nitrate (measured as nitrite equivalent) has already surpassed the ADI of nitrite (0.07mg/kg bw/day) solely through the consumption of processed meat. The study findings indicate that the likelihood of surpassing the ADI was notably greater among children within the age range of 0-9 years. The primary sources of nitrite intake in the diet were found to be processed meat products from pork and turkey. These products were most commonly consumed as components of mixed dishes, including but not limited sandwiches, toasts, and pizzas.

Moreover, despite the absence of evidence linking nitrite and nitrate from processed meat with cardiovascular disease (CVD) in our literature review, the association between processed meat consumption and increased risk of CVD, coupled with the absence of international research on the subject, has prompted us to undertake a more comprehensive investigation of this relationship. Our research took into consideration established CVD risk factors, including dyslipidemia, hypertension, diastolic blood pressure (DBP), systolic blood pressure (SBP), low-density lipoprotein (LDL) cholesterol, diabetes, smoking, obesity, physical activity, and others. The consumption of nitrite and nitrate through processed meat products exhibited a correlation with BP. Specifically, elevated levels of nitrite equivalent intake were found to be associated with an increase in DBP, when accounted for sodium intake. The probability of elevated DBP in individuals who self-report as normotensive (without awareness of hypertension) was found to increase with greater nitrite and sodium intakes.

Extrapolating from the latest data provided by the Hellenic Statistical Authority, it can be inferred that approximately 160625 children are at risk of experiencing adverse health effects due to the consumption of nitrite and nitrate ingested from processed meat. Furthermore, it is projected that the DBP of 5155 to 9613 Greek -unaware of being hypertensive- adults could be adversely affected by the consumption of processed meat products. Therefore, our research outcomes hold significance in the context of public health and thus have been disseminated through various social media platforms, as

indicated in Appendix 2. In order to optimize the advantages of research after all, it is recommended to expeditiously disseminate the findings [3]. Social media platforms have the potential to facilitate rapid and global networking among researchers, doctors, policymakers, the public, and other stakeholders [4].

## 1.2. COMPARISON WITH LITERATURE

Each published paper includes a comprehensive analysis of the findings in relation to the current literature. Consequently, the iteration of related outcomes is deemed unproductive within this chapter. Generally, our research follows reviews on the physiologic context for potential health benefits [5] and nitric oxide biology [6] and complements reviews on benefits and risks for human health [6–10], especially those related to nitrite and nitrate derived from processed meat and meat products [11,12], as well as those indicating a possible relation between dietary nitrite and nitrate and CVD [13,14]. It is important to acknowledge that the discrepancies in the methodology of research studies that evaluate the consumption of nitric compounds in processed meat products present a difficulty in the comparison of outcomes. This is due to variations in the origins of intake, statistics and models involved, age grouping, approaches to dietary exposure assessment, conversion coefficients employed, and other related factors.

Research in the field persists, with a continuous stream of new data being published to either support or challenge prior findings. Savin et al. (2022) indicated recently that nitrate and nitrite are among the most frequently present additives in the food consumed by children, raising the risk that their negative effects will manifest, due to the specificity of the child's metabolic system [15]. A review of Shakil et al. (2022) on health risk issues of nitrite in cured meat also acknowledged the risk of methemoglobinemia in children and colorectal cancer in adults and suggested that nitrite replacements like plant extracts, organic acids and salts and High Hydrostatic Pressure (HHP) could be employed efficiently instead [16]. Chazelas et al. (2022) also provided a fresh perspective in the context of the active debate surrounding the banning of these additives from the food industry by identifying the relationship between nitrate and nitrite intakes and cancer risk using data from the French NutriNet-Santé cohort (2009-ongoing, median follow-up 6.7 years), a large prospective cohort with detailed dietary assessment [17]. Six (6) alternative methods of reducing or partially replacing nitrite in meat processing have been described

in the review of Zhang et al. (2023), suggesting that alternative additives may be the most successful methods of replacing nitrite in meat processing [18]. Finally, in a recently published brief communication by Srour et al. (2022) [19], the authors also underlined a positive association between nitrite from food additives and hypertension risk, which is in accordance with our findings.

## 1.3. STRENGTHS AND LIMITATIONS

A notable advantage of this study lies in the use of nationally representative samples sourced from the HNNHS to assess the consumption of nitrite and nitrate in processed meat products, employing also survey-specific food propensity questionnaires (FPQs) to account for the amount of processed meat consumed over a period of time. The maximum conversion factor of 9% was utilized to account for the endogenous transformation of nitrate to nitrite, so as a comprehensive assessment of the highest potential risk to consumers to be ensured [1]. As regards in particular the methodology for examining associations with BP, the utilization of direct BP measurements effectively eliminated the potential for masking effects resulting from various treatments and addressed the possibility of selection and reverse causality biases.

Regarding the limitations, the term 'processed meat' encompasses a diverse range of products that may exhibit varying levels of potential hazards [20]. Also, the variability of nitrite and nitrate content in foods and water has significant implications for the creation of food-composition databases and evaluation of nitrate intake in the diet [21]. Therefore, the absence of refined nationwide data pertaining to the utilization and occurrence of nitrite and nitrate salts in processed meat products provided a constraint to this research. The regulatory scenario is acknowledged as the worst case one, as it has a tendency to overestimate intakes in a systematic manner [22]. Consequently, our findings could represent an overestimation of nitrite consumption from processed meat. However, the levels of nitrite and nitrate in meat products, as reported by the European industry, were found to be equivalent to the Maximum Permitted Levels (MPLs) [1,2] and furthermore, the compounds could be used at levels exceeding those permitted by legislation as indicated by the levels reported by European Industry [22]. Nevertheless, the methodology used complies with the stepwise approach suggested by the European Commission which recommends utilizing Tier 3 to estimate additive intakes, involving

analyzing individual food consumption data and measured data on additives occurrence, only in cases where Tier 2 has been surpassed [23].

Some other methodological limitations regarding the risk assessment process include the challenge in FoodEx2 codification and mapping with the categories outlined in the legislation [24], as thorough meat product classification may have not been allowed by the information available in the Survey. EFSA has also acknowledged this challenge, where limitations or exemptions in the Regulation are not factored into such projects [1,25]. Moreover, residual levels might be affected by the processing time and temperature, the primary additive dosage, pH, the addition of ascorbate and/or other antioxidant components, the existence of microorganisms [26], but such factors could not be considered, as no relevant data was available.

Some concerns about the methodology employed in our study of the correlation with blood pressure are further addressed in Paper IV of this thesis. On top of that, the limited number of participants categorized as hypertensive according to the ESC/ESH criteria (with a DBP/SBP≥140/90 mmHg, n=36) precluded the possibility of conducting a more comprehensive sensitivity analysis. Finally, a significant segment of the population was not subjected to measurement. However, this limitation was addressed by utilizing questionnaires that were methodologically sound for epidemiological research [27].

## 1.4. FUTURE RESEARCH

Dietary shifts towards a more Westernized diet in developing economies has been linked to a reduction in the intake of plant-based foods and an increase in the consumption of animal-derived products [28]. The escalation of nitrite and nitrate intake from processed meat is a significant concern in the context of the nutrition transition, as it heightens the likelihood of surpassing ADI thresholds established (Jain and Mathur, 2015a). Finding ways to mitigate this transition and encourage a less processed diet is essential [28,29].

The importance of a collaborative endeavor aimed at establishing standards to improve and harmonize the assessment of nitrate and nitrite intake across all demographic segments should be underlined [21,30]. Although it is mandatory for member states to conduct monitoring of consumer intakes in order to facilitate dietary exposure and risk assessment [24], the Reg. (EU) 178/2002 [31] does not define when and how to conduct a risk assessment regarding food or feed safety. The Regulation establishes that the

European Food Safety Authority (EFSA) serves as the designated entity responsible for conducting risk assessments on behalf of the European Union and its constituent Member States. Several member states of the European Union, such as the German Federal Institute for Risk Assessment (BfR), the Austrian Agency for Health and Food Safety (AGES), the French Agency for Food, Environmental and Occupational Health Safety (ANSES) and the Food Safety Authority of Ireland (FSAI), have already established National Risk Assessment Organizations. However, these Organizations do not constitute a legal obligation. In Greece, the Hellenic Food Authority serves as the official Food Control Body and is responsible for implementing enhanced and proactive systems to monitor, evaluate, and regulate potential health risks through the application of risk analysis and risk assessment principles [32].

EFSA determined that the ADIs of nitrite and nitrate could be surpassed if all dietary sources were taken into account at the EU level. Specifically, the ADI of nitrite would be exceeded in infants, toddlers, and children at the mean level of exposure, and in all age groups at the highest level of exposure [1]. Similarly, the ADI of nitrate would be surpassed in all age groups at both the mean and highest levels of exposure [2]. Generally, for an assessment of nitrate levels in the body, an investigation of a vast variety of plant-based foods, meats, as well as drinking water and water-based beverages is suggested to be required [33]. It is also necessary to develop methodologies for estimating nitrate levels in various foods that would also take into account processing factors that can have an impact on nitrate content [34]. The application of food-composition tables exhibits significant heterogeneity, which may potentially impact the precision of estimated daily nitrate consumption [21]. More research is also required on the quantification of nitrate-reducing bacteria, the evaluation of dietary factors, and other factors that affect nitrosation, as well as enhanced exposure assessment for communities connected to public water supplies [35]. Moreover, in order to attain consistency and consequently enhance the dependability of nitrite exposure estimates, the establishment of a more precise definition of the conversion factor may be necessary [36,37].

The literature review conducted has indicated the necessity of conducting separate assessments of nitrite and nitrate intakes from processed meat, due to the potential adverse health impacts associated with their consumption, although it is acknowledged that it is impossible to differentiate between nitrosamines generated from permissible levels of added nitrite and those naturally occurring in the food itself [1]. The research on

carcinogenicity in the future should prioritize the examination of population subgroups that have a higher risk of endogenous N-nitrosamine production, such as smokers or high nitrate and nitrite consumers through food supplements, particularly if beneficial dietary components, such as polyphenols or vital nutrients, are absent (Ahluwalia et al., 2016). Research may potentially result in evaluations of larger groups of chemicals that result in endogenous nitrosation or circadian disturbances [38]. With the new Regulation 2023/2108 of 6 October [39], the EU lowered the limits for nitrite and nitrate used as food additives, following the recent opinion of the European Food Safety Authority (EFSA) that raised the alarm about nitrosamines in food [40].

Additional research is also required to explore the biological mechanisms and uses of dietary nitrate and nitrite [9], to enable a greater understanding of their impact on the prevention of CVD incidents [41]. Future studies should examine how nitrate intake from food can improve health in the context of overall nutritional quality, ensuring that the general public receives consistent health messages [42]. Finally, prior to making any new regulatory or public health recommendations for dietary nitrite and nitrate exposures, the risk-benefit balance should be thoroughly considered [6].

Finally, the HNNHS has much more information to extract from. Further research areas that have been already identified based on data from HNNHS may include:

i.    Nitrite and nitrate intakes from drinking water.

ii.   Cumulative nitrite and nitrate intakes from diet.

iii.  A more refined risk assessment of nitrite and nitrate intakes by type and manufacturer, based on labeling of processed meat products.

iv.   Risk assessment of the most commonly used additives intakes.

# 2. GENERAL CONCLUSIONS

Dietary nitrate and nitrite are plant nutrients, authorized food additives, water contaminants, and nutritional supplements. No evidence links fruit and vegetable nitrate and nitrite consumption to carcinogenicity. The evidence suggests that nitrate-rich vegetables may benefit metabolic and vascular health by improving endothelial function and lowering BP. However, processed animal products with nitrate and nitrite have been linked to increased cancer risk, especially in the gastrointestinal tract. Drinking water may also contribute to nitric compound-related health problems.

Median nitrite and nitrate intake (assessed as nitrite equivalent) from processed meat products revealed that a significant portion of the Greek populace is susceptible to surpassing the ADI of nitrite solely from the consumption of processed meat, primarily pork and turkey, consumed in mixed dishes such as toasts, sandwiches, and pizza. Children aged 0 to 9 years had the highest proportion of exceeding among those of the same age group in the study. Additionally, the findings of our investigation indicate that an increase in processed meat consumption leads to an elevation in DBP, due to the intake of nitric compounds, after controlling for the mediating effect of total sodium intake. No association was observed with SBP.

The outcomes of this thesis indicate a significant cause for concern, given the cumulative impact of chronic exposure to other dietary sources of nitrite and nitrate. Further prospective research is required to validate the correlation between dietary nitrate, nitrite, and nitrosamines and their potential risks and benefits to human health. Authorities need to introduce educational programs with the objective of enhancing public awareness regarding the plausible health risks linked to the consumption of processed meat and nitric salts. Alternative methods should be explored to substitute these additives for ensuring safety. In addition, it is recommended that proficient regulatory bodies establish and sustain a surveillance strategy pertaining to the levels of nitrite and nitrate present in diverse commodities within the Greek marketplace. Subsequent studies may evaluate the dietary consumption of nitrite and nitrate, using more refined occurrence data. Finally, future research, aiming at enhancing comprehension of the potential mechanisms that underlie the correlation between the principal components of processed meat and hypertension, should take into account the interaction between dietary nitric compounds and total sodium intake, to accurately interpret their respective associations with BP.

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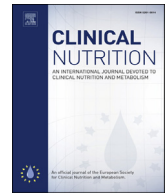
# D. APPENDIXES

**APPENDIX 1 ▪ REPLY-LETTER TO THE EDITOR: "NITRITE AND NITRATE INTAKE FROM PROCESSED MEAT IS ASSOCIATED WITH ELEVATED DIASTOLIC BLOOD PRESSURE (DBP)"**

Letter to the Editor

# Reply-letter to the editor: "Nitrite and nitrate intake from processed meat is associated with elevated diastolic blood pressure (DBP)"

*Dear Editor,*

We would like to thank the Authors Dr Ayçiçek and Dr Koca for their interest in our research [1]. The Authors raised points that are addressed below.

Firstly, although non-adherence of hypertensives to medical and/or nutrition recommendations could have misled the conclusion, this is not a concern as a sensitivity analysis was performed. Specifically, the same multiple logistic regression analyses were used for those self-reporting their status, pre-hypertensives, and those unaware of high Blood Pressure (BP) levels. In all cases, clear definition for their classification was reported (section-2.2.1) and results were properly interpreted by group (section-4.3).

The second concern raised was that omitting added salt could skew the results. We agree, and thus identify it in the discussion as a limitation that may have exacerbated the effect (section-4.3). However, 70–75% of salt/sodium intake comes from processed foods, 15% occurs naturally in foods and only 10% comes from added salt [2]. Furthermore, although the interaction attenuated DBP effects, this remained substantial in the higher salt tertile intakes (section-4.2, Table S3).

The third concern was the inclusion of >80-year-olds, due to therapy being recommended if SBP≥160 mmHg, although this is the case for fit and independent older adults only [3]. Furthermore, adults were categorized by mean BP and not by age, as associations per age group were not within the scope of our research, and thus, results were not interpreted for this age group specifically.

A major concern was raised regarding the methodology used for nutritional assessment, suggesting use of "nutrition"/food diaries. Although the last are the gold standard in intervention trials, they are not preferred in nutrition and health surveys, due to the burden they entail in reporting all foods/drinks consumed which leads to a simplification of a usual diet and to a reporting error [4]. The aim was not only to achieve accurate portion sizes but to report actual usual intake. Our methodology for consumption data followed EFSA's guidelines [5] using two non-consecutive 24-h- recalls with specific probing methods, validated food atlases, standardized household measurements as portion anchors, and a validated Frequency Propensity Questionnaires (FPQ) (section-2.3).

Finally, white-coat effect (WCE) was taken into account when BP was measured as per the guidelines of ESC/ASH [6]. Additionally, individuals with WCE are already on antihypertensive treatment, have an increased risk of CVD and should be closely monitored for transition to sustained hypertension [7]. Therefore, including hypertensives and prehypertensives, aware or unaware, in our study was important and actually added to current knowledge.

In conclusion, all the concerns raised by the Authors were fully addressed in our paper. It is worth mentioning that our findings regarding the effect of added nitrite on hypertension risk are also consistent with the results of a recently published brief communication [8]. We acknowledge that future research on the subject is required to enhance knowledge.

## Funding statement

## Author contribution

SK, AZ and EM drafted and reviewed this reply. All authors attest they meet the ICMJE criteria for authorship.

## Conflict of interest

The authors declare no conflict of interest. The authors SK and AZ alone are responsible for the content and views expressed in this publication and they do not necessarily represent the decisions, policy, or views of the Hellenic Food Authority.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2023.06.008.

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25 May 2023

# APPENDIX 2 ▪ 10th NATIONAL GONGRESS OF THE HELLENIC ATHEROSCLEROSIS SOCIETY

## 2.1.ABSTRACT

### HAA94

## ΔΙΑΤΡΟΦΙΚΗ ΕΚΘΕΣΗ ΣΕ ΝΙΤΡΙΚΑ ΚΑΙ ΝΙΤΡΩΔΗ ΑΛΑΤΑ ΑΠΟ ΕΠΕΞΕΡΓΑΣΜΕΝΟ ΚΡΕΑΣ ΚΑΙ ΣΥΣΧΕΤΙΣΗ ΜΕ ΑΡΤΗΡΙΑΚΗ ΠΙΕΣΗ: ΑΠΟΤΕΛΕΣΜΑΤΑ ΠΑΝΕΛΛΑΔΙΚΗΣ ΜΕΛΕΤΗΣ ΔΙΑΤΡΟΦΗΣ ΚΑΙ ΥΓΕΙΑΣ (ΠΑ.ΜΕ.Δ.Υ.)

**Σ. Κοτοπούλου[1,2], Α. Ζαμπέλας[1,2], Ε. Μαγριπλή[1]**

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**ΣΚΟΠΟΣ:** Η κατανάλωση επεξεργασμένου κρέατος έχει συσχετιστεί θετικά με την υπέρταση, η οποία αποτελεί σημαντικό παράγοντα κινδύνου εμφάνισης καρδιαγγειακών νοσημάτων (ΚΝ). Σκοπός της παρούσας εργασίας ήταν να διερευνηθεί η συσχέτιση μεταξύ διατροφικής πρόσληψης νιτρικών/νιτρωδών αλάτων από επεξεργασμένο κρέας και της αρτηριακής διαστολικής (DBP) και συστολικής (SBP) πίεσης.

**ΥΛΙΚΟ-ΜΕΘΟΔΟΙ:** Μελετήθηκαν δεδομένα 1774 ενήλικων καταναλωτών επεξεργασμένου κρέατος (≥18 ετών). Η κατανάλωση εκτιμήθηκε βάσει δύο 24ωρων ανακλήσεων και ερωτηματολογίων συχνότητας κατανάλωσης τροφίμων. Η διατροφική έκθεση εκτιμήθηκε συνδυάζοντας τα δεδομένα ατομικών καταναλώσεων με τα νομοθετημένα Μέγιστα Επιτρεπόμενα Επίπεδα (ADIs) για προάσπιση υγείας. Δεδομένου ότι ενήλικες με εγκατεστημένη υπέρταση πιθανά ακολουθούν ήδη ειδική διατροφή, για την αποφυγή σφαλμάτων επιλογής (selection bias) και αντίστροφης αιτιότητας (reverse causality) διερευνήθηκε η συσχέτιση με την DBP και την SBP στο σύνολο του πληθυσμού. Η διερεύνηση έγινε με κατανομή του πληθυσμού σε τριτημόρια κατανάλωσης νιτρικών/νιτρωδών αλάτων λαμβάνοντας υπόψιν πιθανή συνεργιστική δράση με τη διατροφική πρόσληψη νατρίου.

**ΑΠΟΤΕΛΕΣΜΑΤΑ:** Η διάμεση συνολική πρόσληψη νιτρικών/νιτρωδών αλάτων (ως ισοδύναμο νιτρωδών) ήταν 0.007 (0.003, 0.017)mg/kg σωματικού βάρους/ημέρα και η μέση τιμή της 0.021(±0.058)mg/kg σωματικού βάρους/

ημέρα. 5,7% (n=102) των καταναλωτών υπερέβησαν το ADI για τα νιτρώδη λαμβάνοντας ≥0,07mg/kg σωματικού βάρους/ημέρα. Κύριες πηγές πρόσληψης ήταν τα επεξεργασμένα προϊόντα από χοιρινό κρέας (39.7%) και γαλοπούλα (34.4%).

Προσαρμοσμένα μοντέλα παλινδρόμησης έδειξαν ότι αύξηση ανά τριτημόριο πρόσληψης νιτρικών/νιτρωδών σε συνάρτηση με αύξηση πρόσληψης νατρίου αυξάνουν συνολικά σημαντικά την DBP κατά 3.03mmHg (95% CI:0, 6.06) και 4.41mmHg (95%CI: 0.17, 8.66) σε σχέση με κάθε παράγοντα αντίστοιχα. Συνυπολογίζοντας όμως και τη σημαντική συνεργιστική δράση των δύο παραγόντων (Coef: -2.11, 95%CI: -4.14, -0.08), η DBP εκτιμάται ότι αυξάνεται τελικά κατά 0.92mgHg συνολικά και κατά 2.20mgHg για τα άτομα με πρόσληψη στο τρίτο τριτημόριο σε σχέση με το πρώτο. Αντίστοιχα ανά περίπου 800mg αύξηση στην πρόσληψη νατρίου, άνω των 1500mg, βρέθηκε αύξηση κατά 2.3mgHg της DBP. Δεν διαπιστώθηκε σημαντική συσχέτιση της πρόσληψης νιτρικών/νιτρωδών και νατρίου με την SBP.

