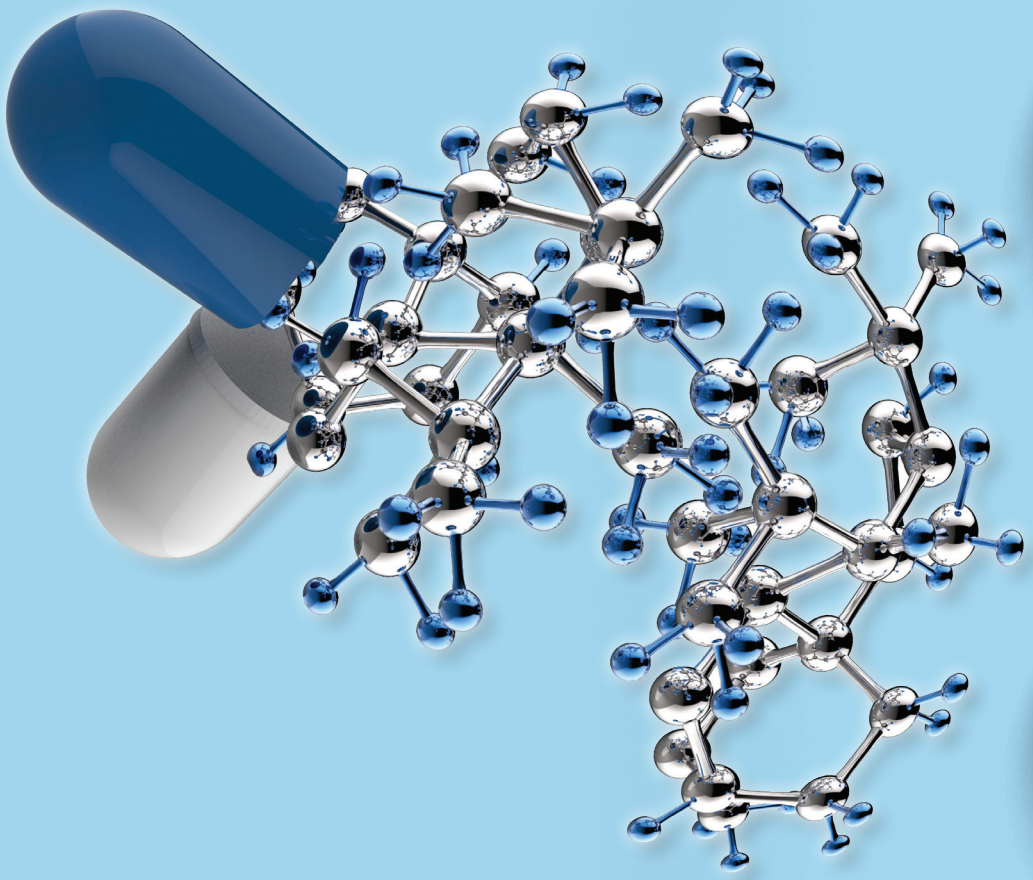
ISSN 1105-4999

ΦΑΡΜΑΚΕΥΤΙΚΗ
PHARMAKEFTIKI

ΤΡΙΜΗΝΙΑΙΑ ΕΚΔΟΣΗ ΜΕ ΘΕΜΑΤΑ ΦΑΡΜΑΚΕΥΤΙΚΩΝ ΕΠΙΣΤΗΜΩΝ
A QUARTERLY EDITION ON PHARMACEUTICAL SCIENCES' TOPICS



ΤΟΜΟΣ VOLUME 33 • ΤΕΥΧΟΣ ISSUE II

ΑΠΡΙΛΙΟΣ - ΙΟΥΝΙΟΣ APRIL - JUNE 2021

6ο ΔΥΟ FORUM