**ΣΥΜΠΕΡΑΣΜΑΤΑ:** Η κατανάλωση επεξεργασμένου κρέατος συμβάλλει στην αύξηση της DBP στον γενικό πληθυσμό ενηλίκων λόγω συγκέντρωσης νιτρικών/νιτρωδών, αλλά για την ορθή ερμηνεία των συσχετίσεων πρέπει να λαμβάνεται υπόψη η αλληλεπίδραση αυτής με τη συνολική διατροφική πρόσληψη νατρίου, σημαντικό μέρος της οποίας οφείλεται επίσης στα επεξεργασμένα τρόφιμα.

## 2.2. POSTER



Poster: ΔΙΑΤΡΟΦΙΚΗ ΕΚΘΕΣΗ ΣΕ ΝΙΤΡΙΚΑ ΚΑΙ ΝΙΤΡΩΔΗ ΑΛΑΤΑ ΑΠΟ ΕΠΕΞΕΡΓΑΣΜΕΝΟ ΚΡΕΑΣ ΚΑΙ ΣΥΣΧΕΤΙΣΗ ΜΕ ΑΡΤΗΡΙΑΚΗ ΠΙΕΣΗ: ΑΠΟΤΕΛΕΣΜΑΤΑ ΠΑΝΕΛΛΑΔΙΚΗΣ ΜΕΛΕΤΗΣ ΔΙΑΤΡΟΦΗΣ ΚΑΙ ΥΓΕΙΑΣ (ΠΑ.ΜΕ.Δ.Υ.)

# APPENDIX 3 ▪ DISSEMINATION OF THESIS RESULTS

The following is a non-exhaustive, indicative compilation of social media platforms/sites where our research findings were hosted:

- ✦ The EC reduces the limits of nitrite and nitrate use in processed meat products and other foods. CIBUM food experts society. 9 October 2023. [Η ΕΕ μειώνει τα όρια νιτρωδών και νιτρικών αλάτων σε αλλαντικά και άλλα τρόφιμα. CIBUM food experts society. 9 Οκτωβρίου 2023]. https://cibum.gr/nea/i-ee-meionei-ta-oria-nitrodon-kai-nitrikon-alaton-sta-allantika-kai-alla-trofima/

- ✦ Nitrates: New study changes the picture about the risks involved. CIBUM food experts society.24 May 2023 [Νιτρικά άλατα: Νέα μελέτη αλλάζει την εικόνα σχετικά με τους κινδύνους που ελλοχεύουν. CIBUM food experts society.24 Μαΐου 2023]. https://cibum.gr/nea/nitrika-alata-ofelima-i-karkinogona/

- ✦ High dietary intake of nitrite and nitrate from the diet-Research data for Greece. Hellenic Food Authority. 24 January 2023 [Υψηλή πρόσληψη νιτρωδών και νιτρικών αλάτων από τη διατροφή-Ερευνητικά δεδομένα για την Ελλάδα. ΕνιαίοςΦορέας Ελέγχου Τροφίμων. 24 Ιανουαρίου 2023].

https://www.facebook.com/efetofficial/photos/a.118682067514230/185910507458052/



Ενιαίος Φορέας Ελέγχου Τροφίμων
24 Ιανουαρίου · 🌐

Υψηλή πρόσληψη νιτρωδών και νιτρικών αλάτων από τη διατροφή – Ερευνητικά δεδομένα για την Ελλάδα

Η μακροχρόνια υψηλή πρόσληψη νιτρωδών και νιτρικών αλάτων μέσω της υπέρμετρης κατανάλωσης επεξεργασμένου κρέατος έχει συσχετιστεί με δυσμενείς επιπτώσεις στην υγεία του ανθρώπου και κυριότερα με εμφάνιση καρκίνου του γαστρεντερικού συστήματος. Η σχετική μελέτη βρίσκεται στην ακόλουθη διεύθυνση: https://doi.org/10.1093/nutrit/nuab113.
Τα νιτρώδη άλατα (νιτρώδες κάλιο – E249 και νι... **Δείτε περισσότερα**

- ➢ Reposted by:

- o Iatropedia. 25 January 2023. https://www.iatropedia.gr/diatrofi/nitrodi-kai-nitrika-alata-apo-ti-diatrofi-erevnitika-dedomena-gia-tin-ellada/162922/

- o K3-Cancer Guidance Centre. 24 January 2023 [Κ3-Κέντρο Καθοδήγησης Καρκινοπαθών. 24 Ιανουαρίου 2023].https://www.facebook.com/kapa3.k3

  - o Race for Life. 25 January 2023 [Αγώνας Ζωής. 25 Ιανουαρίου 2023]. https://www.facebook.com/AgonasZois

- o Kalymento. 24 January 2023. https://www.facebook.com/KalymentoGR

⊕ Dietary intake of nitrate and nitrite - its relationship to health. Hellenic Food Authority. 17 January 2023 [Διατροφική πρόσληψη νιτρικών και νιτρωδών αλάτων-Σχέση της με την υγεία. Ενιαίος Φορέας Ελέγχου Τροφίμων. 17 Ιανουαρίου 2023].

https://www.facebook.com/efetofficial/photos/a.118682067514230 /18419622429 6147/

**ΕΦΕΤ Ενιαίος Φορέας Ελέγχου Τροφίμων**
17 Ιανουαρίου · 🌐 ⋯

Διατροφική πρόσληψη νιτρικών και νιτρωδών αλάτων – Σχέση της με την υγεία

Ο Διεθνής Οργανισμός Ερευνών για τον Καρκίνο (IARC) έχει κατατάξει τα νιτρικά και νιτρώδη άλατα ως πιθανώς καρκινογόνους παράγοντες για τον άνθρωπο (https://monographs.iarc.who.int/list-of-classifications). Στη διατροφή, τα άλατα αυτά μπορεί να τα βρει κανείς ως θρεπτικά στοιχεία στα τρόφιμα φυτικής προέλευσης. Η συγκέντρωσή τους μπορεί να αυξηθεί εξαιτίας της χρήσης τους στα επεξεργασμένα τρόφιμα ζωι... **Δείτε περισσότερα**

  ➢ Reposted by:
    o Aliment Lab. 18 January 2023.
      https://www.facebook.com/alimentlab/?locale=el_GR

    o EATSMART. https://eatsmart.gr/dimosiefseis/

    o Dimitra Ioannou Clinical Dietitian - Nutritionist BSc , MSc. 17 January 2023. https://www.facebook.com/healthyroutinebydi

⊕ Which food safety topics topped the most visited list in 2022. CIBUM foodexperts society. 3 January 2023 [Ποια θέματα ασφάλειας τροφίμων βρέθηκανστην κορυφή της λίστας με την μεγαλύτερη επισκεψιμότητα το 2022. CIBUM food experts society. 3 Ιανουαρίου 2023]. https://cibum.gr/nea/poia-themata-asfaleias-trofimon-vrethikan-stin-koryfi-tis-listas-me-tin-megalyteri-episkepsimotita-to-2022/

⊕ *Αχ, αυτό το παριζάκι...* Γιάννης Ζαμπετάκης. CIBUM food experts society. 21 Οκτωβρίου 2022. https://cibum.gr/aristero-extrem/ach-ayto-to-parizaki/

⊕ Concern for the health of Greeks from nitrites in meat - Study by EFET and the Agricultural University of Athens. CIBUM food experts society. 10 October 2022.[Ανησυχία για την υγεία των Ελλήνων από τα νιτρώδη στο κρέας-Μελέτη τουΕΦΕΤ και του Γεωπονικού Πανεπιστημίου Αθηνών. CIBUM food experts society. 10 Οκτωβρίου 2022]. https://cibum.gr/nea/anisychia-gia-tin-ygeia-ton-ellinon-apo-ta-nitrodi-sto-kreas-meleti-toy-efet-kai-toy-geoponikoy-panepistimioy-athinon/.

- ➢ Reposted by:

  - o Geotechnical Chamber of Greece. 10 October 2022. [ΓΕΩΤΕΕ. 10 Οκτωβρίου 2022]. https://www.facebook.com/groups/13095464628

  - o B and C EPAL Karlovassi Agricultural - Food Technology https://www.facebook.com/groups/2421705204714519/



- ➕ Guest 23: Sakis Spiliotopoulos-Cold Cuts. The food Processors. 14 March 2023 [Guest 23: Σάκης Σπηλιωτόπουλος-Αλλαντικά. The food Processors]. 14 Μαρτίου2023. https://open.spotify.com/episode/5L33qp7IL5GylDD11nzEyP

  - ➢ Reposted by:

  - o Food Science & Technology. 14 March 2023 [Επιστήμη & Τεχνολογία Τροφίμων. 14 Μαρτίου 2023] https://www.facebook.com/groups/271282913067761?hoisted_section_header_type=recently_seen&multi_permalinks=2055125094683525

# APPENDIX 4 ▪ ADDITIONAL PAPERS

Original Research

# Trans fatty acid intake increases likelihood of dyslipidemia especially among individuals with higher saturated fat consumption

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Academic Editor: Grant Pierce
Submitted: 24 November 2021    Revised: 9 January 2022    Accepted: 24 January 2022    Published: 7 April 2022

## Abstract

**Background**: Evidence points to adverse effects of trans fatty acids (TFA) on health. The aim of this study was to estimate total TFA intake, evaluate major food contributors and its effect on dyslipidemia. **Methods**: A total of 3537 adults (48.3% males) were included. Total TFA intake was assessed using two 24-hour dietary recalls. Foods were categorized into specific food groups. Adjusted Logistic Regression analysis was performed to assess the likelihood of dyslipidemia by tertile of TFA aand Saturated Fatty Acid (SFA) level. **Results**: Median TFA intake was 0.53% of energy (from 0.34 to 0.81) ranging from 0.27 (Q1) to 0.95 (Q3) ($p < 0.001$, for trend), and 16% of individuals consumed TFA above 1% of their total energy. Cheese was the main contributor to TFA intake, with processed/refined grains and fried fish following. The latter was the main contributor in older adults (51+ years). Adjusted logistic regression analysis showed that individuals at the highest tertile of trans consumption were 30% more likely to have dyslipidemia compared to the lowest (OR$_{(Q3-Q1)}$: 1.3; 95% CI: 1.02–1.66 and OR$_{(Q2-Q1)}$: 1.3; 95% CI:1.01–1.66, respectively). This increased by 10% when stratified by SFA intake (OR: 1.4; 95% CI: 1.061–1.942) and remained significant only in individuals at the highest tertile and with higher than recommended SFA intake. **Conclusions**: A high intake of TFA combined with high SFA intakes further increase the likelihood of dyslipidemia and should be accounted for in public health prevention programs. Monitoring and evaluation of the recent EU legislative measures on TFA levels in foods is also necessary.

**Keywords**: trans fatty acid intake; dyslipidemia; dietary intake; saturated fat intake; cardiovascular disease; food contribution

## 1. Introduction

Cardiovascular diseases (CVD) are a leading cause of death worldwide, including Europe [1,2]. Ischemic heart disease and cerebrovascular disease have been the two leading causes of death in Greece during the past decade [2], mainly attributed to unfavorable changes in modifiable risk factors such as dyslipidemia [3]. Since the 1990s, accumulating and overwhelming evidence points to the detrimental effects of trans fatty acids (TFA) on human health, particularly with respect to cardiovascular health and total mortality [1,4–7].

Higher TFA intakes have been associated in general with a 20–30% increased risk of all-cause mortality, irrespective of replacement nutrients [8]. TFA are unsaturated fatty acids that contain at least one double bond in the trans configuration and can be of natural origin or industrially produced. The latter have been widely used in food manufacturing, such as bakery products and margarine, due to their increased plasticity and chemical stability. TFA, how-

ever, have been associated with adverse health effects, disrupting circulating lipid biomarkers; specifically increasing LDL-cholesterol, lipoprotein (little) a (Lp$\alpha$) and triacylglycerol levels, decreasing HDL-cholesterol levels and LDL-cholesterol particle size [9,10], but also increasing total-/HDL-cholesterol ratio [11]. TFA intake has also been shown to accentuate systemic inflammation, with a positive relationship being found between TFA intake and c-reactive protein (CRP) levels [12,13], adversely affecting endothelial function. This may partially explain the higher than expected cardiovascular disease risk as a result of abnormal lipid profile [12].

Other to the direct effects of TFA's to the cardiovascular system, they may also exert an indirect effect on it. Specifically, a linear association has been reported between higher TFA intake and increased weight gain and fat adiposity, as well as with impaired glucose tolerance [14,15]. Based on the above adverse physiological effects, it has been reported that a 2% absolute energy intake from TFA is

associated with a substantial increase in coronary heart disease (CHD) incidence, and specifically with 23% increase in CVD risk [16].

The World Health Organization (WHO) recommends that energy intake from TFA should not exceed 1%, including TFA of natural origin [17], and since 2015 it encourages TFA elimination in the food supply [18]. The European Food Safety Authority [19] also suggests that the intake of TFA should be as low as possible within the context of a nutritionally adequate diet. A study conducted between June 1995 and April 1996, assessed TFA intake in 14 Western European countries, one of which was Greece, and found that the population median of TFA consumption in Greece was among the lowest in Europe, ranging between 0.5% and 0.8% of total energy intake, for men and women respectively [20]. Since then a major transition has occurred towards a more Western type dietary pattern, with a simultaneous decreased adherence to a Mediterranean type diet [21]. Also, recently published [22] TFA concentration data of commonly consumed foods in Greece, indicated that certain foods can have TFA content exceeding 2% of total fat. It is therefore of great importance to acquire up-to-date information on total TFA intakes from a nationally representative sample of Greek adults.

Consequently, the aim of the present study was to conduct a TFA exposure assessment in Greek adults, identifying major contributing foods to this exposure and assess the association of TFA intake with likelihood of dyslipidemia and prevalence of other CVD risk factors, using a nationally representative sample.

## 2. Methods

### 2.1 Study design

This study included adults who were enrolled in the Hellenic National Nutrition and Health Survey (HNNHS), a population-based survey conducted between September 2013 and May 2015. The study was designed to assess the health and nutritional status of Greek residents, excluding individuals residing in institutions, members of the armed forces, pregnant and lactating women, and individuals with mental disabilities. Individuals were selected following a multi-stage stratified sampling design, by geographical region, area, sex, and age group. Study details have been published elsewhere [23]. A total of 3775 adults were enrolled in HNNHS and a total of 3537 individuals ≥19 years were included in this study (48.7% males) for which data on TFA intake were available. All work was carried out upon obtaining individual consent and approval by the Ethics Committee of the Department of Food Science and Human Nutrition of the Agricultural University of Athens and by the Hellenic Data Protection Authority (HDPA).

All individuals enrolled in the study were interviewed by trained personnel. An interviewer-administered questionnaire was used to obtain information on sociodemographics, anthropometric characteristics, medication in-take, and lifestyle choices (such as smoking habits and level of physical activity).

### 2.2 Dietary & trans fatty acid assessment

Two 24 hr-recalls were collected; one during the first face-to-face interview, and the second through a telephone interview after 8–20 days on a different non-consecutive day, using the Automated Multiple Pass Method. For optimal intake assessment specific, validated food atlases and standardized household measures were used as portion anchors. The TFA content of the food groups used for this study was derived from two sources. The primary data source was the Nutrition Data System for Research (NDSR) developed by the University of Minnesota which is an integrated data system providing extended nutrient profile data [3] for globally consumed food. This system, however, does not contain ethnic consumed foods. Due to the high sensitivity requirements of TFA measurement, data from chemical analysis of 140 samples from different foods frequently consumed by the population residing in Greece, including fast food, pies and pastries, were used [22]. Details can be found in Appendix Table 3. To estimate the contribution of each food group (FG) to total TFA intake, foods reported in 24 hr were organized into 37FG's, based on their composition (Appendix Table 4). Foods included in recipes/mixed dishes were assigned to multiple food groups according to the different foods that they consisted of and were then grouped as stated above.

The percentage of the contribution of each FG to TFA intake was derived by the following formula: % contribution of FG to TFA = (sum of TFA intake for that FG/sum of total TFA) * 100. This was calculated separately for each age and sex group. Total TFA intake (grams/day) was then transformed to total energy from TFA (TFA in grams * 9 kcal/per gram) and the latter was standardized by total mean energy consumption.

Data on total fat, SFA, Poly- and Mono- unsaturated fatty acids (PUFA & MUFA, respectively) and added sugars were also calculated as per total energy intake. Total fiber and cholesterol intake was measured in grams per day. Sodium from foods alone was also estimated and was reported in total grams per day.

### 2.3 Definition of dyslipidemia

Individuals with dyslipidemia were defined as those reported having high plasma cholesterol and/or triglycerides levels, or on medication, or those who were classified as dyslipidemic based on the European Society of Cardiology cut-offs of lipid levels. These include (either/or): LDL-cholesterol ≥116 mg/dL; HDL-cholesterol ≤35 mg/dL in females and ≤40 mg/dL for males; total triglycerides >150 mg/dL; Total Cholesterol >200 mg/dL; or on antilipidemic medication. Therefore, those who were unaware of their status were also accounted for. The Friedewald Formula was used to calculate LDL-cholesterol [24]

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and since it is known that the Friedewald Formula is not sensitive for triglyceride values >400 mg/dL, individual data were checked and only 4 individuals (out of 1088) had such blood triglyceride levels.

LDL = Total Cholesterol – HDL-cholesterol – (TG/5), in mg/dL

(Where TG, Triglycerides; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein.)

All blood samples were collected in the morning, between 8:00 and 10:00 AM, after fasting for at least 10 hours. All biochemical examinations listed above, as well as fasting plasma glucose were carried out using enzymatic methods in Cobas Integra 400 analyzer (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

### 2.4 Other parameters

BP measurements were taken with individuals rested for at least 5 minutes, seated with their back upright, and their arm well-supported at a 45° angle from the trunk at the heart level [25]. Three consecutive measurements taken on a single occasion were used to assess individuals blood pressure. The average of these measurements was used to describe and report the study populations mean SBP and DBP levels.

Sociodemographic and anthropometric data were collected by trained health professionals using Computer Assisted Personal Interview (CAPI) software. Specifics on age, sex and educational level were acquired by highly trained health professionals. Educational level was classified into 3 groups: <6 years of schooling; ≥6–11 years; and ≥12 years.

Smoking habits and physical activity level were also assessed. Individuals were classified as ex-smokers' if they had stopped smoking at least for 30 days, smokers, or never-smokers. Physical activity (PA) was defined as light, moderate or high, according to the International Physical Activity Questionnaire (IPAQ), as per calculation guidelines [26]. Individuals scoring below the light activity level were categorized as sedentary. Weight (kg) and height (m) were measured from which Body Mass Index (BMI) was derived [weight/height$^2$ (kg/m$^2$)]. Weight status was categorized as "underweight <18.5 kg/m$^2$", "18.5 ≤ normal weight < 25 kg/m$^2$", "25 ≤ overweight < 30 kg/m$^2$", and "obese ≥30 kg/m$^2$".

### 2.5 Statistical analysis

Data were analyzed using appropriate methodology for survey design to have generalizable results to the reference population. Specifically, data were weighted by area, age group, and sex (as per sampling frame), using the 2011 Population Census. Continuous variables were presented as mean ± standard deviation (sd) when normally distributed, and median (IQR) for skewed distributions. Categorical variables were presented as frequencies with 95% confidence intervals (95% CI). CI's were reported as informative

for the population distribution, since this is a National representative study. Group differences were tested using chi square test for proportions, and ANOVA or Kruskal Wallis rank sum test for continuous data, depending on data distribution. $p$ for trend was tested post hoc. Survey specific logistic regression model [with linearized SE's] was used to assess the likelihood of dyslipidemia, by tertile of TFA intake. The model was adjusted for a priori known risk factors. Specifically, weight status, sex, age, smoking status, and sodium intake were introduced as categorical in the model, whereas saturated fat intake, physical activity level (IPAQ), educational level, and fiber intake as continuous. This was decided following a preliminary assessment on group differences. Logistic regression model was also stratified by SFA intake, to account for potential mediating effect between TFA and SFA intakes. All $p$-value estimates were based on two-sided tests. A $p$-value <0.05 was considered statistically significant. STATA 14.0 (StataCorp, Texas ltd., Texas, USA) statistical package was used for the analysis.

## 3. Results

The description of the main demographic, anthropometric dietary and other personal characteristics is depicted in Table 1. Overall, TFA intake did not differ by sex, age (in total and category), BMI, weight status and total energy consumption. Median TFA intake was 0.53% as energy (0.34% to 0.81%) in the total population but ranged from 0.27% in the first tertile to 0.95 in the 3rd, with a significant increasing trend ($p < 0.001$). A total of 16% of individuals consumed TFA above 1% of their total energy intake while a weighted 33.7% of the population had a TFA median intake of 0.95% of total energy, with an Interquartile range distribution of 0.81% to 1.31%. Individuals consuming highest TFA levels also had significant higher intakes of total fat, SFA, PUFA and MUFA (all expressed as % of total energy intake). Large differences were observed in SFA intake with individuals at the 1st tertile (Q1) of TFA intake consuming on average 10%, individuals at the 2nd TFA tertile consuming 13% (Q2) and those at the 3rd, 14.6% 3rd(Q3). This increasing trend was also observed in total cholesterol and sodium intakes ($p < 0.001$ for between group differences and for trend). Total fiber intake was significantly lower in the highest tertile of TFA intake with a significant decreasing trend found ($p < 0.001$). Mean systolic and diastolic blood pressures (SBP and DBP respectively), smoking, marital and professional status, as well as educational level, did not significantly differ.

In Fig. 1 the main food groups that contribute to TFA intake are depicted, including dairy and meat (poultry and red), in which TFA are found naturally. Cheese was by far the main contributor to TFA intake, with processed/refined grains and fried fish following.

In Fig. 2 main food contributors by age group are presented, in this case excluding food groups with naturally

3

**Table 1. Distribution of demographic, anthropometric, dietary, and other personal characteristics of the HNNHS population in total and by tertile of TFA intake.**

| Variables | Total Population N = 3537 | Tertile of Trans fatty acid intake | | | $p$ for differences | $p$ for trend |
|---|---|---|---|---|---|---|
| | | 1st Tertile N = 1163 | 2nd Tertile N = 1196 | 3rd Tertile N = 1178 | | |
| Trans fatty acid intake, as % energy | | 0.27 (0.1, 0.34) | 0.53 (0.47, 0.61) | 0.95 (0.81, 1.31) | <0.001 | <0.001 |
| Sex, % (95% CI) | | | | | | |
| Males | 48.7% | 50.4 (47.7, 53.1) | 47.2 (44.3, 50.2) | 47.4 (44.5, 50.2) | 0.26 | |
| Age in years, mean (sd) | | 44.1 (18.5) | 42.9 (18.3) | 43.8 (19.1) | 0.202 | 0.499 |
| Age category, % (95% CI) | | | | | 0.254 | |
| 18–39 years | 40.0 (45.1, 48.9) | 31.1 (28.7, 33.6) | 35.9 (33.5, 38.5) | 33.0 (30.6, 35.5) | | |
| 40–59 years | 32.1 (30.4, 33.9) | 37.3 (34.1, 40.6) | 30.0 (27.0, 33.1) | 32.7 (29.6, 36.0) | | |
| ≥60 years | 20.9 (19.2, 22.7) | 31.3 (27.2, 35.8) | 32.1 (28.2, 36.3) | 36.5 (32.3, 41.1) | | |
| BMI (kg/m$^2$) | 25.5 (4.7) | 25.6 (4.8) | 25.5 (4.8) | 25.3 (4.6) | 0.816 | 0.161 |
| BMI category, % (95% CI) | | | | | 0.176 | 0.358 |
| Healthy weight | 88.2 (46.3, 50.1) | 47.8 (44.6, 51.1) | 37.2 (33.9, 40.4) | 15.1 (12.9, 17.6) | | |
| Overweight | 34.7 (32.9, 36.6) | 47.8 (44.7, 51.2) | 33.1 (30.1, 36.3) | 18.9 (16.4, 21.8) | | |
| Obesity | 17.1 (15.6, 18.7) | 48.8 (45.6, 52.1) | 33.9 (31.0, 37.0) | 17.3 (14.8, 20.0) | | |
| Total energy in kcals, mean (sd) | 1937 (859) | 1956 (904) | 1915 (817) | 1942 (856) | 0.022 | 0.501 |
| Total fat, % energy, mean (sd) | 38.1 (10.3) | 35.0 (11.9) | 38.1 (9.3) | 41.0 (8.7) | <0.001 | <0.001 |
| Trans fat, % energy, median, IQR | 0.53 (0.34, 0.81) | 0.27 (0.17, 0.34) | 0.53 (0.47, 0.61) | 0.95 (0.81, 1.31) | <0.001 | <0.001 |
| SFA, % energy, mean (sd) | 12.6 (4.3) | 10.1 (3.8) | 13.0 (3.6) | 14.6 (4.3) | <0.001 | <0.001 |
| PUFA, % energy, median IQR | 4.9 (3.9, 6.4) | 4.8 (3.7, 6.3) | 4.8 (3.8, 6.3) | 5.2 (4.1, 6.5) | <0.001 | <0.001 |
| MUFA, % energy, mean (sd) | 17.1 (6.1) | 16.9 (7.3) | 16.7 (5.6) | 17.6 (5.2) | 0.003 | <0.001 |
| Added sugars, % energy, median IQR | 9.9 (4.9, 16.8) | 8.9 (3.9, 15.8) | 10.6 (5.3, 17.4) | 10.3 (5.6, 17.2) | 0.232 | |
| Fiber (gr), median IQR | 18.4 (12.1, 33.9) | 22.6 (13.9, 47.8) | 18.1 (12.3, 31.3) | 14.3 (6.1, 22.2) | <0.001 | <0.001 |
| Cholesterol (gr), median IQR | 203 (126, 313) | 162 (90, 274) | 202 (133, 305) | 238 (158, 360) | <0.001 | <0.001 |
| Total sodium (mg), mean (sd) | 2087 (738) | 1927 (737) | 2109 (702) | 2222 (746) | <0.001 | <0.001 |
| Total METS, median IQR | 2226 (984, 4986) | 2466 (990, 5280) | 2160 (942, 4746) | 2148 (990, 4764) | 0.242 | 0.201 |
| Smoking status, % (95% CI) | | | | | 0.633 | - |
| Ex-smoker | 16.8 (15.4, 18.3) | 33.6 (29.2, 38.2) | 33.6 (29.4, 38.2) | 32.8 (28.5, 37.4) | | |
| Current smoker | 34.2 (32.3, 36.2) | 34.1 (31.0, 37.3) | 33.9 (30.8, 37.0) | 32.1 (29.1, 35.3) | | |
| Never smoker | 49.0 (47.0, 51.0) | 31.5 (28.9, 34.2) | 33.5 (31.0, 36.2) | 34.9 (32.3, 37.6) | | |
| Systolic BP in mmHg, mean | 118.6 (15.3) | 119.1 (14.4) | 117.3 (15.5) | 119.5 (15.8) | 0.754 | 0.804 |
| Diastolic BP in mmHg, mean | 72.0 (10.6) | 71.5 (10.8) | 72.1 (11.0) | 72.5 (10.0) | 0.422 | 0.276 |
| Dyslipidemia, % (95% CI) | 27.6 (26.0, 29.3) | 28.0 (23.3, 28.9) | 28.2 (25.4, 31.1) | 28.7 (25.8, 31.8) | 0.393 | - |
| Professional status, % (95% CI) | | | | | 0.232 | - |
| Employed | 49.1 (47.1, 51.2) | 31.3 (28.7, 34.0) | 35.6 (32.9, 38.3) | 33.1 (30.6, 35.9) | | |
| Unemployed | 20.0 (18.5, 21.5) | 35.6 (31.7, 39.7) | 32.7 (29.0, 36.6) | 31.7 (28.0, 35.6) | | |

**Table 1. Continued.**

| Variables | | Tertile of Trans fatty acid intake | | | | |
|---|---|---|---|---|---|---|
| | Total Population N = 3537 | 1st Tertile N = 1163 | 2nd Tertile N = 1196 | 3rd Tertile N = 1178 | *p* for differences | *p* for trend |
| Homeworkers | 7.7 (6.8, 8.7) | 33.9 (27.8, 40.5) | 28.4 (22.8, 34.9) | 37.7 (31.3, 44.5) | | |
| Pensioners | 23.2 (21.4, 25.0) | 32.4 (28.4, 36.6) | 32.2 (28.5, 36.2) | 35.4 (31.3, 39.7) | | |
| Educational Level in school years, % (95% CI) | | | | | 0.383 | - |
| <6 | 14.9 (13.3, 16.6) | 36.0 (30.6, 41.7) | 31.5 (26.6, 36.8) | 32.5 (27.5, 38.0) | | |
| ≥6–11 | 35.9 (34.0, 37.8) | 33.9 (30.9, 37.1) | 32.9 (29.9, 36.0) | 33.2 (30.2, 36.4) | | |
| ≥12 | 49.2 (47.2, 51.3) | 30.9 (28.5, 33.5) | 34.9 (32.4, 37.3) | 34.2 (31.7, 36.8) | | |
| Marital status, % (95% CI) | | | | | | |
| Single/Divorced/Separated | 44.1 (42.1, 46.1) | 31.7 (29.1, 34.3) | 34.7 (32.1, 37.4) | 33.6 (31.1, 36.3) | 0.692 | - |
| Widowed | 7.5 (6.5, 8.7) | 32.5 (26.2, 39.4) | 30.6 (24.3, 37.7) | 36.9 (30.2, 44.2) | | |
| Married/Cohabitation agreement | 48.4 (46.3, 50.5) | 33.5 (30.6, 36.5) | 33.5 (30.8, 36.2) | 33.0 (30.2, 35.9) | | |

All proportions are weighted by area, sex and age. Group differences were tested using chi square test for proportions, and ANOVA or Kruskal Wallis rank sum test for continuous data, depending on data distribution. *p* for trend was tested post hoc.
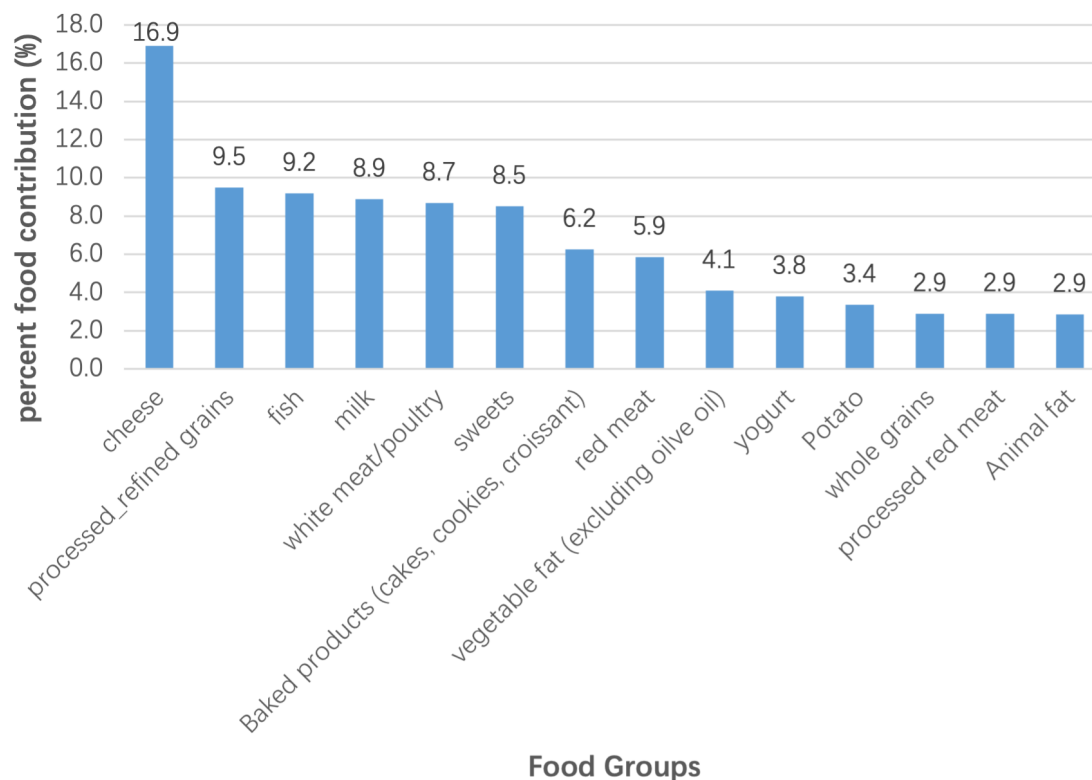
**Fig. 1. Main food group contribution to TFA in total population.** The three most contributing food groups to TFA intake in adults in Greece are cheese, processed/refined grains such as pies/pastries and fried fish.

occurring TFA's. Although total TFA intake did not differ by age group, the weight of the major contributing foods highly differed. Specifically, processed refined grains (mainly from savory pastries & pies) and sweets were the main food contributors in younger adults, whereas fried fish clearly picked in older adults, with a 13% contribution in adults 71+ years and 18% in those between 51 and 70 years.

A descriptive presentation of the proportion of the population with specific CVD risk factors among those consuming above the recommended levels of TFA intake ($>1\%$ of total energy) for the total population and by sex is presented in Fig. 3. Overall hypercholesterolemia and total dyslipidemia affected more than 50% of the population with TFA consumption above recommended intakes, with prevalence being significantly higher in males, although total distribution intakes did not differ (as per Table 1). Clinically significant prevalence was also observed in those with abnormal plasma glucose ($>110$ mg/dL) and LDL-cholesterol levels ($\geq 130$ mg/dL).

Dyslipidemia likelihood in total and by level of SFA intake is shown in Table 2. A fully adjusted model, accounting for weight status saturated fat intake, sex, age, smoking status, physical activity level (IPAQ), educational level, and fiber intake, showed that dyslipidemia was 30% more likely for those at the 2nd tertile or the 3rd tertile compared to the lowest intakes (OR: 1.3; 95% CI: 1.02–1.66 and 1.01–1.66, respectively). When the logistic regression was stratified

by SFA intake above and below recommended guidelines (10% of total energy), the likelihood of dyslipidemia increased to 40%, (OR: 1.4; 95% CI: 1.06–1.94). The results remained significantly higher only among those with $>10\%$ SFA intakes. Higher physical activity, and never smoking significantly reduced the odds of dyslipidemia but did not null the risk attributed to higher TFA and SFA intakes. Overweight and obesity as well as increasing age categories significantly increased the odds of dyslipidemia in all cases.

## 4. Discussion

The present study showed that higher TFA intakes were significantly associated with an increased likelihood of dyslipidemia with prevalence of dyslipidemia reaching 63% among adults that consumed TFA above the recommended intake which is set to 1% of total energy intake. Also, although approximately 16% of the population exceeded the recommended levels of TFA intake, the median intake of the population at the highest tertile was 0.95% This means that approximately 1/3 of the population had an intake borderline to the recommended cut-off level. This proportion of the population also greatly exceeded SFA recommended intakes by 4.6%, a factor that showed to further increase likelihood of dyslipidemia by an additional 10%. The major food groups contributing to TFA intakes were a mix of natural and industrially produced TFA's, with an emphasis on cheese, processed grains, fried fish, and baked

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**Table 2. Likelihood of Dyslipidemia by tertile intake of trans fatty acids, in total and by level of saturated fat intake.**

| Dyslipidemia | | | | <10% Saturated fats | | | ≥10% Saturated fat | | |
|---|---|---|---|---|---|---|---|---|---|
| | Odds Ratio | Std. Err. | [95% Conf. Interval] | Odds Ratio | Std. Err. | [95% Conf. Interval] | Odds Ratio | Std. Err. | [95% Conf. Interval] |
| Trans intake % energy 1st Tertile the reference level | | | | | | | | | |
| 2nd tertile | 1.3 | 0.2 | 1.02, 1.66 | 1.4 | 0.3 | 0.94, 2.22 | 1.3 | 0.2 | 0.97, 1.80 |
| 3rd tertile | 1.3 | 0.2 | 1.01, 1.66 | 0.8 | 0.2 | 0.49, 1.34 | 1.4 | 0.2 | 1.06, 1.94 |
| Weight status[1] | 1.7 | 0.2 | 1.44, 2.11 | 2.0 | 0.4 | 1.38, 2.80 | 1.7 | 0.2 | 1.34, 2.11 |
| Sex[2] | 1.1 | 0.1 | 0.87, 1.27 | 1.0 | 0.2 | 0.70, 1.40 | 1.1 | 0.1 | 0.85, 1.35 |
| Age category[3] | | | | | | | | | |
| 40–59 | 3.7 | 0.4 | 2.93, 4.51 | 5.0 | 1.1 | 3.23, 7.66 | 3.3 | 0.4 | 2.60, 4.29 |
| ≥60 | 5.4 | 0.9 | 3.70, 7.02 | 7.4 | 2.3 | 3.98, 13.72 | 4.6 | 0.9 | 3.20, 6.68 |
| Smoking status[4] | | | | | | | | | |
| current | 0.8 | 0.1 | 0.66, 1.13 | 0.9 | 0.2 | 0.56, 1.52 | 0.9 | 0.1 | 0.62, 1.18 |
| never | 0.7 | 0.1 | 0.52, 0.86 | 0.8 | 0.2 | 0.47, 1.26 | 0.6 | 0.1 | 0.48, 0.85 |
| Physical activity level[5] | 0.7 | 0.1 | 0.52, 0.81 | 0.5 | 0.1 | 0.35, 0.83 | 0.7 | 0.1 | 0.53, 0.88 |
| Educational level[5] | 1.1 | 0.1 | 0.95, 1.30 | 1.2 | 0.2 | 0.88, 1.52 | 1.1 | 0.1 | 0.92, 1.32 |
| Total Saturated fat intake, % energy | 0.9 | 0.1 | 0.70, 1.11 | - | - | - | - | - | - |
| Sodium intake (>1500 compared to <1500) | 0.9 | 0.1 | 0.79, 1.05 | 1.0 | 0.1 | 0.74, 1.231 | 0.9 | 0.1 | 0.75, 1.07 |
| Total MUFA intake, % energy | 1.0 | 0.0 | 0.99, 1.02 | 1.0 | 0.0 | 0.99, 1.05 | 1.0 | 0.0 | 0.98, 1.02 |

Reference categorization: [1]overweight & obesity vs healthy weight; [2]baseline level 19–39.9; [3]females compared to males; [4]compared to ex-smokers; [5]assessed as continuous variables.

model adjusted for weight status, saturated fat intake, sex, age, smoking status, physical activity level (IPAQ), educational level, and fiber intake

MUFA, Monounsaturated fatty acids.
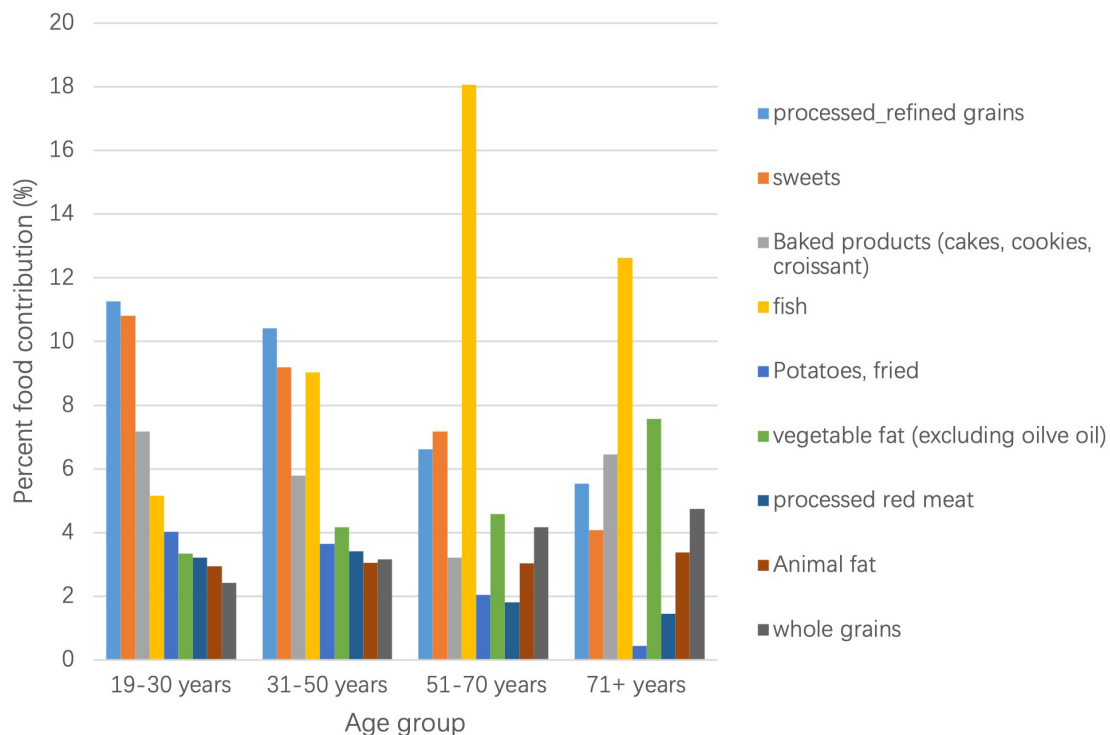
**Fig. 2. Main food group contribution to TFA by age group.** The most contributing food group to TFA intake (excluding naturally ocurring TFA) for the age groups 19–30 y and 31–50 y was processed/refined grains, while for the age groups 51–70 y and 71+ y this was fried fish.

goods & sweets. These results are in agreement with other studies [27] who reported a higher CVD risk with increasing TFA intakes and underline the need for public health prevention programs.