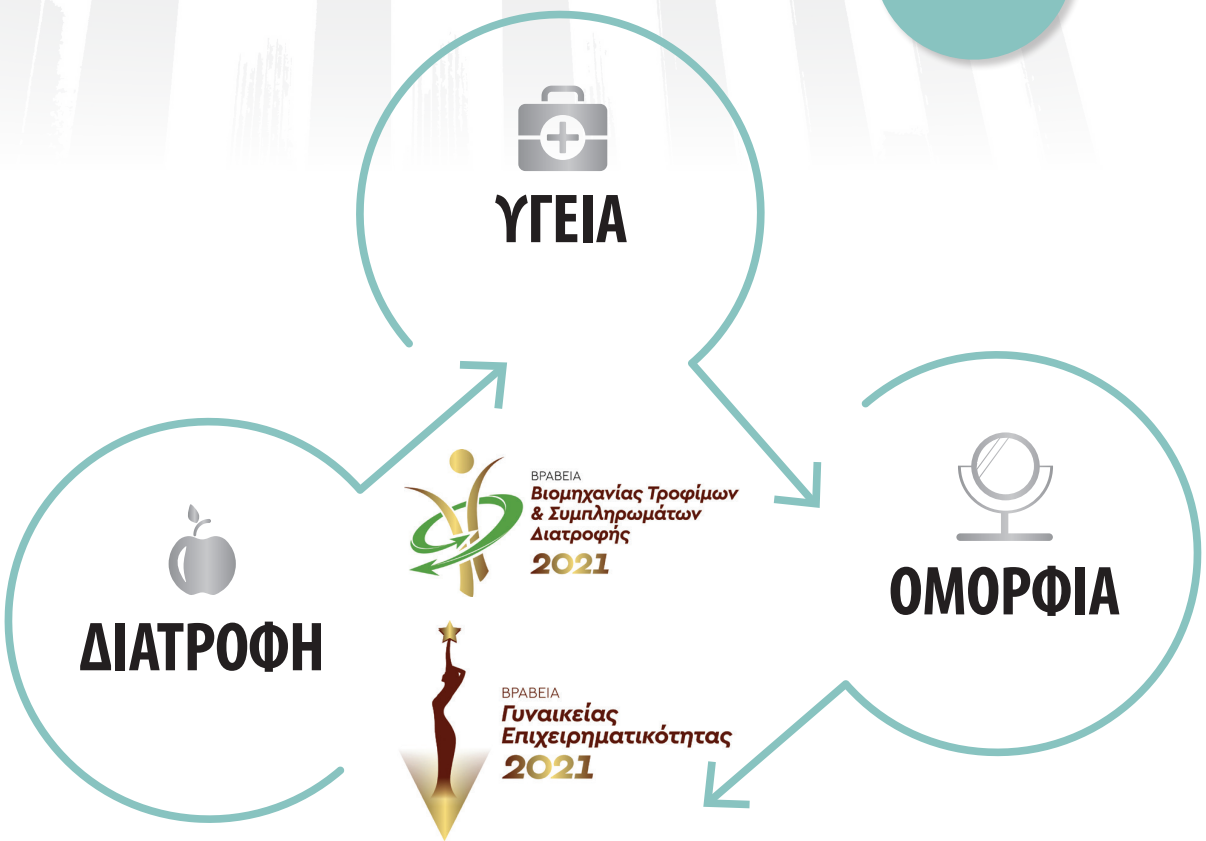
& ΕΚΘΕΣΗ

Διατροφής | Υγείας | Ομορφιάς

23-24 ΟΚΤΩΒΡΙΟΥ 2021

ΖΑΠΠΕΙΟΝ ΜΕΓΑΡΟ

Είσοδος Ελεύθερη



Υπεύθυνος επικοινωνίας: Γεράσιμος Κουλουμπής
Τηλ.: +30 22994 40962, Email: k.ge@zita-congress.gr, www.dyoforum.gr

Follow us

Το γεγονός που θα αλλάξει τη ζωή σου! Σε αφορά!!

Ενημερώσου στο www.dyoforum.gr

Σε συνεργασία με τον

ΕΜΠΟΡΙΚΟΣ ΣΥΛΛΟΓΟΣ ΑΘΗΝΩΝ

Διοργάνωση

ZITA GROUP
ISO 9001-14001 since 1982

ΠΕΡΙΟΔΙΚΟ ΔΥΟ

Υπό την Αιγίδα των:

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
ΥΠΟΥΡΓΕΙΟ ΤΟΥΡΙΣΜΟΥ

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
ΠΕΡΙΦΕΡΕΙΑ ΑΤΤΙΚΗΣ

ΔΗΜΟΣ ΑΘΗΝΑΙΩΝ

ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
AGRICULTURAL UNIVERSITY OF ATHENS

ΙΑΤΡΙΚΟΣ ΣΥΛΛΟΓΟΣ ΑΘΗΝΩΝ

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
ΕΜΠΟΡΙΚΟ & ΒΙΟΜΗΧΑΝΙΚΟ ΕΠΙΜΕΛΗΤΗΡΙΟ ΑΘΗΝΩΝ

ΕΛΛΗΝΙΚΗ ΦΑΡΜΑΚΕΥΤΙΚΗ ΕΤΑΙΡΕΙΑ

UNESCO
Οργανισμός των Ηνωμένων Εθνών για την Εκπαίδευση, την Επιστήμη και τον Πολιτισμό

unitwin
Έδρα UNESCO Εφηβικής Υγείας και Ιατρικής Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών Ελλάδα

Χορηγός Επικοινωνίας: Deal.gr DEAL

ΦΑΡΜΑΚΕΥΤΙΚΗ

ΤΡΙΜΗΝΙΑΙΑ ΕΚΔΟΣΗ ΜΕ ΘΕΜΑΤΑ
ΦΑΡΜΑΚΕΥΤΙΚΩΝ ΕΠΙΣΤΗΜΩΝ
ΤΟΜΟΣ 33, ΤΕΥΧΟΣ ΙΙ,
ΑΠΡΙΛΙΟΣ - ΙΟΥΝΙΟΣ 2021

ΔΙΕΥΘΥΝΤΗΣ ΣΥΝΤΑΞΗΣ

Α. Τσαντίλη

Ομοτ. Καθηγήτρια, Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών (ΕΚΠΑ)

tsantili@pharm.uoa.gr

ΑΡΧΙΣΥΝΤΑΚΤΗΣ

Γ.Α. Καρίκας

Ομότιμος καθηγητής, Πανεπιστήμιο Δυτικής Αττικής, karikasg@uniwa.gr

ΣΥΝΤΑΚΤΙΚΗ ΕΠΙΤΡΟΠΗ

Κ. Δεμέτζος

Καθηγητής, ΕΚΠΑ

Β. Δημόπουλος

Καθηγητής, Πανεπιστήμιο Θεσσαλονίκης, ΑΠΘ

Ν. Κόλμαν

Galenica SA

Χ. Κοντογιώργης,

Επ. Καθηγητής, Δ.Π.Θ.

Π. Κουρουνάκης

Ομοτ. Καθηγητής,
Πανεπιστήμιο Θεσσαλονίκης, ΑΠΘ

Π. Μαχαίρας

Ομοτ. Καθηγητής, ΕΚΠΑ

Σ. Νικολαρόπουλος

Αναπλ. Καθηγητής, Πανεπιστήμιο Πατρών

Γ. Πάιρας

Αναπλ. Καθηγητής, Πανεπιστήμιο Πατρών

Ε. Παντερή

Καθηγήτρια, ΕΚΠΑ

Δ. Ρέκκας

Αναπλ. Καθηγητής, ΕΚΠΑ

PHARMAKEFTIKI

A QUARTERLY EDITION
ON PHARMACEUTICAL SCIENCES' TOPICS
VOLUME 33, ISSUE II,
APRIL - JUNE 2021

EDITOR

A. Tsantili

Emeritus Professor, National and Kapodistrian University of Athens (NKUA)

tsantili@pharm.uoa.gr

CO EDITOR

G.A. Karikas

Emeritus professor, University of West Attica, Greece, karikasg@uniwa.gr

EDITORIAL BOARD

C. Demetzos

Professor, NKUA

V.J. Demopoulos

Professor, University of Thessaloniki, AUTh

N. Kolman

Galenica SA

Ch. Kontogiorgis

Assistant Professor, D.U.Th.