In 2020 WHO created a Certification Programme for Trans Fat Elimination in order to recognize countries that have eliminated industrially produced TFA's, with 14 countries, including USA, being recognized with best-practice and monitoring and enforcement systems in place already in effect policies [18]. TFA's can be of natural (ruminant) origin (r-TFA) or industrially produced (i-TFA). Industrial sources of TFA are mainly of concern, since the consumption of r-TFA on average contributes to less than 0.5% of total energy intake [14]. Recently, the EU Regulation 2019/649 (EC, 2019) set a limit of 2% i-TFA per 100 g of fat in processed foods, in an effort towards further i-TFA reduction in the food supply in the EU, a regulation fully implemented since April 2021. A reduction of i-TFA intake has been a global public health priority. However, the question remains whether a banning policy alone will effectively decrease CVD risk, since the present study revealed that even levels close to but below 1% TFA intake with respect to energy consumption are associated with increased likelihood of dyslipidemia, especially among individuals that have SFA intakes >10% of their total energy consumption. This is of major importance since studies that have evaluated implemented mandatory TFA limit

policy, showed that in foods that had decreased their TFA content to adequate levels, in some cases SFA content increased [28–31], while in other cases unsaturated fats increased [32]. Of course, it should be mentioned that a recent meta-analysis of epidemiological studies did not find a significant increased risk of CVD outcomes with SFA intake, but it was associated with TFA [8]. The studies included however, did not assess TFA intake by level of SFA consumption, hence the results are not comparable. Mazidi *et al.* [33], reported that SFA intake was associated with all-cause mortality in the National Health and Nutrition Examination Survey (NHANES). When the authors performed a meta-analysis on end point associated with SFA intake they found a significant association with CHD only [33]. Another study showed that non-optimal SFA and TFA intakes accounted for 3.6% and 7.7% of global CHD mortality, with important between country heterogeneity [7]. The type of fat consumed, may therefore, affect health outcome [7] and may also be population specific based on dietary, lifestyle and other variables.

In the present study r-TFA's were not distinguished from i-TFA's since a recent systematic review reported that both sources of TFA can increase cardiometabolic risk parameters, especially lipid profile [6]. Specifically, although rTFA seems to be less harmful than iTFA for HDL cholesterol, in the case of total cholesterol and LDL cholesterol it may be worse. This is of great importance consider-

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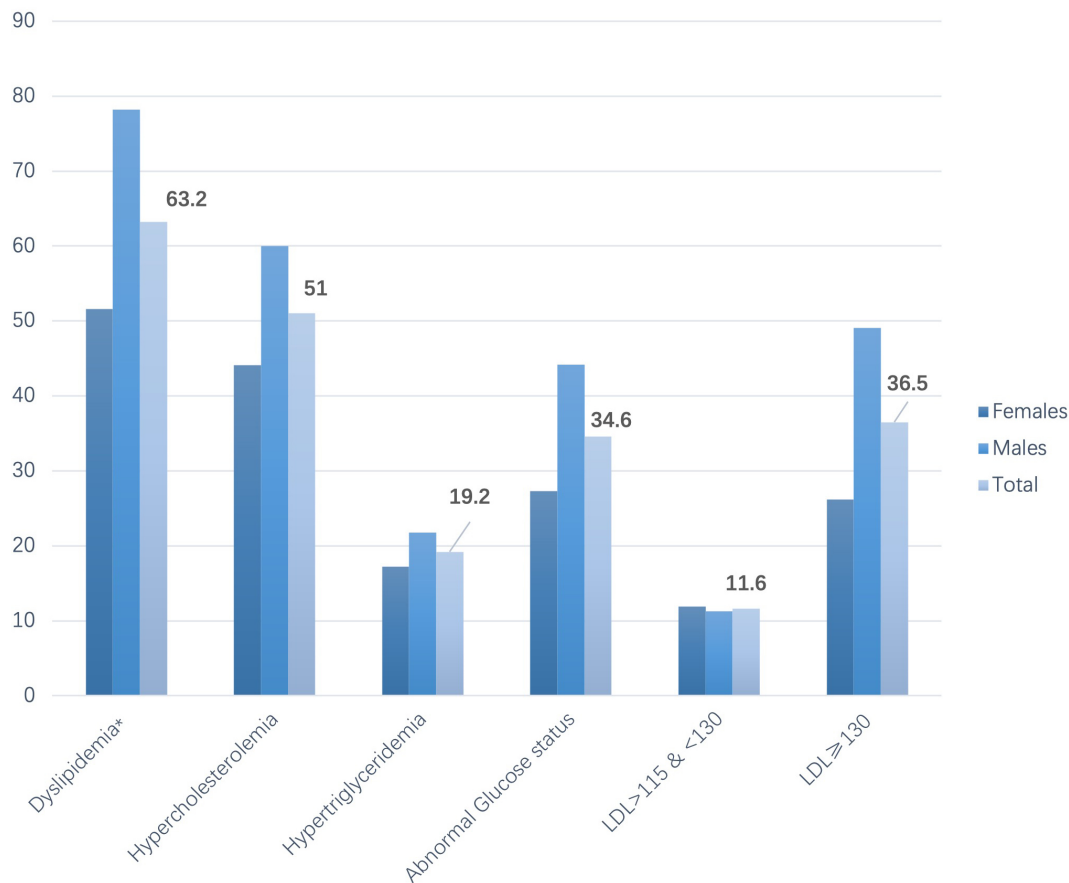
**Fig. 3. Proportion of population with specific disease status that consume over 1% of total energy from trans-fat, in total and by sex (N = 577, 46.35% of population).** LDL, Low density Lipoprotein, all in md/dL. Dyslipidemia as per measured abnormal lipid profile. Hypercholesterolemia: >200 mg/dL. Abnormal Glucose status: Fasting plasma glucose >110 mg/dL.

ing that LDL is one of the strongest determinants of CVD risk and high levels of LDL were found in 36.5% of this study's population that consumed TFA >1% of their total energy. In addition, considering the potential mediating effect of SFA on dyslipidemia, the fact that cheese was the main food item contributing to TFA with an approximately 7% marked difference compared to the 2nd main contributor (refined/processed grains) raises concerns on the effectiveness of the implemented TFA policy, if educational and other promotional campaigns are not administered.

TFA's are also present in baked and fried foods and significantly intake in the Greek population. Interestingly, results from the present representative study showed that apart from cheese, another major contributor to total TFA food was processed grains and their products and in particular baked goods such as savory and sweet pastries and pies.

Finally, the third major food which contributed highly to total TFA intake was fried fish, in all age groups, and primarily in adults over 50 years of age, indicating that the method of cooking could also significantly contribute to total TFA intake. This is of special interest since fish is a food that is perceived by most individuals as healthy and

can be consumed in restaurants or at home. Since there are no available occurrence data on TFA content of fried fish prepared at home and/or out of home in Greece, these results should be viewed with caution and point to a need to focus on sampling fried fish, particularly from the catering sector, in future official food controls in Greece.

The results of this large, national cross-sectional study have been presented with caution due to the nature of the design and should be treated accordingly. Specifically, only the likelihood of outcomes can be evaluated with respect to specific risk factors, in this case dyslipidemia and level of TFA intake, and no temporal effects can be established. Other strong end points were not included, such as CVD outcomes since people tend to change their dietary and lifestyle habits after a specific event. This would have included systematic exposure measurement error in the analysis. The study however also has strong points, since it is a strategically designed study that aimed to evaluate the nutritional and health status using a national representative sample. Furthermore, TFA analysis was performed using country specific data, particularly for baked goods consumed, obtained during an official control program by the Hellenic Food Authority.

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**Table 3. Content of Trans Fatty Acids (g/100g food). total (TFA) and industrial (i-TFA) per food group.**

| Food Groups | Samples analyzed | HNNHS (n) | HNNS unique foods list (n) | TFA g/100 g food | i-TFA g/100 g food |
|---|---|---|---|---|---|
| Savoury (i.e., non-sweet) foods and snacks | 70 | 2703 | 39 | | |
| Cheese pies | 30 | 712 | 6 | 0.58 | 0.49 |
| Cheese pies kaseropita | 2 | 14 | 2 | 0.87 | 0.72 |
| Spinach pies | 5 | 235 | 4 | 0.09 | 0.09 |
| Sausage pies | 5 | 67 | 3 | 0.43 | 0.40 |
| Pizza | 12 | 451 | 3 | 0.16 | 0.01 |
| Pizza tomato. cheese margarita | 4 | 55 | 2 | 0.21 | 0.04 |
| Meat products | 10 | 1094 | 15 | | |
| Pork skewers | 3 | 265 | 3 | 0.05 | 0.05 |
| Pork gyros | 3 | 201 | 4 | 0.09 | 0.09 |
| Chicken gyros | 1 | 88 | 3 | 0.11 | 0.11 |
| Burger (no bread) bifteki | 1 | 498 | 1 | 0.08 | 0.08 |
| Kebab | 2 | 42 | 4 | 0.26 | 0.18 |
| French Fries | 5 | 44 | 7 | 0.05 | 0.05 |
| Pop corn | 3 | 100 | 1 | 0.12 | 0.12 |
| Dessert/sweet foods and snacks | 70 | 2854 | 58 | | |
| Cakes | 20 | 608 | 21 | 0.09 | 0.07 |
| Cakes | 10 | 531 | 15 | | |
| Cake vanilla-chocolate/cocoa | 2 | 22 | 1 | 0.08 | 0.08 |
| Cake cocoa | 2 | 23 | 1 | 0.05 | 0.05 |
| Gateaux type layer cakes | 10 | 77 | 6 | 0.2 | 0.00 |
| Gateaux cake almond | 2 | 6 | 1 | 0.172 | 0.00 |
| Gateaux cake vanilla-chocolate | 2 | 9 | 1 | 0.322 | 0.00 |
| Gateaux cake chocolate | 1 | 33 | 1 | 0.28 | 0.00 |
| Cookies/biscuits | 15 | 1113 | 15 | | |
| Cookies | 10 | 940 | 12 | 0.23 | 0.13 |
| Butter cookies | 1 | 55 | 1 | 0.21 | 0.00 |
| Cinnamon cookies | 2 | 58 | 1 | 0.07 | 0.03 |
| Grape must cookies | 1 | 79 | 1 | 0.08 | 0.08 |
| Chocolate/cocoa cookies | 1 | 41 | 1 | 0.11 | 0.10 |
| Biscuits | 5 | 173 | 3 | 0.07 | 0.07 |
| Stuffed biscuits | 4 | 169 | 1 | 0.08 | 0.08 |
| Croissants | 10 | 382 | 8 | 0.18 | 0.07 |
| Doughnuts | 10 | 112 | 2 | | |
| Doughnuts Loukoumas | 5 | 53 | 1 | 0.07 | 0.04 |
| Doughnuts Donuts | 5 | 59 | 1 | 0.08 | 0.08 |
| Sweet Pastries (Bougatsa)) | 5 | 54 | 1 | 0.48 | 0.47 |
| Wafers | 5 | 104 | 3 | 0.61 | 0.59 |
| Ice creams | 5 | 481 | 8 | 0.13 | 0.01 |
| Ice cream parfait | 1 | 9 | 1 | 0.14 | 0.00 |
| Sum | 140 | 5557 | 97 | | |

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**Table 4. Total Food Groups used for TFA study analysis.**

| Food Groups | | |
| --- | --- | --- |
| **Fruits** | **Egg** | **Artificially-sweetened beverages** |
| Fresh fruits, cooked or dried | Eggs | Carbonated artificially-sweetened beverages |
| **Fruit juice, 100%** | **Fish and Shellfish** | **Salty snacks** |
| Natural fruit juices unsweetened | Fish fresh and frozen | Chips, crackers, pop corn |
| **Non-starchy vegetables** | Shellfish | **Desserts and Sweets** |
| Green leafy vegetables | **Red meat** | Sweets, candy, chocolate |
| Tomatoes, carrots, lettuce | Lamb, pork, veal, game | Milk desserts |
| Mixed and other vegetables | **White meat** | Sugary foods (i.e., baklavas) |
| Vegetable juice | Poultry | **Condiments and spices** |
| **Starchy vegetables** | **Processed meat** | **Salt all types** |
| Corn, beans, green beans | Sausages, ham, salami, beacon of red meat origin | **Water from mixed recipes** |
| Pumpkin | **Processed white meat** | Water natural, mineral and carbonated |
| Sweet potatoes | Sausages of white meat origin | **Coffee** |
| **Potato** | Chicken nuggets | **Tea** |
| Potatoes | **Processed fish** | **Artificially sweeteners** |
| **Wholegrain cereals** | Smoked, caned and salted fish | **Sugar** |
| Wholegrain cereal products | Fish sticks | Sugar, honey, syrup |
| **Processed cereals** | **Olive oil and olives** | **Baked products** |
| All refined grains and cereal products | Olive oils | Cake, biscuit, pie, muffin, doughnuts |
| **Legumes** | Oils | **Artificially sweetened Fruit juices** |
| Legumes, (i.e., beans) | **Vegetable fat** | Artificially sweetened fruit juices |
| Meat alternatives, soy, tofu | Vegetable oils, vegetable fat, vegetable oil-based salad dressing | **Baby food** |
| **Nuts** | **Animal fat** | Baby food |
| Nuts, almonds, seeds | Butter, mayonnaise | |
| Peanut butter | White sauce, cream | |
| Almond milk | Alcoholic beverages | |
| **Milk** | Alcoholic beverages | |
| Milk and milk drinks | **Sugar-sweetened beverages** | |
| **Yogurt** | Carbonated and non-carbonated sugar-sweetened beverages | |
| Yogurt | | |
| **Cheese** | | |

## 5. Conclusions

Dyslipidemia prevalence increased with higher total TFA intake, especially among those with high SFA intakes, underlining the need for stricter adherence to dietary guidelines following educational programs along with set public health policies. These are both highly modifiable factors and can greatly serve as vehicles to reduce dyslipidemia, a major cardiovascular risk factor. Both r-TFA and i-TFA should be monitored and further evaluated by level of SFA intake. Although i-TFA is expected to decrease following the implemented TFA elimination policy, monitoring the lipid profile of processed foods, particularly non-branded/non-prepackaged foods such as bakery foods and fried fish, and checking the abidance of the food and catering sector to the new EU legislation on i-TFA is necessary and important.

### Abbreviations

TFA, Trans Fatty Acids; Itfa, industrialized Trans Fatty Acids; rTFA, ruminant Trans Fatty acids; PUFA, Polyunsaturated fatty Acids; MUFA, Mono unsaturated Fatty acids; SFA, Saturated Fatty Acids; BMI, Body Mass Index; FG, Food groups; HNNHS, Hellenic National Nutritiona and Helath Survey; EU, European Union; CVD, Cardiovascular Diseae; CRP, C Reactive Protein; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; Lp$\alpha$, lipoprotein (little) a; TG, Triglycerides; PA, Physica activity; WHO, World Health Organization; IPAQ, International Physical Activity Questionnaire; CI, Confidence Interval.

### Author contributions

EM, GMa and AZ, designed the research study. EM, GMi, RM and AZ, performed the research. SK, AN, GMi, DP and RM provided help and advice on study methodology and statistical analyzis. EM, AN, SK and DP analyzed the data. EM, GMa and AZ wrote the manuscript. AN, DP and GMi significantly edited the manuscript. All authors contributed to other editorial changes in the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All work was carried out upon obtaining individual consent and approval by the Ethics Committee of the Department of Food Science and Human Nutrition of the Agricultural University of Athens and by the Hellenic Data Protection Authority (HDPA) (MIS 374143).

### Acknowledgment

Special thanks to all the fieldworkers.

### Conflict of interest

The authors declare no conflicts of interest. Demosthenes Panagiotakos is serving as one of the Guest editors of this journal. We declare that Demosthenes Panagiotakos had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Grant N. Pierce.

### Appendix

*TFA Content of Foods*

Food items sampled and analyzed are shown in Table 3. The mean TFA from multiple measurements was calculated and used for ethnically consumed food.

The TFA was recalculated for 5557 out of 87953 food consumed by the HNNHS sample. representing a percentage of 6.3% and for 97 out of 1915 unique foods (5.1%).

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Original Research Article

# Changes of *trans* and saturated fatty acid content in savory baked goods from 2015 to 2021 and their effect on consumers' intake using substitution models: A study conducted in Greece

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A B S T R A C T

**Background:** In Greece, nearly a third of savory baked goods (SBGs) exceeded the limit of 2 g of nonruminant or industrial *trans* fatty acids (i-TFA) per 100 g fat in 2015. The impact of the Commission Regulation (European Union) 2019/649 on exposure to *trans* fatty acids (TFA), i-TFA, and saturated fatty acid (SFA) from SBGs has not been previously evaluated.

**Objectives:** The study aimed to explore fatty acid reformulation of SBG products and assess differences in TFA, i-TFA, and SFA intakes using a sample of Greek SBG consumers from a nationally representative survey.

**Methods:** In 2021, 140 samples of SBGs were collected in the greater metropolitan area of Athens, and their fat profile and content were compared to those from 2015. Based on these measurements, food consumption substitution models were employed to examine TFA and SFA intake differences, and the percent contribution from SBG among consumers was calculated ($N = 1008$). Nutrient densities were calculated by adjusting all fat intakes by individual mean energy intake (percentage of daily total energy intake).

**Results:** The 2% i-TFA legislative limit/100 g of fat in measured SBGs was exceeded by 11.4% in 2021 compared to 31.1% in 2015 (19.7% increase in compliance). Median i-TFA and TFA intakes from SBGs were reduced from 0.05 (0.01, 0.12)% and 0.13 (0.03, 0.27)% in 2015 to 0.03 (0.01, 0.09) and 0.06 (0.03, 0.13)% in 2021, respectively. In terms of SFA, a mean increase/100 g was calculated, resulting in an increased intake in 2021 compared to 2015 [5.18% (2.78, 8.37) and 3.55 (1.99, 5.73), respectively].

**Conclusions:** Despite the reductions seen in i-TFA content of SBGs, food product reformulation efforts in Greece should focus not only on TFA content but also on SFA reduction to improve public health.

*Keywords*: i-TFA, TFA, dietary exposure, dietary intake, SFA, savory baked goods, EU regulation, fat intake

## Introduction

Dietary *trans* fatty acids (TFAs) can be of natural origin (r-TFA) or of nonruminant origin (usually referred to as industrially produced, i-TFA). Although evidence of the differences between the 2 as per their health impact remains insufficient [1,2], the elimination of i-TFA is regarded as a relatively straightforward, cost-effective policy measure that can save lives [3]. In fact, a recent systematic review that assessed the link between food reformulation and health outcomes showed that mortality from cardiovascular diseases (CVDs) was reduced between 4.3–6.2% in 4 of the 5 studies which evaluated the effect of TFA bans in packaged or restaurant foods [4]. In 2004, Denmark became the first

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country in the world to adopt legislation that set a maximum amount on the content of i-TFA in processed foods [5], and its example was followed later by other countries. Following these initiatives, in 2019, the European Commission adopted Regulation European Union (EU) 2019/649, which set a legal limit for nonruminant TFA of 2 g/100 g fat to restrict the i-TFA concentration in foods. However, despite the importance of this measure, exposure to foods containing i-TFA, even at amounts less than the legislative limit, could still place consumers at risk if these are consumed frequently and in large portions [6]; hence, estimation of the population exposure to main food sources of i-TFA is necessary for the evaluation of this legislative measure.

Commercially baked products are easily accessible and among the main sources of i-TFA [7]. Intake assessment using data from adults participating in the Hellenic National Nutrition and Health Survey (HNNHS) indicated that bakery products were the main contributors to TFA intake in the age group 18–50 y [8] and 1 of the top 3 in the older population, most probably due to the partially hydrogenated margarines and shortenings that are used in dough preparation. Previous occurrence data on TFA and i-TFA in processed foods available in the Greek market, collected in 2015, indicated the presence of i-TFA above the currently imposed legal limits for several products [9]. The Regulation (EU) 2019/649 on i-TFA has been fully implemented in all EU member states since April 1, 2021 [10], but the replacers of the i-TFA have not been defined. It has been observed that in some countries where this measure has already been implemented, the mean fat content of the products remained constant; however, the majority of the i-TFA were substituted by SFA [11], a fat type that has also been associated with cardiovascular events [12,13] and all-cause mortality [14].

Consequently, the primary aim of the present study was to explore fatty acid composition in commercial savory baked goods (SBGs), following the Regulation (EU) 2019/649 and assess intake differences of TFA, i-TFA, and SFA in 2021 compared to 2015, using food consumption substitution models. The secondary aim was to assess TFA, i-TFA, and SFA contributions from SBGs. Our hypothesis was that TFA's were substituted with SFA's to maintain organoleptic characteristics and/or to minimize cost of production.

## Methods

### Study design and population

Participants from the HNNHS, a population-based survey carried out between September 2013 and May 2015 among noninstitutionalized individuals and nonpregnant nor lactating females, were included in this study. Details of the HNNHS sampling and methodology, including questionnaires used, have been published elsewhere [15]. All HNNHS participants who were SBG consumers were included in this study. The Hellenic Data Protection Authority and the ethics committee of the Department of Food Science and Human Nutrition of the Agricultural University of Athens approved the study, and individual consent to participate was signed prior to commencement.

Dietary intake data were retrieved in 2013–2015. For the purpose of this study, data from SBG consumers ($n = 1008$, 54.5% females), which constituted 22.7% of the 4450 HNNHS participants, were retrieved from the HNNHS database and were included. Specific SBG measurements of SFA and TFA (r-TFA and i-TFA) content were performed in 2 different periods: in 2015 and in 2021. Based on these measurements and intake data, TFA and SFA intakes from SBGs were estimated. Intakes from the different time points were compared using

food consumption substitution models (from now on called substitution models with details provided below). Participants were categorized into 3 age groups: children and adolescents (≤19 y), adults (20–59 y), and elderly (≥ 60 y) (see also Supplementary Table 1). The flow of the study population selection process and the final sample size included can be seen in Figure 1.