P. Kourounakis

Emeritus Professor,
University of Thessaloniki, AUTh

P. Macheras

Emeritus Professor, NKUA

S. Nikolaropoulos

Associate Professor, University of Patras

G. Pairas

Associate Professor, University of Patras

I. Panderi

Professor, NKUA

D. Rekkas

Associate Professor, NKUA

Οδηγίες προς συγγραφείς/Authors guidelines: https://www.hsmc.gr/author-guidelines/

E-mail για υποβολή εργασιών:
tsantili@pharm.uoa.gr, karikasg@uniwa.gr
Για την ηλεκτρονική έκδοση της «Φαρμακευτικής» και οδηγίες προς συγγραφείς επισκεφτείτε την διεύθυνση: www.hsmc.gr

Τα άρθρα που δημοσιεύονται στην «Φαρμακευτική» καταχωρούνται στα Chemicals Abstracts, EMBASE, SCOPUS και EBSCO

E-mail for manuscript submission:
tsantili@pharm.uoa.gr, karikasg@uniwa.gr
For "Pharmakeftiki" electronic edition and instructions to authors please visit www.hsmc.gr

Articles published in "Pharmakeftiki" are indexed in Chemical Abstracts, EMBASE, SCOPUS and EBSCO

ΠΕΡΙΕΧΟΜΕΝΑ / CONTENTS

ΦΑΡΜΑΚΕΥΤΙΚΗ
ΤΡΙΜΗΝΙΑΙΑ ΕΚΔΟΣΗ
ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΕΤΑΙΡΕΙΑΣ ΦΑΡΜΑΚΟΧΗΜΕΙΑΣ
& ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΦΑΡΜΑΚΕΥΤΙΚΗΣ
ΕΤΑΙΡΕΙΑΣ

PHARMAKEFTIKI
A QUARTERLY JOINT EDITION OF
THE HELLENIC SOCIETY OF
MEDICINAL CHEMISTRY &
THE HELLENIC PHARMACEUTICAL SOCIETY

ZITA MEDICAL MANAGEMENT

ZITA MEDICAL MANAGEMENT, Ομήρου 29Α, Πέτα Σαρωνικού, Ελλάδα
Τηλ.: + 30 22994 40962, Fax: 22990 66029, E-mail: g.kouloumpis@zitamanagement .com

ZITA MEDICAL MANAGEMENT

www.hsmc.gr/en/pharmakeftiki-journal/

ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, II, 2021 | 72-87 ΑΡΘΡΟ ΕΠΙΣΚΟΠΗΣΗΣ

PHARMAKEFTIKI, 33, II, 2021 | 72-87 REVIEW ARTICLE

Το Μικροβίωμα του Εντέρου και η Συσχέτισή του με Ψυχικές Διαταραχές

Χαράλαμπος Τριάντης[1*], Παναγιώτης Θεοδόσης-Νόμπελος[1], Ευανθία Ασημακοπούλου[2], Αθανάσιος Σπαθής[1,3]

[1]Τμήμα Φαρμακευτικής, Πανεπιστήμιο Frederick, Λευκωσία, Κύπρος

[2]Τμήμα Νοσηλευτικής, Πανεπιστήμιο Frederick, Λευκωσία, Κύπρος

[3]Synoesis Therapeutics LTD, Λευκωσία, Κύπρος

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ:
Μικροβίωμα; άξονας μικροβιώματος-εντέρου-εγκεφάλου; φλεγμονή; συναισθηματικές διαταραχές; αγχώδεις διαταραχές; ψυχώσεις

***ΣΥΓΓΡΑΦΕΑΣ ΑΛΛΗΛΟΓΡΑΦΙΑΣ:**
Χαράλαμπος Τριάντης
Email: hsc.tc@frederick.ac.cy
Tel: +357 22394394
Γ. Φρειδερίκου 7, Λευκωσία, 1036, Κύπρος