### Estimation of trans and SFAs content of SBGs

#### *Sampling of SBGs*

Seven categories of commercially produced SBGs were included in this study and were selected based on previously recorded frequency of consumption [8] and availability. Specifically, SBGs included: *1)* cheese pies made with puff pastry, *2)* cheese pies made with shortcrust pastry, *3)* cheese pies made with phyllo pastry (characterized by thin sheets of unleavened dough of crispy texture when baked, made from flour and water; typically prepared by brushing with olive oil or other types of vegetable oil between sheets of dough), *4)* vegetarian pies (spinach or leek pies without cheese typically made with phyllo pastry and olive or other types of vegetable oil), *5)* processed meat pies (sausage or ham pies) typically made with puff pastry, *6)* savory "bougatsa" (made with special type of dough containing butter/solid fat and filled with cheese) and *7)* "peinirli" (i.e., pizza boat with cheese and vegetables and/or processed meat). The choice of these food categories was based on the results of a previous TFA evaluation program of the Hellenic Food Authority, which showed that particularly the non-prepacked SBGs were more likely to have TFA exceeding the 2% limit [9]. Approximately 20 samples were obtained for each product category. Samples were purchased from 123 different food businesses in Attiki prefecture (Athens greater metropolitan area) during the last 2 wk of March 2021, just before the full implementation of the Regulation (EU) 2019/649 [10]. We sought to collect samples not only from artisan bakeries (small businesses) ($n = 111$) but also from chain bakeries (located all over Greece) ($n = 12$) to optimize measured sample accuracy. Sampling was stratified according to product category and geographical location of Attiki prefecture, aiming to collect as equal as possible numbers and categories of bakery products from randomly selected bakeries in each of the regional units of Attiki prefecture. The sampling of products in 2015 has been previously described [9].

#### *Chemical analysis of SBGs.*

The chemical analysis of SBGs sampled in 2021 was identical to the chemical analysis of SBGs sampled in 2015 and was performed by the same scientific personnel. In brief:

- Sample preparation

Each sample was homogenized and separated into 2 sub-samples that were stored at 4°C and analyzed within 2 d.

- Lipid extraction for the determination of Fatty Acid Methyl Ester (FAME) profiles

The fat extraction was carried out by a mild in-house method based on AOAC 966.06 (Association of Official Analytical Chemists 2005) and Roese-Gottlieb method (AOAC 905.02) to avoid any possible alteration of the FAME profile. Briefly, 5 g of each sample was diluted in a flask with a 10% ammonia ($NH_3$) solution (v/v) and refluxed in a water bath at 60°C for half an hour. After cooling, the flask content was
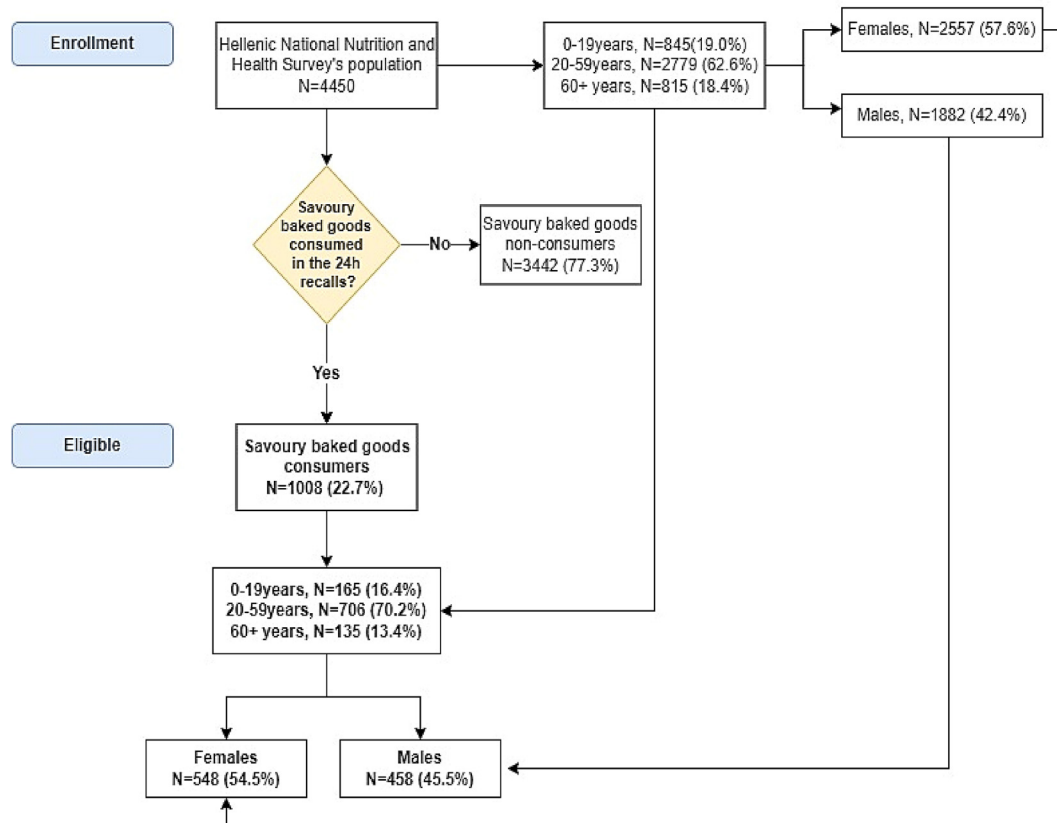
**FIGURE 1.** Participant flow chart.

poured into a separating funnel and extracted with 3 portions of a diethyl ether-petroleum ether solution (1:1). All ether phases were collected into a second separating funnel and washed with 3 portions of water. Finally, the remaining ether phase was filtered and evaporated at a moderate temperature (40°C). ~0.1 g of the resulting fat was treated with 3 mL heptane and 0.2 mL KOH methanolic solution 2 N in a vial and mixed in a vortex. After 30 min, the upper heptane phase was ready for injection into a gas chromatograpfhy with a flame ionization indicator (GC-FID) system (Agilent 7890A Network Gas Chromatograph; Agilent Technologies).

- Fatty acid analysis

The gas chromatography system was equipped with a TR-FAME capillary column (50 m × 0.22 mm, 0.25 μm film thickness Thermo Fisher Scientific). Helium was used as carrier gas at a flow rate of 3.0 mL/min. The setting temperatures for the injector and the detector were 260°C and 280°C, respectively. The oven temperature was initially set at 100°C for 6 min, then raised at a rate of 4°C/min to 160°C, remaining stable for 10 min. Afterwards another gradual temperature rise was held with a rate of 4°C/min ≤240°C and retained stable for 4 min, resulting in a total run time of 55 min. Each sample was injected twice by an autosampler unit (Agilent 7683B Series injector) in split mode (30:1) with an injection volume of 1μL.

Four commercial standards purchased from Supelco (Sigma) were used for the identification of the chromatograph peaks [i.e., a FAME Mix C4–C24 ($n = 37$ components), a *trans*-9-elaidic methyl ester standard (18:1), a linoleic acid methyl ester isomer mix (18:2) ($n = 4$) and linolenic acid methyl ester isomer mix (18:3) ($n = 8$)]. Fatty acids and *trans*-isomers content were expressed as a percentage of the sum of

the fatty acids. Both r-TFA and i-TFA were estimated according to the European Commission's analytical approach of 2018 [10]. Specifically, r-TFA was calculated using the following equation: r-TFA (g/100 g) = [butyric acid (g/100 g) × 29.4 × 6]/100. Then, the i-TFA content was estimated from the subtraction of r-TFA from total TFA: i-TFA (g/100 g) = total-TFA − r-TFA. Any negative value of i-TFA was replaced by 0 as recommended by the European Commission [10].

### TFA and SFA intake assessment

Using the Automated Multiple Pass Method, 2 24-h recalls were obtained per participant on a different nonconsecutive day: 1 at the initial in-person interview and the second through a telephone interview 8–20 d later. Individuals were asked to select the amount consumed using validated food atlases and standardized grids and mounts. Intake reported was converted to specific grams represented by these pictures, as previously described [15].

For the substitution models, the HNNHS, which is the latest food consumption dataset from a nationally representative sample of the Greek population and was previously used in research to assess TFA intake among Greek adults [8], was updated to include both measured TFA and SFA content of SBGs sampled. Specifically, results from the current fatty acid analysis (year 2021) of SBG samples regularly consumed by Greeks were included in the database, adding an extra column with the new nutritional content of these foods, as well as the previously measured values (2 models based on real measurements from 2 different time points). These newly measured nutritional values were used to derive the (new) estimated concentrations of TFA and SFA that would be consumed from SBGs for each individual, and in total, after food reformulation as per the policy (models of intake based

on measured reformulated SBGs). Nutrient densities for TFA's and SFA were calculated by adjusting all fat intakes by individual mean energy intake (percentage of daily total energy intake). Differences in energy-adjusted TFA and SFA intakes for SBG consumers were then calculated, comparing new estimated values from 2021 measurements with those based on 2015 measurements, maintaining SBG consumption unchanged. Food composition of other foods and amounts of foods consumed were assumed unchanged during this process.

The different types of nonprepacked commercial SBGs involved are presented in Supplementary Table 3. The daily TFA and SFA intakes from these products (g/d) were calculated by multiplying the mean content of TFA/SFA per SBG (g in 100 g) with the individual daily consumption of each product (g/d). Subsequently, the estimated daily intakes were summed per individual and averaged over the number of reporting days (the majority of the populations had 2 24-h recalls). TFA and SFA estimates were adjusted for energy intake and were expressed as nutrient densities (percentage contribution to daily total energy intake) to have comparable data. The percentage contribution of each SBG to the overall TFA intake from savory bakery products was additionally estimated.

## Other parameters

Qualified, trained professionals collected data on anthropometric and sociodemographic characteristics, including lifestyle preferences, using the Computer Assisted Personal Interview method. Health professionals collected data on disorders and medication use.

Area of residence was classified into 3 groups: Attiki and Thessaloniki, islands (including Crete), and mainland. Marital status was categorized into 4 groups: single, married/cohabiting, divorced/separated, and widowed. Educational level was grouped as follows: ≤6 y of schooling, 12 y of schooling, and higher education (including colleges). Employment status was grouped into 3 categories: unemployed, employed, and pension. Physical activity status was defined as sedentary, low, moderate, and high, based on the International Physical Activity Questionnaire as per calculation guidelines [16]. Individuals were also categorized as ex-smokers if they had stopped smoking for ≥30 d, were never smokers, and were current smokers, as per the definition by the National Center for Health Statistics [17]. BMI (kg/m$^2$) of adults was calculated based on measured weight and height [weight/height$^2$(kg/m$^2$)]. Weight status was classified into 3 groups: healthy weight <25, 25 ≤ overweight <30, and obese ≥30. Children and adolescents were classified using the extended International Obesity Task Force tables [18]. Differences between the total study sample (n = 1008) and sub-totals are attributed to 2 missing values in the sex and age variables.

## Statistical analysis

Data were analyzed by STATA software version 13.0 (StataCorp; Texas Ltd.). Continuous variables were presented as mean (SD) when normally distributed and median (25th, 75th percentile) for skewed distributions. Categorical variables were presented as frequencies. Group differences were tested by the $\chi^2$ test for proportions and analysis of variance or Kruskal-Wallis rank sum test for continuous data, depending on their distribution. The Wilcoxon signed-rank test was used to compare our results with those of previous research [8]. Level of significance was set at 5%, and trend analysis was also assessed for continuous variables. Survey-specific analyses were not employed since the aim of the study was to examine intake TFA and SFA differences and estimate SBG contribution to TFA and SFA intakes, using

substitution models based on measurements from 2 different years, and not to estimate mean population intakes.

## Results

The contents of total fat and fatty acids in different types of SBGs in 2021 compared to 2015 are depicted in Table 1. An increase in total fat content was found in all SBGs other than cheese pies with phyllo pastry, with most of the products having a concomitant increase in SFA content (g/100 g of product). Mean i-TFA values were reduced in almost all product categories in 2021 compared to 2015 (Table 1). Table 2 presents the amounts of SFA, total TFA, and i-TFA per 100 g of fat and indicates that the number and percentage of products for which i-TFA was found to be above the legal limit of 2% of fat was reduced in most categories. There was a ~19.7% reduction in the number of products exceeding the 2% limit between the 2 sampling periods (31.1% in 2015 compared with 11.4% in 2021).

The baseline characteristics of SBG consumers are depicted in Table 3, whereas their dietary characteristics are separately presented in Table 4, overall, and by total SFA consumption tertiles of the reformulated SBGs. The median (25th, 75th percentile) TFA contribution to energy intakes of SBG consumers, regarding sampling 2021, were 0.61 (0.47, 0.77)%, 0.50 (0.34, 0.75)% and 0.52 (0.35, 0.77)% per age group (≤19 y, 20–59 y and ≥60 y) respectively (Supplementary Table 4). The mean (SD) contribution of SFA to energy intakes of children/adolescents, adults, and elderly consumers of SBGs were 17.32 (4.40)%, 14.71 (4.49)%, and 15.10 (3.86)% respectively (Supplementary Table 4).

Significant differences were observed in the age of SBG consumers, as well as in age categories and in area of residence ($P < 0.001$ for between groups and for trend). Specifically, more children and adolescents were categorized in the third tertile compared to the first for SFA intake, whereas the opposite was observed among adult consumers ($P < 0.001$) (Table 3). In urban areas (Attiki and Thessaloniki), there was a significantly higher percentage of consumers in the first tertile of SFA intake compared to that of the third, a finding that was not observed in the mainland nor in the islands (Table 3). No differences were found for sex, marital status, education level, employment status, physical activity status, and smoking status between tertiles of SFA (Table 3).

Based on 2021 models, the mean total SFA intake for SBG consumers was 15.2% of total energy and ranged from 10.42% in the first tertile to 20.26% in the third tertile (Table 4). Median (25th, 75th percentile) total TFA intake was 0.52% (0.35–0.76%) of daily total energy intake and ranged from 0.39% (0.26%, 0.59%) in the first tertile to 0.66% (0.48%, 0.89%) in the third with a significant increasing trend ($P < 0.001$). Individuals consuming the highest SFA concentrations also had significantly higher intakes of total TFA from all foods and total TFA, SFA, and i-TFA from SBGs (all expressed as % of daily total energy intake). Significant differences were observed in TFA, SFA, and i-TFA intakes from SBGs, with consumers in the first tertile of total SFA intake consuming on average 0.05%, 2.53%, and 0.02%, at the second tertile consuming 0.07%, 3.55%, and 0.03%, and at the third tertile 0.10%, 5.18%, and 0.06%, respectively. Total energy intake, BMI, and weight status, both in adults and children, did not significantly differ.

Estimated median total TFA intake from SBGs, based on the SBG reformulation and for the same intake, was reduced from 0.13% in 2015 to 0.06% of energy intake in 2021 ($P < 0.01$) (Table S5). However, estimated total SFA intake from SBGs increased from 2.66% in 2015 to 3.50% of energy intake in 2021 ($P < 0.01$) (Supplementary Table 5).

**TABLE 1**

Contents of total fat, SFA, *trans* fatty acid, and industrial *trans* fatty acid (g/100 g product as purchased) in different types of nonprepacked savory baked goods (mean and SD) from bakeries in Greece in 2015 and 2021

| Type of product | Total fat (2015) | Total fat (2021) | SFA (2015) | SFA (2021) | TFA (2015) | TFA (2021) | i-TFA (2015) | i-TFA (2021) |
|---|---|---|---|---|---|---|---|---|
| Cheese pies with phyllo pastry | 19.52 (4.49) | 19.21 (4.96) | 7.70 (3.59) | 7.25 (3.60) | 0.26 (0.12) | 0.12 (0.05) | 0.15 (0.11) | 0.01 (0.02) |
| Cheese pies with shortcrust pastries | 19.68 (4.51) | 25.08 (3.83) | 9.60 (2.07) | 12.13 (2.52) | 0.51 (0.40) | 0.55 (0.47) | 0.45 (0.39) | 0.44 (0.46) |
| Cheese pies with puff pastry | 22.54 (2.30) | 25.65 (3.04) | 12.40 (1.31) | 14.13 (1.74) | 0.94 (0.53) | 0.31 (0.29) | 0.86 (0.56) | 0.24 (0.29) |
| Bougatsa with cheese | - | 17.71 (3.76) | - | 8.09 (2.16) | - | 0.28 (0.22) | - | 0.16 (0.21) |
| Pizza/peinirli[1] | 11.7 (1.4) | 12.46 (2.32) | 6.30 (0.77) | 6.83 (1.86) | 0.12 (0.03) | 0.18 (0.06) | 0.0 (0.0) | 0.02 (0.06) |
| Vegetarian pies (e.g., spinach or leek pie) | 11.4 (0.46) | 17.63 (3.98) | 2.30 (0.80) | 4.03 (2.21) | 0.09 (0.08) | 0.06 (0.04) | 0.09 (0.07) | 0.05 (0.03) |
| Meat-containing pies (e.g., sausage pies, ham pies)[2] | 22.2 (1.96) | 23.14 (4.70) | 11.20 (0.53) | 11.14 (3.51) | 0.43 (0.30) | 0.18 (0.21) | 0.40 (0.29) | 0.15 (0.22) |
| All SBGs | 18.8 (5.2) | 20.1 (5.8) | 8.9 (3.7) | 9.1 (4.2) | 0.47 (0.45) | 0.24 (0.28) | 0.39 (0.45) | 0.15 (0.27) |

i-TFA, industrial *trans* fatty acid; SBG, savory baked good; SD, standard deviation; SFA, saturated fatty acid; TFA, *trans* fatty acid.

[1] In 2015, pizza slice samples from bakeries were collected, whereas, in 2021, pizza boat (peinirli) samples from bakeries were collected.

[2] In 2015 only sausage pies were collected ($N = 5$), whereas in 2021 sausage pies ($N = 16$), cheese and ham pies ($N = 3$) and cooked beef in tomato sauce pies ($N = 1$) were collected.

**TABLE 2**

Percentage of SFA, *trans* fatty acid, and industrial *trans* fatty acid (g/100 g fat) in different types of nonprepacked savory baked goods (mean and SD) collected in 2015[3] and 2021

| Type of product | SFA (g/100 g fat) (2015)[3] | SFA (g/100 g fat) (2021) | total TFA (g/100 g fat) (2015)[3] | total TFA (g/100 g fat) (2021) | i-TFA (g/100 g fat) (2015)[3] | i-TFA (g/100 g fat) (2021) | No. of samples exceeding the legal limit (i-TFA>2%) (2015) | No. of samples exceeding the legal limit (i-TFA>2%) (2021) |
|---|---|---|---|---|---|---|---|---|
| Cheese pies with phyllo pastry | 37.90 (11.62) | 36.42 (9.86) | 1.28 (0.50) | 0.66 (0.25) | 0.76 (0.57) | 0.06 (0.11) | 0 (0%) | 0 (0%) |
| Cheese pies with shortcrust pastries | 49.13 (5.53) | 48.21 (6.33) | 2.75 (2.24) | 2.24 (1.83) | 2.36 (1.98) | 1.78 (1.81) | 5 (50%) | 6 (31.6%) |
| Cheese pies with puff pastry | 55.03 (2.59) | 55.11 (2.55) | 4.09 (2.17) | 1.21 (1.06) | 3.72 (2.31) | 0.96 (1.06) | 7 (63.6%) | 2 (9.1%) |
| Bougatsa with cheese | - | 45.44 (5.91) | - | 1.60 (1.19) | - | 0.93 (1.17) | - | 5 (25%) |
| Pizza/Peinirli[1] | 54.1 (5.9) | 54.20 (9.14) | 1.02 (0.36) | 1.52 (0.65) | 0.00 (0.01) | 0.24 (0.71) | 0 (0%) | 2 (10%) |
| Vegetarian pies (e.g., spinach or leek pie) | 19.9 (6.9) | 23.14 (12.03) | 0.77 (0.58) | 0.34 (0.20) | 0.76 (0.63) | 0.30 (0.20) | 0 (0%) | 0 (0%) |
| Meat-containing pies (e.g., sausage pies, ham pies)[2] | 50.5 (2.4) | 47.15 (9.59) | 1.93 (1.27) | 0.77 (0.77) | 1.81 (1.23) | 0.63 (0.82) | 2 (40%) | 1 (5.26%) |
| All SBGs | 45.78 (12.78) | 44.34 (13.29) | 2.28 (1.97) | 1.19 (1.14) | 1.87 (1.98) | 0.69 (1.11) | 14 (31.1%) | 16 (11.4%) |

i-TFA, industrial *trans* fatty acids; SBG, savory baked good; SD, standard deviation; SFA, saturated fatty acids; TFA, *trans* fatty acids.

[1] In 2015, pizza slice samples from bakeries were collected, whereas in 2021, pizza boat (peinirli) samples from bakeries were collected.

[2] In 2015, only sausage pies were collected ($N = 5$), whereas in 2021, sausage pies ($N = 16$), cheese and ham pies ($N = 3$), and cooked beef in tomato sauce pies ($N = 1$) were collected.

[3] Reproduced from reference [9] with permission.

Among SBG consumers, 11.5% ($N = 116$) consumed TFA at levels above 1% of their daily total energy intake (Supplementary Table 1). No individual exceeded this level from the consumption of SBGs alone. All the data regarding the population enrolled and those consuming total TFA ≥1% of daily total energy intake, regarding sampling 2021, in total and per age group as proportions of the general population and consumers of SBGs, are provided in Supplementary Table 1. Information on the distribution of baseline characteristics (demographic, anthropometric, and other personal characteristics) for the total HNNHS population and by SBG consumption is shown in Supplementary Table 2.

Figure 2 illustrates the distribution of TFA and SFA intakes in total and from SBG consumers, and Figure 3 depicts median i-TFA intakes from SBGs in total and by age group in 2015 and 2021, respectively, assuming that the composition and consumption of other foods remained the same. Supplementary Table 4 shows the distributions of total TFA and SFA intakes from all foods, and Supplementary Table 5 the TFA, SFA, and i-TFA intakes from SBGs for SBG consumers by sex and age group, as a proportion of their daily total energy intake considering sampling of 2015 and 2021.

The primary sources of TFA intake (percentage energy) from different categories of SBGs for the highest consumers of each age group and in total are shown in Figure 4. In total, the main contributor for percentiles (p) p90, p95, and p99 was cheese pie made with shortcrust pastry, followed by cheese pie with puff pastry, and cheese pie with phyllo. These differed, however, for older age groups at the 90th percentile, with participants ≥60 y not consuming any cheese pies made with shortcrust pastry and having vegetarian pies as their main TFA contributor. Peinirli (pizza boat) contributed only among the highest consumers (p99) in total and between all age groups, and

**TABLE 3**

Distribution of baseline characteristics (demographic, anthropometric, and other personal characteristics) for savory baked goods consumers from the Hellenic National Nutrition and Health Survey (conducted between 2013–2015) overall and by tertile of SFA[1] intake using substitution models[1]

| Variables | Population N = 1008; SFA intake as %energy, mean (SD): 15.20 (4.5) | Tertiles of total SFA intakes among consumers (2021) | | | P for between Tertile differences[2] | P-trend[3] |
|---|---|---|---|---|---|---|
| | | 1st Tertile N = 335; SFA intake as %energy, mean (SD): 10.42 (1.85) | 2nd Tertile N = 337; SFA intake as %energy, mean (SD): 14.92 (1.09) | 3rd Tertile N = 336; SFA intake as %energy, mean (SD): 20.26 (2.78) | | |
| Age groups, n (%) | | | | | <0.001 | <0.001 |
| 0–19 y | 165 (16.4) | 33 (9.9) | 44 (13.1) | 88 (26.3) | | |
| 20–59 y | 706 (70.2) | 261 (77.9) | 243 (72.1) | 202 (60.5) | | |
| 60+ y | 135 (13.4) | 41 (12.2) | 50 (14.8) | 44 (13.2) | | |
| Sex, n (%) | | | | | 0.275 | 0.276 |
| Females | 548 (54.5) | 189 (56.4) | 189 (56.1) | 170 (50.9) | | |
| Males | 458 (45.5) | 146 (43.6) | 148 (43.9) | 164 (49.1) | | |
| Area of residence, n (%) | | | | | <0.001 | <0.001 |
| Attiki and Thessaloniki | 711 (70.8) | 261 (77.9) | 239 (71.1) | 211 (63.5) | | |
| Islands (including Crete) | 98 (9.8) | 24 (7.2) | 25 (7.5) | 49 (14.8) | | |
| Mainland | 194 (19.4) | 50 (14.9) | 72 (21.4) | 72 (21.7) | | |
| Marital status, n (%) | | | | | 0.213 | 0.601 |
| Single | 422 (50.2) | 146 (48.3) | 149 (50.9) | 127 (51.6) | | |
| Married/cohabiting | 352 (41.9) | 136 (45.0) | 113 (38.6) | 103 (41.9) | | |
| Divorced/separated | 34 (4.0) | 7 (2.4) | 18 (6.1) | 9 (3.7) | | |
| Widowed | 33 (3.9) | 13 (4.3) | 13 (4.4) | 7 (2.8) | | |
| Education level, n (%) | | | | | 0.142 | 0.066 |
| ≤6 y of school | 68 (8.0) | 23 (7.5) | 17 (5.8) | 28 (11.2) | | |
| 12 y of school | 309 (36.3) | 115 (37.6) | 102 (34.6) | 92 (36.6) | | |
| Higher education (including colleges) | 475 (55.7) | 168 (54.9) | 176 (59.6) | 131 (52.2) | | |
| Employment status, n (%) | | | | | 0.648 | 0.972 |
| Unemployed | 241 (28.3) | 81 (26.5) | 81 (27.4) | 79 (31.7) | | |
| Employed | 485 (57.0) | 178 (58.2) | 169 (57.1) | 138 (55.4) | | |
| Pension | 125 (14.7) | 47 (15.3) | 46 (15.5) | 32 (12.9) | | |
| Physical activity status[4], n (%) | | | | | 0.182 | <0.05 |
| Sedentary | 67 (7.9) | 23 (7.5) | 21 (7.2) | 23 (9.2) | | |
| Low | 114 (13.5) | 35 (11.5) | 51 (17.6) | 28 (11.2) | | |
| Moderate | 337 (39.5) | 128 (42.0) | 116 (40.0) | 93 (37.2) | | |
| High | 327 (38.7) | 119 (39.0) | 102 (35.2) | 106 (42.2) | | |
| Smoking status, n (%) | | | | | 0.107 | 0.060 |
| Never smoker | 447 (52.0) | 153 (49.7) | 146 (49.0) | 148 (58.3) | | |
| Current smoker | 309 (35.9) | 120 (39.0) | 115 (38.6) | 74 (29.1) | | |
| Ex-smoker | 104 (12.1) | 35 (11.3) | 37 (12.4) | 32 (12.6) | | |

EU, European Union; HNNHS, Hellenic National Nutrition and Health Survey; SD, standard deviation; SFA, saturated fatty acid; TFA, *trans* fatty acid.

[1] Substitution models: measured SFA and TFA content in savory baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake amounts post Regulation (EU) 2019/649 if intakes remained unaltered.

[2] P for between group distributions differences (between tertiles).

[3] P-trend across tertiles.

[4] Physical activity was defined according to the International Physical Activity Questionnaire, as per calculation guidelines [4]. Individuals scoring below the light activity level were categorized as sedentary.

savory bougatsa was the least consumed. The main contributors to TFA intake from SBGs per age group and in total can be viewed in the supplementary material (Supplementary Figure 1). In total, cheese pies made with puff pastry were the main contributor to TFA intake (29.5%), followed by cheese pies with shortcrust pastry (25.6%) and cheese pies with phyllo pastry (24.1%). However, for consumers over 60 y, vegetarian pies (17.2%) contributed more to TFA intake than cheese pies with puff pastry (16.6%).

## Discussion

This study aimed to assess the level of compliance of SBG producers in Greece, the main TFA contributor, with the Regulation (EU) 2019/649 for TFA reduction and evaluate the effect on TFA, i-TFA, and SFA intakes following the Regulation. Measured SBG fatty acid content showed that most SBG producers complied with the TFA upper limit of 2 g of i-TFA/100 g of fat in 2021, with a 19.7% increase in compliance compared to 2021 (11.4% of SBG's exceeded the limit in 2021 compared to 31.1%), but an increase in SFA content/100 g resulted. As per findings from substitution models, this would lead to a reduction in TFA and i-TFA intake expressed as percentage of daily energy in all age groups, with the contribution from SBGs being almost halved for all consumption amounts (p25–p99). The increase in SFA content, however, would lead to increased consumption, resulting in over half of the SBG consumers exceeding 15% SFA of daily total energy intake compared to 13.7% prior to reformulation.

The main SBG contributor to i-TFA and SFA was cheese pie in total, with children and adolescents preferring cheese pies with shortcrust pastry and adults aged 20–59 y cheese pies with puff pastry, 2 types of foods that had the highest mean i-TFA content among the food

**TABLE 4**
Distribution of dietary characteristics of savory baked good consumers extrapolated from Hellenic National Nutrition and Health Survey consumption data (2013–2015); results overall and by tertile of SFA intake adjusted for individual mean energy intake using savory baked goods using substitution models[1]

| Variables | SFA intake as %energy, mean (SD): 15.20 (4.5) | Total SFA tertiles of consumers (2021) N = 1008 | | | P for between group differences[2] | P-trend[3] |
| --- | --- | --- | --- | --- | --- | --- |
| | | 1st tertile N=335; SFA intake as %energy, mean (SD): 10.42 (1.85) | 2nd tertile N = 337; SFA intake as %energy, mean (SD): 14.92 (1.09) | 3rd tertile N = 336; SFA intake as %energy, mean (SD): 20.26 (2.78) | | |
| Total energy intake (kcal/d), mean (SD) | 2127.3 (916.3) | 2184.6 (1025.6) | 2097.3 (881.0) | 2100.2 (831.7) | 0.3745 | 0.86 |
| Total TFA from all food intake as % energy, median (25th, 75th percentile) | 0.52 (0.35, 0.76) | 0.39 (0.26, 0.59) | 0.53 (0.39, 0.74) | 0.66 (0.48, 0.89) | <0.001 | <0.001 |
| Total TFA intake from savory bakery products as %energy, median (25th, 75th percentile) | 0.06 (0.03, 0.13) | 0.05 (0.02, 0.08) | 0.07 (0.03, 0.13) | 0.10 (0.05, 0.20) | <0.001 | <0.001 |
| SFA intake from savory bakery products as %energy, median (25th, 75th percentile) | 3.50 (1.89, 6.04) | 2.53 (1.42, 3.99) | 3.55 (1.99, 5.73) | 5.18 (2.78, 8.37) | <0.001 | <0.001 |
| i-TFA intake from savory bakery products as %energy, median (25th, 75th percentile) | 0.03 (0.01, 0.09) | 0.02 (0.01, 0.04) | 0.03 (0.01, 0.09) | 0.06 (0.01, 0.14) | <0.001 | <0.001 |
| BMI adults (kg/m$^2$), mean (SD) | 25.2 (4.6) | 25.1 (4.4) | 25.2 (4.7) | 25.5 (4.8) | 0.183 | 0.776 |
| BMI adults categories,[4] n (%) | | | | | 0.747 | 0.59 |
| Healthy weight | 457 (54.9) | 161 (53.8) | 162 (56.3) | 134 (54.7) | | |
| Overweight | 243 (29.2) | 95 (31.8) | 79 (27.4) | 69 (28.2) | | |
| Obese | 132 (15.9) | 43 (14.4) | 47 (16.3) | 42 (17.1) | | |
| BMI children categories, n (%) | | | | | 0.704 | 0.185 |
| Healthy weight | 108 (75.5) | 23 (82.1) | 31 (79.5) | 54 (71.0) | | |
| Overweight | 28 (19.6) | 4 (14.3) | 7 (17.9) | 17 (22.4) | | |
| Obese | 7 (4.9) | 1 (3.6) | 1 (2.6) | 5 (6.6) | | |

EU, European Union; HNNHS, Hellenic National Nutrition and Health Survey; i-TFA, industrial *trans* fatty acids; SD, standard deviation; SFA, saturated fatty acids; TFA, *trans* fatty acids (total from all foods in this analysis).

[1] Substitution models: measured SFA and TFA content in savory baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake amounts post Regulation (EU) 2019/649 if intakes remained unaltered.

[2] P for between group distributions differences (between tertiles).

[3] P-trend across tertiles.

[4] Weight (kg) and height (m) were measured from which BMI was derived [weight/height$^2$ (kg/m$^2$)]. Weight status was categorized as "healthy weight if BMI <25 kg/m$^2$", "25≥overweight<30 kg/m$^2$", and "obese≥30 kg/m$^2$." Children and adolescents were classified using the extended international obesity task force tables [5]. For adults only.



**FIGURE 2.** Distribution of TFA and SFA intakes in total and from SBGs as a percentage of daily total energy intake for consumers of SBGs using substitution models.[1]
EU, European Union; HNNHS, Hellenic National Nutrition and Health Survey; SBG, savory baked good; SFA, saturated fatty acids; TFA, *trans* fatty acids.
[1]Substitution models: measured SFA and TFA content in savory baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake amount post Regulation (EU) 2019/649 if intakes remained unaltered.
The x-axis (p25–p99) refers to the percentile intakes of SBG consumers, from the lowest consumers (25th percentile to the highest 99th percentile), of SFA and TFA as percent of total energy intake.
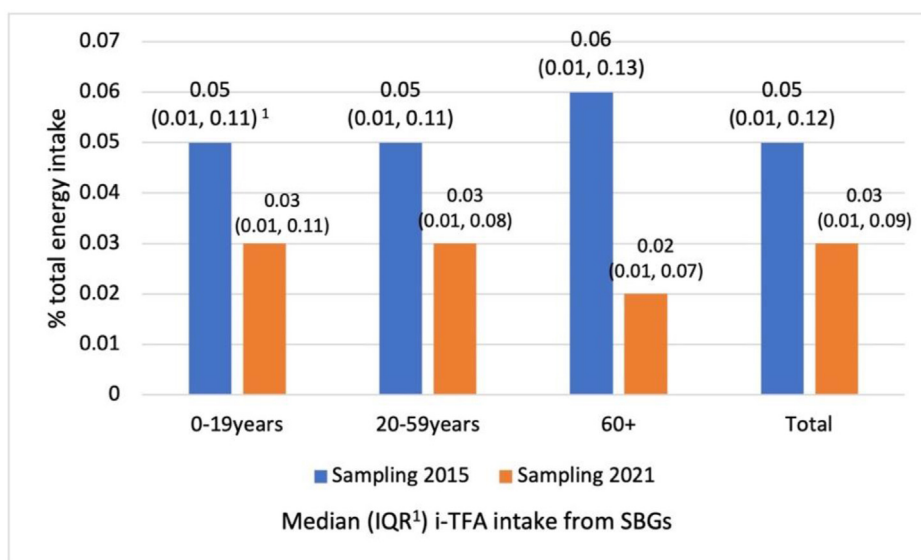
**FIGURE 3.** i-TFA intake from SBGs, as a percentage of daily total energy intake, in total and by age group using substitution models.[2]
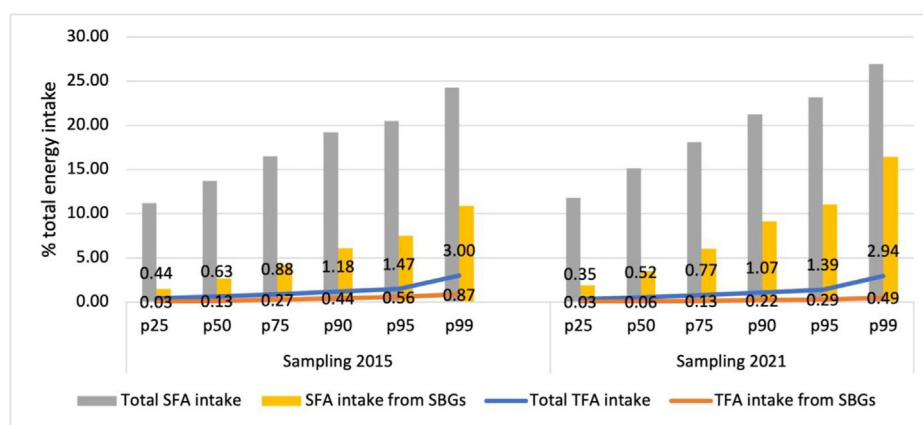EU, European Union; HNNHS, Hellenic National Nutrition And Health Survey; i-TFA, industrial *trans* fatty acids; IQR, interquartile range; SBG, savory baked good; SFA, saturated fatty acids; TFA, *trans* fatty acids.
[1]25th percentile, 75th percentile.
[2]Substitution models: measured SFA and TFA content in savory baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake amounts post Regulation (EU) 2019/649 if intakes remained unaltered.
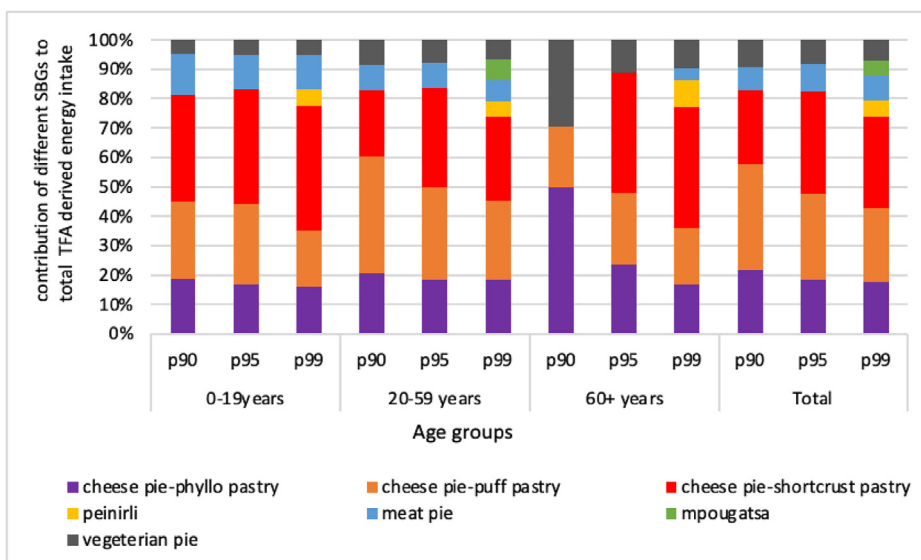


**FIGURE 4.** TFA intake (percentage daily total energy intake) among high consumers (upper 10% bound of the distribution) from different SBGs per age group and in total using substitution models.[1]
[1]Substitution models: measured SFA and TFA content in savory baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake amounts post Regulation (EU) 2019/649 if intakes remained unaltered.
X-axis SBG consumers at 90th, 95th, and 99th percentile (p90, p95 and p99, respectively).

categories investigated on the eve of the full implementation of the Regulation (EU) 2019/649. Cheese pie prepared with phyllo pastry, a choice lower in i-TFA and within limits compared to puff pastry, was the main TFA contributor among the SBGs for older adults. These results are important because other than the health risks from frequent consumption, they also show the trend of food preference toward a more processed diet [19] and higher saturated fat choices. Therefore,

depending on the type of fat that manufacturers will choose to replace hard margarine, the nutritional profile backbone of the SBGs will be provided, and potentially the public health effectiveness on the Regulation (EU) 2019/649 policy of TFA.

Recently, a reduction of i-TFA intake has been a global public health priority, with the WHO creating a Certification Programme for Trans Fat Elimination. This Programme aims to recognize countries

that eliminate i-TFA [20] and will soon be launching a Validation Programme for i-TFA elimination for countries that have demonstrated implementation of a best practice TFA policy and effective monitoring and enforcement systems [21]. Questions and concerns, however, have been raised in the past on the type of fat that will replace TFA, saturated compared to *cis*-unsaturated, following TFA elimination actions [22]. Consequently, the question remains whether an upper i-TFA limit policy from processed food alone will effectively help decrease TFA intake and overall CVD risk. The decrease and ultimate elimination of i-TFA is regarded as a relatively straightforward, low-cost, and effective policy measure [3,23]. This study showed that the majority of the SBGs sampled in 2021 followed the Regulation (EU) 2019/649, and in practice, this resulted in a decrease in TFA intake among SBG consumers. Similar findings have been reported by other countries that have also examined the effects of the 2 g/100 g fat policy following its implementation in i-TFA in many industry-manufactured processed foods [24]. Despite these first positive results, challenges in TFA reduction in food content must be acknowledged, such as the availability of suitable replacement fats/oils with healthier fatty acid profiles, reformulated food quality and taste, operational alterations, consumer acceptance, and cost [25]. Nevertheless, even small increases in either TFA or SFA intake translate to large health impacts at the population level [26], and the opposite is true for small risk reductions. This study showed a small decrease in TFA and an increase in SFA between all population age groups based on the food reformulation made. Despite the controversies between SFA intake and CVD risk, it has been shown that replacing SFA with any *cis*-unsaturated fatty acids has been shown to improve metabolic control [12] and decrease CVD events [13]. Furthermore, higher SFA intake may be associated with higher all-cause mortality [27] and heighten the effect even of lower TFA consumption amounts on serum lipid concentrations [8]. Hence, monitoring intake of food products high in both SFA and TFA is essential, especially based on the results obtained in this study using substitution models in population-based usual intakes from measured fat content in SBGs.

TFA reduction is part of Greece's National Action Plan on Food Reformulation [28] following the European Council Conclusions on food product improvement in 2016 [29], although replacers have not been specified and no actions have been taken for SFA reduction. Studies that have evaluated the implementation of mandatory TFA ban policy showed that SFA content TFA in foods decreased to adequate amounts, but SFA concentrations varied by type of food, with a slight increase seen in supermarket foods and a decrease in restaurants [30]. A Dutch modeling study on the impact of food reformulation of processed food reported a decrease of total TFA by 0.2% in young adults, without changes observed in SFA intake [31].

Most oils and fats are not composed of only 1 group of fatty acids, and hence, increases in fat content alone can increase SFA content of foods. Therefore, it is imperative to raise awareness among members of the Hellenic Federation of Bakers not only of the importance of fatty acid composition of the fat that they use in SBGs but also of the amount of fat and the portion sizes of the final product. SFA reduction goals similar to those in other EU countries [32] could also be set so that the Regulation (EU) 2019/649 on TFA would achieve meaningful results in terms of public health.

Study limitations that need to be accounted for include the small differences in sampling between the 2 y tested. Although some differences may have resulted, these mainly reduced both SFA and TFA intake since sausage is relatively higher in fat content than minced meat or ham.