ΠΕΡΙΛΗΨΗ

Το μικροβιωμα αποτελεί ένα πολυποίκιλο σύστημα, τόσο σε είδη όσο και σε ποσότητα μικροβίων, το οποίο εμπλέκεται σε διάφορες φυσιολογικές λειτουργίες. Διατάραξη, μείωση ή ανισορροπία των ειδών του μικροβιώματος αποτελεί παράγοντα για διάφορες παθοφυσιολογικές διαταραχές, όπως σακχαρώδη διαβήτη, παχυσαρκία και καρδιαγγειακά νοσήματα. Η φλεγμονή και το οξειδωτικό στρες δείχνουν να είναι κύριοι αλληλοσυνδεόμενοι μηχανισμοί που οδηγούν στην εμφάνιση αυτών των διαταραχών μέσω του μικροβιώματος. Κύριο ρόλο σε αυτές τις διαδικασίες παίζει ο άξονας μικροβιώματος-εντέρου-εγκεφάλου, ενός περίπλοκου πολυ-οργανικού συστήματος αμφίδρομης σηματοδότησης μεταξύ της εντερικής χλωρίδας και του εγκεφάλου, επηρεάζοντας τη φυσιολογία του ξενιστή, την ομοιόσταση, την ανάπτυξη και το μεταβολισμό και τροποποιώντας το συστηματικό και εντερικό ανοσοποιητικό σύστημα. Στη βάση αυτών των μηχανισμών, το μικροβίωμα φαίνεται ότι σχετίζεται και με την εκδήλωση και εξέλιξη σοβαρών ψυχικών νόσων, όπου το μικροβίωμα αλλά και δείκτες της φλεγμονής βρίσκονται τροποποιημένα σε αυτές τις παθήσεις, και πιθανόν σε αυτό να εμπλέκεται και η εμφάνιση λοιπών επιπλοκών των νόσων αυτών στην περιφέρεια. Επίσης, η φαρμακευτική αγωγή έναντι των νόσων αυτών μπορεί να τροποποιήσει τη χλωρίδα του εντέρου που εμπλέκεται στη σύνθεση νευροδραστικών, φλεγμονωδών, και άλλων ουσιών. Οι μεταβολικές επιπλοκές διαφόρων φαρμάκων δείχνει να συνδέεται εν μέρει και με το τροποποιημένο μικροβίωμα. Με βάση τα ανωτέρω προκύπτει ότι τα μικρόβια του εντέρου και τα προβιοτικά θα μπορούσαν να παίξουν σημαντικό ρόλο στην ανάπτυξη, ρύθμιση και λειτουργία του εγκεφάλου καθώς και στην απορρύθμιση του. Παρόλα αυτά είναι δύσκολο να υπάρξει συγκεκριμένη οριοθέτηση και απόλυτη συσχέτιση μεταξύ μικροβιώματος και ψυχικών διαταραχών λόγω πολυπλοκότητας και ποικιλίας των συστημάτων που εμπλέκονται και απαιτούνται περαιτέρω μελέτες.

1. Εισαγωγή

Το μικροβίωμα του εντέρου είναι πολυποίκιλο και αποτελείται από τρισεκατομμύρια μικροοργανισμών, αλλά κυριαρχείται κυρίως από τέσσερα είδη, τα Firmicutes (φιρμοειδή), τα Bacteroidetes (βακτηροειδή), τα Actinobacteria (ακτινοβακτήρια) και τα Proteobacteria (πρωτεοβακτήρια).[1,2] Η σύσταση του μικροβιώματος μεταβάλλεται ανάλογα την ηλικία. Επίσης μεταβάλλεται και ο λόγος φιρμοειδή προς βακτηροειδή (Firmicutes/Bacteroidetes ratio), τα οποία αποτελούν το μεγαλύτερο ποσοστό.[3] Επιπλέον παράγοντες που μπορούν να επηρεάσουν τη σύσταση του μικροβιώματος είναι γενετικοί και περιβαλλοντικοί όπως πιθανές λοιμώξεις, χρήση αντιβιοτικών, διατροφή, στρες και τρόπος τοκετού κτλ.[4] Το εντερικό μικροβίωμα έχει σημαντικές μεταβολικές δράσεις, όπως τη σύνθεση θρεπτικών συστατικών, βιταμινών και ενέργειας μέσω άπεπτων πολυσακχαριτών, όπως φυτικές ίνες και άμυλο.[5]