Random measurement errors between years cannot be excluded since samples from 2015 were not retained to be re-analyzed in 2021. This was minimized by employing exact methodological analyses, the same instruments, and laboratories. Our results rely on a sample of SBG consumers identified through a nationally representative nutrition survey. This may limit the extrapolation of our study results to SBG consumers in Greece. In addition, since the study did not employ a nationally representative sample of SBG consumers, survey design analysis was not performed to correct variance estimates. Another limitation of this study is the lack of recent food consumption data from a representative population sample. For this reason, substitution models were employed using data collected from HNNHS based on measurements performed in 2021. Finally, the authors acknowledge that data from 24-h dietary recalls are affected by random measurement error, which may cause bias to the estimated distribution, percentile values, or regression coefficients [33], particularly in the case of episodically consumed foods. It is for this reason that the present analysis focused on individuals who reported SBG consumption (consumers only). This is a conservative approach, which may lead to higher median intakes, but it is the recommended method for risk characterization purposes, as per the aim of this study. This is an acknowledged assumption, although random within-person variance can affect intakes estimated through short-term measurements in any direction.

In view of the validated and robust methods followed for dietary data collection and sample analysis, to our knowledge, this study provides a first evaluation of the effectiveness of the Regulation (EU) 2019/649 TFA policy in Greece, confirming low TFA intake in Greece. The Regulation (EU) 2019/649 to eliminate i-TFA was found effective in its primary goal, but vigilance and monitoring of the concentrations of SFA following product reformulation is also essential. It is also important to examine processed packaged foods that contain mandatory food labels to assess the type of fat that replaced TFA reformulation following the *trans*-fat policy implementation in Greece. A further risk assessment of processed brand-named foods that are frequently consumed is also recommended to monitor the population that may remain at risk for chronic disease because of increased SFA consumption, regardless of the implementation of the TFA policy.

## Author contributions

The authors' responsibilities were as follows – GM, AZ, CP, EM: designed research; EM, SK, GM, AP: conducted research; CP, SS, AP, GB: provided essential materials and conducted the analysis; CP, MC, ZM: provided essential materials; SK and EM: analyzed data and performed statistical analysis; E-MK: updated database; GM, EM, SK, AP: wrote paper; EM, SK, GM, AZ, AN, SS, CP, GB, ZM, DP: had primary responsibility for final content (writing—review, and editing); EM, ZM, AZ: supervised; and all authors: read and approved the final manuscript.

## Conflict of interest

The authors report no conflicts of interest.

## Funding

Union (European Social Fund) and Greece (Ministry of Health) under the Operational Program "Human Resources Development 2007–2013."

## Data availability

Data described in the manuscript, codebook, and analytic code will be made available upon request, pending application and approval.

## Acknowledgments

We thank HNNHS and Hellenic Food Authority teams and to all HNNHS fieldworkers.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2023.08.014.

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**SUPPLEMENTARY MATERIAL**

**Title page**

**Comparisons of trans and saturated fatty acid content in savoury baked goods and their effect on consumers' intake using substitution models.**

**Georgios Marakis et al,.**

**Supplementary Data:**

Table S1: General population, consumers of SBGs and exceeders of 1% of their daily total energy intake from TFA consumption in general population and SBG consumers;

Table S2: Distribution of baseline characteristics (demographic, anthropometric and other personal characteristics) for Hellenic National Nutrition and Health Survey (HNNHS) population and SBGs consumers and non-consumers;

Table S3: Types and number of SBGs analyzed in 2015 and in 2021 and their frequency of consumption based on Foods from Hellenic National Nutrition and Health Survey used for TFA analysis;

Table S4. Distribution of TFA, SFA and i-TFA intakes* from SBGs, as % of daily total energy intake, by sex and age group of SBGs consumers only;

Figure S1. The contribution of different SBGs to the total daily intake of TFA by age group and in total.

**Table S1:** General population, consumers of savoury baked goods and exceeders of 1% of their daily total energy intake from trans fatty acid consumption in general population and consumers.

| Age group, y | Population in HNNHS | | Consumers of SBGs | | | | Population in HNNHS with total TFA intake≥1%energy | | | Consumers of SBGs with total TFA intake ≥1% energy | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N (a) | % [(a)/Total(a)*100] | N (b) | % per age group [(b)/Total(b)*100] | % per age group in general population [(b)/ (a)]*100 | % in total general population [(b)/Total (a)]*100 | N (c) | % per age group [(c)/Total(c)*100] | % in total general population [(c)/Total (a)]*100 | N (d) | % per age group [(d)/Total(d)]*100 | % in total general population [(d)/Total (a)]*100 | % per age group in general population [(d)/ (a)]*100 | % in consumers of SBGs [(d)/Total (b)]*100 | %in general population with total TFA intake ≥1% [(d)/Total(c)]*100 |
| 0-19 | 845 | 19 | 165 | 16.4 | 19.5 | 3.7 | 165 | 25.5 | 3.7 | 20 | 17.2 | 0.5 | 2.4 | 2.0 | 3.1 |
| 20-59 | 2779 | 62.6 | 706 | 70.2 | 25.4 | 15.9 | 350 | 54.1 | 7.9 | 74 | 63.8 | 1.7 | 2.7 | 7.4 | 11.4 |
| 60+ | 815 | 18.4 | 135 | 13.4 | 16.6 | 3 | 132 | 20.4 | 3.0 | 22 | 19.0 | 0.5 | 2.7 | 2.2 | 3.4 |
| **Total** | **4439** | **100** | **1006** | **100.0** | **22.7** | **22.7** | **647** | **100.0** | **14.6** | **116** | **100.0** | **2.6** | **2.6** | **11.5** | **17.9** |
| **Total in study** | **4450** | | **1008** | | **22.7** | | **647** | | **14.5** | **116** | | **2.6** | | **11.5** | **17.9** |

(a): HNNHS population; (b): Population of SBGs consumers; (c) HNNHS population with total TFA intake ≥ 1% of daily total energy intake; (d): Population of SBGs consumers with total TFA intake ≥ 1% of daily total energy intake

**Table S2:** Distribution of baseline characteristics (demographic, anthropometric and other personal characteristics) for Hellenic National Nutrition and Health Survey population and SBG's consumers and non-consumers

| Variables | HNNHS[1] population N=4450 | SBG[2] consumers N=1008 | SBG non-consumers N=3442 | p-for between SBG Consumers and SBG Non-Consumers differences |
|---|---|---|---|---|
| Age groups, n (%) | | | | <0.001 |
| 0-19years | 845 (19.0) | 165 (16.4) | 680 (9.8) | |
| 20-59years | 2779 (62.6) | 706 (70.2) | 2073 (60.4) | |
| 60+ years | 815 (18.4) | 135 (13.4) | 680 (19.8) | |
| Sex, n (%) | | | | <0.05 |
| Females | 2557 (57.6) | 548 (54.5) | 2009 (58.5) | |
| Males | 1882 (42.4) | 458 (45.5) | 1424 (41.5) | |
| Area of residence, n (%) | | | | 0.001 |
| Attiki & Thessaloniki | 2924 (66.1) | 711 (70.9) | 2213 (64.7) | |
| Islands (including Crete) | 477 (10.8) | 98 (9.8) | 379 (11.1) | |
| Mainland | 1025 (23.1) | 194 (19.3) | 831 (24.2) | |
| Marital status, n (%) | | | | <0.001 |
| Single | 1561 (43.5) | 422 (50.2) | 1139 (41.4) | |
| Married/Cohabiting | 1599 (44.5) | 352 (41.9) | 1247 (45.4) | |
| Divorced/Separated | 168 (4.7) | 34 (4.0) | 134 (4.9) | |
| Widowed | 262 (7.3) | 33 (3.9) | 229 (8.3) | |
| Education level, n (%) | | | | <0.001 |
| Up to 6 years of school | 443 (12.1) | 68 (8.0) | 375 (13.4) | |
| 12 years of school | 1328 (36.3) | 309 (36.3) | 1019 (36.3) | |
| Higher education (including colleges) | 1884 (51.6) | 475 (55.7) | 1409 (50.3) | |
| Employment status, n (%) | | | | <0.001 |
| Unemployed | 1129 (30.9) | 241 (28.3) | 888 (31.7) | |
| Employed | 1786 (48.9) | 485 (57.0) | 1301 (46.4) | |
| Pension | 738 (20.2) | 125 (14.7) | 613 (21.9) | |

| Variables | HNNHS[1] population N=4450 | SBG[2] consumers N=1008 | SBG non-consumers N=3442 | p-for between SBG Consumers and SBG Non-Consumers differences |
|---|---|---|---|---|
| Physical activity status[3], n (%) | | | | 0.518 |
|    Sedentary | 252 (6.9) | 67 (7.9) | 185 (6.6) | |
|    Low | 505 (13.9) | 114 (13.5) | 391 (14.0) | |
|    Moderate | 1422 (39.2) | 337 (39.9) | 1085 (39.0) | |
|    High | 1451 (40.0) | 327 (38.7) | 1124 (40.4) | |
| Smoking status, n (%) | | | | <0.05 |
|    Never smoker | 1876 (50.6) | 447 (52.0) | 1429 (50.2) | |
|    Current smoker | 1244 (33.6) | 309 (35.9) | 935 (32.9) | |
|    Ex-smoker | 584 (15.8) | 104 (12.1) | 480 (16.9) | |

[1] HNNHS: Hellenic National Nutrition and Health Survey

[2] SBGs: Savoury Baked Goods

[3] Physical activity (PA) was defined according to the International Physical Activity Questionnaire (IPAQ) , as per calculation guidelines [4]. Individuals scoring below the light activity level were categorized as sedentary.

**Table S3:** Types and number of savoury baked goods analyzed in 2015 and in 2021 and number of consumers per type of SBGs based on Hellenic National Nutrition and Health Survey

| Type of bakery product | No of samples analyzed in 2015 | No of samples analyzed in 2021 | Consumers No of consumers | Percentage (%) |
|---|---|---|---|---|
| Cheese pies with phyllo pastry | 9 | 20 | 305 | 30.26% |
| Cheese pies with puff pastry | 11 | 22 | 189 | 18.75% |
| Cheese pies with shortcrust pastry | 10 | 19 | 98 | 9.72% |
| Pies containing meat (e.g., sausage pies, ham pie) | 5 | 19 | 118 | 11.71% |
| Peinirli (a.k.a. pizza-boat) | 5 | 20 | 33 | 3.27% |
| Mpougatsa with cheese | - | 20 | 6 | 0.60% |
| Vegetarian pies (e.g., spinach or leek pie) | 5 | 20 | 259 | 25.69% |
| **Total sum** | **45** | **140** | **1008** | **100.0%** |

SBG's: Savoury Baked Goods

**Table S4.** Distribution of total TFA and SFA intakes* from all foods, as % of daily energy intake, by sex and age group of SBG consumers.

| | Age groups (years) | N | Total TFA intake | | | | | | | | Total SFA intake | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | p25 | p50 | p75 | p90 | p95 | p99 | mean | SD | p25 | p50 | p75 | p90 | p95 | p99 | mean | SD |
| **Sampling of 2015** | **FEMALES** | **548** | **0.44** | **0.62** | **0.88** | **1.18** | **1.47** | **2.82** | **0.71** | **0.48** | **11.13** | **13.62** | **16.37** | **19.05** | **20.37** | **23.81** | **13.87** | **3.92** |
| | 0-19years | 77 | 0.51 | 0.69 | 0.84 | 1.20 | 1.65 | 4.07 | 0.80 | 0.55 | 12.96 | 15.13 | 18.34 | 20.44 | 21.61 | 28.80 | 15.63 | 3.89 |
| | 20-59years | 390 | 0.41 | 0.58 | 0.88 | 1.11 | 1.47 | 3.00 | 0.69 | 0.48 | 10.79 | 13.20 | 15.93 | 18.55 | 20.07 | 23.81 | 13.48 | 3.91 |
| | 60+ | 81 | 0.47 | 0.61 | 0.89 | 1.35 | 1.44 | 1.95 | 0.71 | 0.37 | 11.66 | 13.52 | 16.80 | 18.61 | 19.41 | 22.36 | 14.06 | 3.51 |
| | **MALES** | **458** | **0.44** | **0.64** | **0.88** | **1.24** | **1.48** | **3.24** | **0.73** | **0.55** | **11.23** | **13.78** | **16.61** | **19.31** | **20.59** | **24.49** | **14.04** | **3.99** |
| | 0-19years | 88 | 0.54 | 0.72 | 0.89 | 1.07 | 1.29 | 3.96 | 0.76 | 0.44 | 12.87 | 15.54 | 18.71 | 20.87 | 22.69 | 24.50 | 15.93 | 3.85 |
| | 20-59years | 316 | 0.41 | 0.58 | 0.84 | 1.18 | 1.45 | 2.12 | 0.68 | 0.41 | 10.83 | 13.20 | 15.55 | 18.59 | 20.22 | 24.28 | 13.42 | 3.90 |
| | 60+ | 54 | 0.55 | 0.73 | 1.07 | 1.62 | 3.36 | 6.16 | 1.01 | 1.08 | 11.51 | 14.72 | 17.39 | 20.21 | 20.78 | 21.35 | 14.60 | 3.75 |
| | **TOTAL** | **1006** | **0.44** | **0.63** | **0.88** | **1.18** | **1.47** | **3.00** | **0.72** | **0.51** | **11.21** | **13.71** | **16.50** | **19.21** | **20.48** | **24.28** | **13.95** | **3.95** |
| | 0-19years | 165 | 0.54 | 0.71 | 0.88 | 1.16 | 1.39 | 3.96 | 0.78 | 0.50 | 12.91 | 15.31 | 18.46 | 20.46 | 22.42 | 24.83 | 15.79 | 3.86 |
| | 20-59years | 706 | 0.41 | 0.58 | 0.87 | 1.16 | 1.46 | 2.39 | 0.69 | 0.45 | 10.83 | 13.20 | 15.80 | 18.58 | 20.07 | 23.81 | 13.45 | 3.90 |
| | 60+ | 135 | 0.47 | 0.68 | 0.97 | 1.37 | 1.62 | 4.69 | 0.83 | 0.75 | 11.66 | 14.25 | 17.11 | 19.17 | 20.48 | 21.35 | 14.28 | 3.61 |
| **Sampling of 2021** | **FEMALES** | **548** | **0.34** | **0.51** | **0.74** | **1.01** | **1.37** | **2.91** | **0.62** | **0.47** | **11.69** | **14.91** | **17.85** | **20.77** | **23.04** | **26.93** | **14.99** | **4.50** |
| | 0-19years | 77 | 0.45 | 0.63 | 0.74 | 1.18 | 1.58 | 4.14 | 0.71 | 0.56 | 13.46 | 16.42 | 20.51 | 22.98 | 25.12 | 29.70 | 17.20 | 4.55 |
| | 20-59years | 390 | 0.33 | 0.51 | 0.74 | 0.98 | 1.36 | 2.94 | 0.60 | 0.47 | 11.29 | 14.53 | 17.41 | 20.35 | 23.08 | 26.93 | 14.60 | 4.53 |
| | 60+ | 81 | 0.34 | 0.50 | 0.73 | 1.13 | 1.43 | 1.93 | 0.59 | 0.37 | 12.17 | 14.75 | 17.45 | 19.67 | 20.46 | 22.71 | 14.76 | 3.66 |
| | **MALES** | **458** | **0.37** | **0.53** | **0.79** | **1.11** | **1.43** | **3.19** | **0.65** | **0.54** | **12.06** | **15.30** | **18.33** | **21.41** | **23.44** | **27.11** | **15.44** | **4.48** |
| | 0-19years | 88 | 0.49 | 0.60 | 0.84 | 1.06 | 1.20 | 3.90 | 0.69 | 0.43 | 13.95 | 18.18 | 20.38 | 23.49 | 23.79 | 29.57 | 17.44 | 4.29 |
| | 20-59years | 316 | 0.34 | 0.50 | 0.78 | 1.08 | 1.43 | 2.14 | 0.60 | 0.41 | 11.60 | 14.74 | 17.36 | 20.69 | 22.49 | 27.11 | 14.85 | 4.44 |
| | 60+ | 54 | 0.40 | 0.56 | 0.89 | 1.45 | 3.22 | 6.16 | 0.90 | 1.08 | 12.07 | 15.33 | 18.95 | 20.74 | 23.14 | 23.98 | 15.63 | 4.13 |
| | **TOTAL** | **1006** | **0.35** | **0.52** | **0.77** | **1.07** | **1.39** | **2.94** | **0.63** | **0.50** | **11.79** | **15.10** | **18.12** | **21.22** | **23.14** | **26.93** | **15.19** | **4.50** |
| | 0-19years | 165 | 0.47 | 0.61 | 0.77 | 1.06 | 1.27 | 3.90 | 0.70 | 0.50 | 13.67 | 17.64 | 20.51 | 23.20 | 23.93 | 29.57 | 17.32 | 4.40 |
| | 20-59years | 706 | 0.34 | 0.50 | 0.75 | 1.01 | 1.37 | 2.26 | 0.60 | 0.44 | 11.50 | 14.59 | 17.37 | 20.47 | 22.99 | 26.93 | 14.71 | 4.49 |
| | 60+ | 135 | 0.35 | 0.52 | 0.77 | 1.25 | 1.57 | 4.68 | 0.71 | 0.75 | 12.09 | 15.09 | 18.03 | 20.43 | 21.54 | 23.87 | 15.10 | 3.86 |

* Intake distribution of total TFA intake in 2021 are from substitution models[1] based on measurements from reformulated SBGs samples post Reg. (EU) 2019/649 on TFA reduction; TFA: trans fatty acids; SFA: saturated fatty acids.

[1]Substitution models: measured SFA and TFA content in savoury baked goods in 2021 were replaced from those measured during the HNNHS study years (2015) to evaluate TFA intake levels post Regulation (EU) 2019/649 if intakes remained unaltered

**Table S5.** Distribution of trans, saturated and industrial fatty acid intakes* from savoury baked goods, as % of daily total energy intake, by sex and age group of SBGs consumers only.

| | Age groups (years) | N | TFA intake from SBGs | | | | | | i-TFA intake from SBGs | | | | | | SFA intake from SBGs | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | p25 | p50 | p75 | p90 | p95 | p99 | p25 | p50 | p75 | p90 | p95 | p99 | p25 | p50 | p75 | p90 | p95 | p99 |
| **Sampling of 2015** | **FEMALES** | **548** | **0.04** | **0.13** | **0.27** | **0.45** | **0.61** | **0.88** | **0.01** | **0.05** | **0.12** | **0.21** | **0.36** | **0.73** | **1.51** | **2.74** | **4.39** | **6.34** | **7.68** | **10.18** |
| | 0-19years | 77 | 0.06 | 0.15 | 0.28 | 0.51 | 0.61 | 0.89 | 0.01 | 0.05 | 0.10 | 0.24 | 0.34 | 0.67 | 1.52 | 2.87 | 4.41 | 6.91 | 9.06 | 12.2 |
| | 20-59years | 390 | 0.04 | 0.12 | 0.25 | 0.43 | 0.59 | 0.87 | 0.01 | 0.05 | 0.11 | 0.20 | 0.36 | 0.75 | 1.48 | 2.60 | 4.26 | 6.09 | 7.50 | 9.91 |
| | 60+ | 81 | 0.04 | 0.17 | 0.33 | 0.45 | 0.65 | 1.16 | 0.01 | 0.04 | 0.13 | 0.21 | 0.36 | 0.97 | 2.00 | 3.32 | 4.84 | 6.93 | 8.36 | 10.74 |
| | **MALES** | **458** | **0.03** | **0.13** | **0.27** | **0.44** | **0.55** | **0.82** | **0.01** | **0.05** | **0.12** | **0.23** | **0.34** | **0.46** | **1.52** | **2.55** | **3.97** | **5.83** | **7.20** | **11.78** |
| | 0-19years | 88 | 0.03 | 0.14 | 0.27 | 0.41 | 0.49 | 0.73 | 0.01 | 0.05 | 0.12 | 0.22 | 0.33 | 0.37 | 1.49 | 2.36 | 3.68 | 5.81 | 7.08 | 8.56 |
| | 20-59years | 316 | 0.03 | 0.13 | 0.26 | 0.44 | 0.55 | 0.82 | 0.01 | 0.05 | 0.11 | 0.23 | 0.35 | 0.46 | 1.51 | 2.49 | 3.87 | 5.83 | 7.17 | 11.24 |
| | 60+ | 54 | 0.06 | 0.16 | 0.33 | 0.51 | 0.55 | 1.18 | 0.01 | 0.07 | 0.13 | 0.27 | 0.34 | 0.51 | 2.33 | 3.07 | 4.81 | 6.11 | 11.27 | 15.02 |
| | **TOTAL** | **1006** | **0.03** | **0.13** | **0.27** | **0.44** | **0.56** | **0.87** | **0.01** | **0.05** | **0.12** | **0.22** | **0.34** | **0.54** | **1.51** | **2.66** | **4.24** | **6.07** | **7.50** | **10.88** |
| | 0-19years | 165 | 0.04 | 0.15 | 0.28 | 0.42 | 0.56 | 0.88 | 0.01 | 0.05 | 0.11 | 0.22 | 0.34 | 0.43 | 1.50 | 2.59 | 4.27 | 6.32 | 7.20 | 10.88 |
| | 20-59years | 706 | 0.03 | 0.13 | 0.26 | 0.44 | 0.56 | 0.82 | 0.01 | 0.05 | 0.11 | 0.22 | 0.35 | 0.59 | 1.49 | 2.56 | 4.04 | 6.01 | 7.43 | 10.23 |
| | 60+ | 135 | 0.05 | 0.16 | 0.33 | 0.46 | 0.65 | 1.16 | 0.01 | 0.06 | 0.13 | 0.26 | 0.36 | 0.51 | 2.15 | 3.15 | 4.84 | 6.93 | 8.82 | 13.46 |
| **Sampling of 2021** | **FEMALES** | **548** | **0.03** | **0.06** | **0.12** | **0.22** | **0.29** | **0.49** | **0.01** | **0.03** | **0.08** | **0.16** | **0.23** | **0.38** | **1.90** | **3.45** | **5.74** | **8.63** | **10.77** | **17.94** |
| | 0-19years | 77 | 0.04 | 0.07 | 0.15 | 0.26 | 0.33 | 0.50 | 0.01 | 0.03 | 0.10 | 0.17 | 0.25 | 0.41 | 2.16 | 3.83 | 6.48 | 10.5 | 13.12 | 23.46 |
| | 20-59years | 390 | 0.03 | 0.06 | 0.12 | 0.21 | 0.28 | 0.51 | 0.01 | 0.03 | 0.08 | 0.16 | 0.22 | 0.37 | 1.80 | 3.20 | 5.58 | 8.30 | 10.85 | 17.94 |
| | 60+ | 81 | 0.04 | 0.06 | 0.12 | 0.18 | 0.24 | 0.38 | 0.01 | 0.02 | 0.05 | 0.14 | 0.18 | 0.29 | 2.36 | 3.85 | 6.32 | 8.28 | 9.34 | 11.8 |
| | **MALES** | **458** | **0.03** | **0.07** | **0.14** | **0.22** | **0.29** | **0.51** | **0.01** | **0.03** | **0.09** | **0.16** | **0.22** | **0.38** | **1.85** | **3.55** | **6.42** | **9.23** | **11.29** | **14.79** |
| | 0-19years | 88 | 0.04 | 0.08 | 0.14 | 0.22 | 0.27 | 0.56 | 0.01 | 0.03 | 0.11 | 0.17 | 0.21 | 0.43 | 1.68 | 3.74 | 6.52 | 9.31 | 10.62 | 15.32 |
| | 20-59years | 316 | 0.03 | 0.07 | 0.14 | 0.22 | 0.29 | 0.42 | 0.01 | 0.03 | 0.09 | 0.16 | 0.23 | 0.33 | 1.84 | 3.45 | 6.39 | 8.92 | 11.25 | 14.43 |
| | 60+ | 54 | 0.03 | 0.07 | 0.13 | 0.22 | 0.29 | 0.66 | 0.01 | 0.02 | 0.08 | 0.16 | 0.22 | 0.52 | 2.38 | 3.65 | 6.39 | 9.66 | 14.53 | 14.79 |
| | **TOTAL** | **1006** | **0.03** | **0.06** | **0.13** | **0.22** | **0.29** | **0.49** | **0.01** | **0.03** | **0.09** | **0.16** | **0.22** | **0.38** | **1.89** | **3.50** | **6.04** | **9.12** | **11.05** | **16.43** |
| | 0-19years | 165 | 0.04 | 0.07 | 0.15 | 0.23 | 0.29 | 0.50 | 0.01 | 0.03 | 0.11 | 0.17 | 0.23 | 0.41 | 1.85 | 3.82 | 6.5 | 9.53 | 11.49 | 20.46 |

| Age groups (years) | N | TFA intake from SBGs | | | | | | i-TFA intake from SBGs | | | | | | SFA intake from SBGs | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | p25 | p50 | p75 | p90 | p95 | p99 | p25 | p50 | p75 | p90 | p95 | p99 | p25 | p50 | p75 | p90 | p95 | p99 |
| 20-59years | 706 | 0.03 | 0.06 | 0.13 | 0.21 | 0.29 | 0.42 | 0.01 | 0.03 | 0.08 | 0.16 | 0.22 | 0.33 | 1.82 | 3.35 | 5.89 | 8.76 | 11.16 | 16.67 |
| 60+ | 135 | 0.03 | 0.06 | 0.13 | 0.21 | 0.29 | 0.49 | 0.01 | 0.02 | 0.07 | 0.14 | 0.22 | 0.38 | 2.36 | 3.85 | 6.39 | 9.19 | 10.27 | 14.56 |

* Intake distribution of total TFA intake in 2021 are from substitution models based on measurements from reformulated SBGs samples post Reg. (EU) 2019/649 on TFA reduction; TFA: trans fatty acids; SFA: saturated fatty acids; i-TFA: industrial trans fatty acids.

SBGs: Savoury Baked Goods

**Figure S1.** The contribution of different SBGs to the total daily intake of TFA by age group and in total.
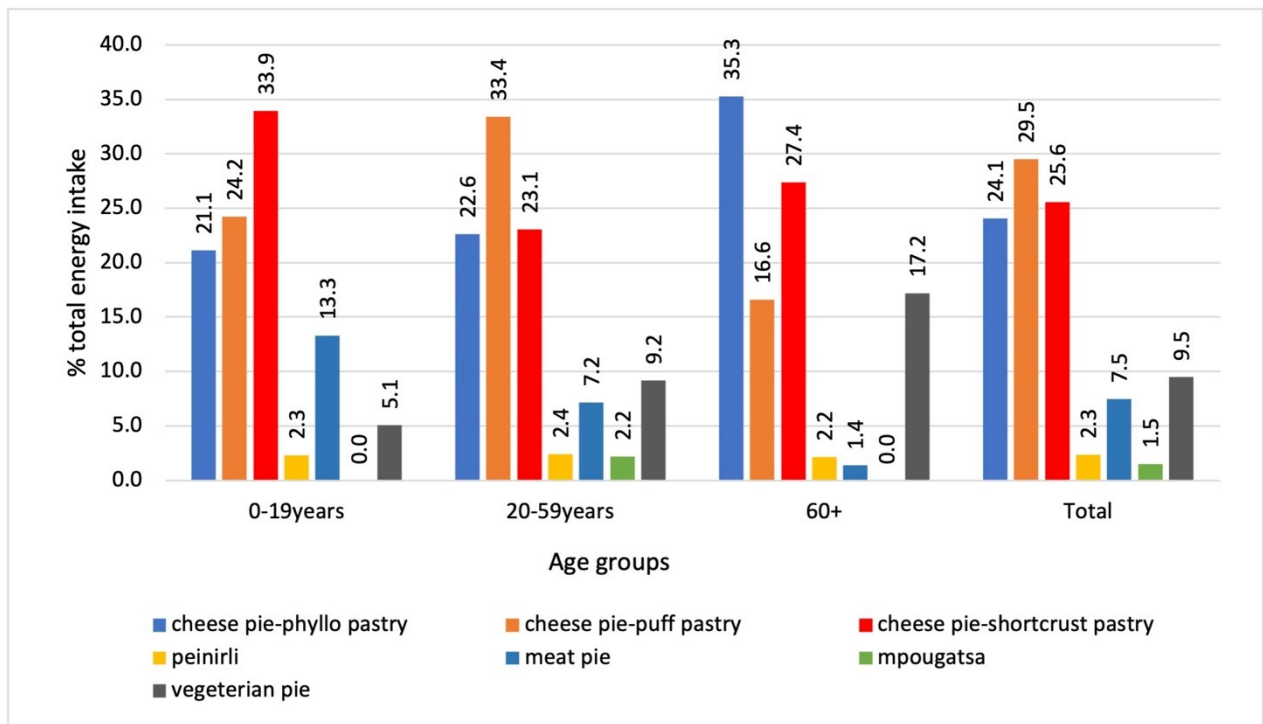


SBGs: Savoury Baked Goods

# APPENDIX 5 ▪ PARTICIPATION IN ADDITIONAL CONFERENCES

## 5.1. 6th GRESPEN CONGRES 2023

- **Abstract**

### ΑΞΙΟΛΟΓΗΣΗ ΤΗΣ ΕΥΡΩΠΑΪΚΗΣ ΠΟΛΙΤΙΚΗΣ ΜΕΙΩΣΗΣ ΤΩΝ TRANS ΛΙΠΑΡΩΝ ΟΞΕΩΝ ΓΙΑ ΤΗΝ ΠΡΟΑΣΠΙΣΗ ΤΗΣ ΔΗΜΟΣΙΑΣ ΥΓΕΙΑΣ

Γ. Μαράκης[1], Σ. Κοτοπούλου[1,2], Χ. Προεστός[3], Ζ. Μούσια[1], Δ. Παπαδημητρίου[1], Α. Νάσκα[4], Α. Ζαμπέλας[1,2], Ε. Μαγριπλή[2*]

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**Εισαγωγή/Σκοπός:** Σύμφωνα με πρόσφατη έρευνα[1], το 16% του Ελληνικού πληθυσμού υπερέβαινε το ανώτατο όριο πρόσληψης trans λιπαρών (Trans Fatty Acids-TFA). Δεδομένου ότι τα επεξεργασμένα τρόφιμα ήταν μία από τις κύριες πηγές έκθεσης, σκοπός της παρούσης μελέτης είναι να εκτιμηθεί η πρόσληψη TFA και κορεσμένων λιπαρών (Saturated Fatty Acids-SFA) από αλμυρά αρτοσκευάσματα εμπορίου (Savoury Baked Goods-SBGs) στο σύνολο του πληθυσμού, ενόψει της πλήρους εφαρμογής του Ευρωπαϊκού Κανονισμού (ΕΕ) 2019/649.

**Υλικό/Μέθοδος:** Μετρήθηκε η περιεκτικότητα σε λιπαρά οξέα 140 δειγμάτων SBGs που συλλέχθηκαν το 2021. Τα αποτελέσματα χρησιμοποιήθηκαν για τον προσδιορισμό της διατροφικής έκθεσης σε βιομηχανικώς παραγόμενα TFA (i-TFA), συνολικά TFA και SFA, χρησιμοποιώντας μοντέλα αντικατάστασης (substitution models) ενός εθνικά αντιπροσωπευτικού δείγματος καταναλωτών SBGs από την Πανελλαδική Μελέτη Διατροφής και Υγείας (ΠΑΜΕΔΥ). Οι εκτιμηθείσες τιμές συγκρίθηκαν με εκείνες της προηγούμενης έρευνας[1].

**Αποτελέσματα:** Η εκτιμώμενη διάμεση πρόσληψη i-TFA και TFA από SBGs μειώθηκε μεταξύ των ετών 2015 και 2021 ενώ η αντίστοιχη SFA αυξήθηκε. Ομοίως, η διάμεση πρόσληψη ολικών TFA μειώθηκε, ενώ η μέση πρόσληψη ολικών SFA αυξήθηκε. Οι μεταβολές στις τιμές παρουσιάζονται στον Πίνακα 1. Το ποσοστό του γενικού πληθυσμού με πρόσληψη TFA>1% μειώθηκε από 15.7% το 2015 σε 14.5% το 2021.

**Συμπεράσματα:** Η μείωση της πρόσληψης i-TFA, TFA από SBGs καθώς και ολικών TFA συνοδεύεται από αύξηση της πρόσληψης SFA, γεγονός που δεικνύει την αναγκαιότητα διερεύνησης του λιπιδαιμικού προφίλ των λοιπών επεξεργασμένων τροφίμων, καθώς και διεξαγωγής αξιολογήσεων κινδύνου-οφέλους, για την εκτίμηση της αποτελεσματικότητας της ευρωπαϊκής πολιτικής μείωσης των TFA στην προάσπιση της δημόσιας υγείας.

**Πίνακας 1.** Πρόσληψη i-TFA, TFA, SFA από SBGs και ολικών TFA, SFA (% ημερήσιας συνολικής ενεργειακής πρόσληψης) μεταξύ 2015 και 2021 για τους καταναλωτές SBGs

| Έτος | i-TFA | | TFA | | SFA | | Ολικά TFA | | Ολικά SFA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **p50 (p25, p75) | p95 | p50 (p25, p75) | p95 | p50 (p25, p75) | p95 | p50 (p25, p75) | p95 | mean (sd) | p95 |
| 2015 | 0.05 (0.01, 0.12) | 0.34 | 0.13 (0.03, 0.27) | 0.56 | 2.66 (1.51, 4.24) | 7.50 | 0.63 (0.44, 0.88) | 1.47 | 13.95 (3.95) | 20.48 |
| 2021 | 0.03 (0.01, 0.09) | 0.22 | 0.06 (0.03, 0.13) | 0.29 | 3.50 (1.89, 6.04) | 11.05 | 0.52 (0.35, 0.77) | 1.39 | 15.19 (4.50) | 23.14 |

**p50: διάμεση τιμή, p25: 25ο εκατοστημόριο, p75: 75ο εκατοστημόριο, p95: 95ο εκατοστημόριο, mean: μέση τιμή, sd: τυπική απόκλιση.

[1] Emmanuella Magriplis, Georgios Marakis, Sotiria Kotopoulou, Androniki Naska, George Michas, Renata Micha, Demosthenes Panagiotakos, Antonis Zampelas. Trans fatty acid intake increases likelihood of dyslipidemia especially among individuals with higher saturated fat consumption. Rev. Cardiovasc. Med. 2022, 23(4), 130. https://doi.org/10.31083/j.rcm2304130

- **Oral Presentation**

# EVALUATION OF THE EUROPEAN POLICY FOR THE REDUCTION OF TRANS FATTY ACIDS (TFA) TO PROTECT PUBLIC HEALTH

G. Marakis [1], S. Kotopoulou [1,2], C. Proestos [3], Z. Mousia [1], D. Papadimitriou [1], A. Naska [4], A. Zampelas [1,2], E. Magriplis [2*]

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## Background

**Reviews in Cardiovascular Medicine**

*Rev. Cardiovasc. Med.* 2022; 23(4): 130
http://doi.org/10.31083/j.rcm2304130

*Original Research*
**Trans fatty acid intake increases likelihood of dyslipidemia especially among individuals with higher saturated fat consumption**

Emmanuella Magriplis[1,*], Georgios Marakis[2], Sotiria Kotopoulou[1,2], Androniki Naska[3], George Michas[1], Renata Micha[4,5], Demosthenes Panagiotakos[6], Antonis Zampelas[1,2]

➢ **16%** of Greeks consumed TFA above 1% of energy intake (WHO recommendation)
➢ **Processed/refined grains** (mainly from savoury pastries and pies): one of the main contributors

- The Commission Regulation (EU) No 2019/649 on TFA specifies that the content of TFA in foodstuffs, other than TFA naturally occurring in fat of animal origin, shall not exceed 2 grams per 100 grams of fat.

## Objectives

- To measure the **content of TFA and SFA of commercial Savoury Baked Goods (SBGs)**

- To estimate **exposure** to **TFA and SFA** from SBGs in a representative **sample** of consumer participants in the **Hellenic National Nutrition and Health Survey (HNNHS)**

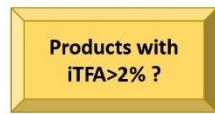- To assess the percentage of **population at risk**

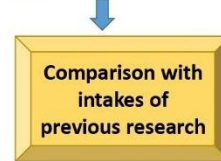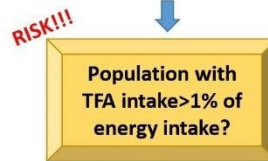in the eve of the full implementation of European Regulation (EU) 2019/649 on TFA (April 2021)
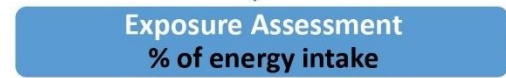
## Methods

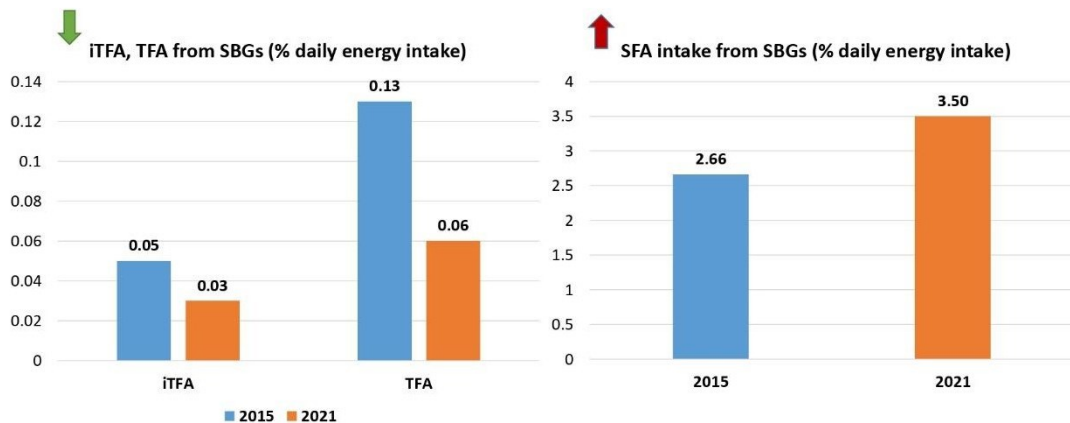**140 SBG samples collected in March 2021 and analyzed**

1. Cheese pies with phyllo pastry
2. Cheese pies with puff pastry
3. Cheese pies with shortcrust pastry
4. Pies containing meat
5. Peinirli
6. Bougatsa with cheese
7. Vegeterian pies

**Concentration Data**
HNNHS updated with new FA measurements

**Consumption Data**
**SBG consumers, n=1008**
(22.7% of HNNHS participants)

**Exposure Assessment**
**% of energy intake**

**Products with iTFA>2% ?**

reformulation assessment

RISK!!!

**Population with TFA intake>1% of energy intake?**

**Comparison with intakes of previous research**

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## Results



iTFA, TFA from SBGs (% daily energy intake)

iTFA: 2015 = 0.05, 2021 = 0.03
TFA: 2015 = 0.13, 2021 = 0.06

■ 2015  ■ 2021

SFA intake from SBGs (% daily energy intake)

2015 = 2.66, 2021 = 3.50

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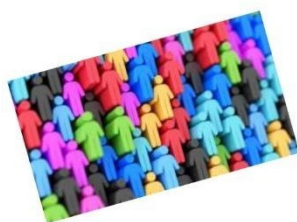
## Results

**A 37% reduction in the number of products above the 2% limit seen between the two sampling periods 2015 and 2021**

**A statistically significant lower proportion of the population in 2021 compared to the corresponding one in 2015 (14.5% vs 15.7%) has TFA intakes above 1% of energy intake**

# Conclusion

The decrease in TFA intakes is accompanied by an increase in SFA intakes.
**Further investigation of food lipid profiles** and **risk-benefit assessments**
are required to evaluate the effectiveness of the
European TFA reduction policy in protecting public health.

Thank you for your attention,
*Sotiria Kotopoulou*
Hellenic Food Authority & Agricultural University of Athens

DISCUSSION

## 5.2. 9th PANHELLENIC CONFERENCE OF THE GREEK LIPID FORUM

- **Abstract**

### Exposure assessment of trans fatty acids among adults in Greece; Results from the Hellenic National Nutrition and Health Survey (HNNHS)

G. Marakis[1], A. Zampelas[1,2], S. Kotopoulou[1,2], A. Naska[3], Z. Mousia[1], E. Magriplis[2]*

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**Abstract:** Trans fatty acid (TFA) intake has been associated with increased cardiovascular disease risk, mainly due to their adverse effects on blood lipids levels and markers of inflammation. In the 90's, the TRANSFAIR Study concluded that TFA intake in Greece was low and below the WHO recommendation of <1% of total energy intake. Since then, there has been lack of information regarding specific population based TFA intake in Greece. The primary aim of this study was to estimate TFA intake in a representative sample of Greek adults from two individual 24-hour recalls and derive the main food groups contributing to TFA intake, by combining the most recent occurrence data on TFA with food consumption data from the Hellenic National Nutrition and Health Survey (HNNHS). Finally, consumer profile of those exceeding intake was evaluated. The population sample of HNNHS consisted of 3726 adults (mean age 45.4±19.1 years, mean BMI 25.8±4.7 kg/m$^2$, 59.3% females). The median intake of TFA among adults was 0.53% (IQR: 0.34-0.81) of total energy intake, with 16.1% of adults consuming ≥1% of total energy. Consumer profile of those exceeding TFA recommendations, in both males and females, was being married or cohabiting, employed, of higher educational level (>12 years) and non-smoker (p for all<0.001). Adjusted logistic regression showed that adults consuming ≥1% of total energy from TFA, were 9.4 times more likely to have higher serum LDL-cholesterol levels, above 130mg/dL, compared to those with <1% TFA (OR = 9.4, 95%CI [2.0-43.7]), which is in line with current knowledge regarding the effects of TFA on blood lipids. Adults who consumed ≥1% of total energy intake from TFA, were also 1.9 times more likely to have sodium intake ≥2300mg/d ([95%CI: 1.4-2.7] and 1.8 times more likely [95%CI: 1.1-3.2] to exceed saturated fatty acid recommendation, than those with <1% TFA. Cheese (16.9%), processed refined grain products (9.5%) and fried fish (9.2%) were the 3 main food contributors, with processed refined grain products being the main source of industrially produced TFA in the age group 18-50 y. These results can provide guidance to public authorities for planning targeted food inspections and developing awareness campaigns.
**Keywords:** trans fat, exposure assessment, HNNHS

*Greek Lipid Forum*