Μια λεπτή ισορροπία στη σύνθεσή των μικροβίων του εντέρου είναι το κλειδί στη διατήρηση της εντερικής ανοσίας και της ομοιόστασης ολόκληρου του σώματος, ενώ οποιαδήποτε διακοπή αυτής της ισορροπίας θα μπορούσε να οδηγήσει σε σημαντικές παθοφυσιολογικές συνέπειες. Η ανισορροπία στο μικροβίωμα είναι κοινώς γνωστή ως δυσβίωση. Αν και ο χαρακτηρισμός της σύστασης ενός υγιούς μικροβιώματος βρίσκεται ακόμα σε πρώιμο στάδιο, η ανάλυση της αλληλουχίας 16S rRNA έχει επιτρέψει τη διερεύνηση και σύγκριση της μικροβιακής σύνθεση μεταξύ ατόμων. Χαρακτηριστικό παράδειγμα αποτελεί ο λόγος Firmicutes/Bacteroidetes, αύξηση του οποίου αποτελεί δείκτη παθοφυσιολογικών διαταραχών συμπεριλαμβανομένης της παχυσαρκίας λόγω αύξησης της αποθήκευσης ενέργειας και της απορρόφησης λιπιδίων.[6] Πλήθος διαταραχών έχουν συνδεθεί με μεταβολές του μικροβιώματος στις οποίες περιλαμβάνονται αλλεργίες, γαστρικός καρκίνος, κοιλιοκάκη, ανορεξία, σύνδρομο ευερέθιστου εντέρου (Irritable bowel syndrome, IBS), νόσος Crohn, παχυσαρκία, σακχαρώδης διαβήτης (ΣΔ) τύπου 2 κτλ.[1,7]

Επιπλέον, τόσο το μικροβίωμα του εντέρου όσο και το ανοσοποιητικό σύστημα εμπλέκονται στην αιτιοπαθογένεση ή την εκδήλωση νευροαναπτυξιακών, νευροψυχιατρικών και νευροεκφυλιστικών ασθενειών, όπως διαταραχές αυτιστικού φάσματος, μείζων καταθλιπτική διαταραχή, αγχώδης διαταραχή, νόσος Parkinson και Alzheimer.[4,8,9] Τα τελευταία χρόνια, υπάρχει έντονη έρευνα πάνω στη μικροχλωρίδα του εντέρου[10] και έχει δειχθεί ο ρόλος της φλεγμονής και η σύνδεσή της με σοβαρές ψυχικές νόσους (Serious mental illness - SMI), εντούτοις υπάρχει ακόμα σχετικό έλλειμμα στη διερεύνηση των παθοφυσιολογικών μηχανισμών που σχετίζονται με τις παραπάνω διαταραχές.

2. Μικροβίωμα, Νευρικό Σύστημα και Φλεγμονή

Η επικοινωνία μεταξύ εντερικού μικροβιώματος και εγκεφάλου είναι αμφίδρομη και πραγματοποιείται κυρίως μέσω νευρικών, ενδοκρινικών και ανοσοποιητικών μονοπατιών. Από άποψη νεύρωσης, ο γαστρεντερικός σωλήνα (ΓΕΣ), το πάγκρεας και η χοληδόχο κύστη νευρώνονται από το πνευμονογαστρικό νεύρο, το οποίο συνδέει το κεντρικό νευρικό σύστημα (ΚΝΣ) με το εντερικό νευρικό σύστημα. Το εντερικό νευρικό σύστημα ή "δεύτερος εγκέφαλος" ή "εγκέφαλος του εντέρου" όπως συχνά αποκαλείται, είναι ένα από τα κύρια τμήματα του αυτόνομου νευρικού συστήματος (ΑΝΣ) και αποτελείται από ένα δίκτυο νευρικών ινών που νευρώνουν τον ΓΕΣ.11 Ο άξονας του υποθαλάμου-υπόφυσης-επινεφριδίων (hypothalamus–pituitary–adrenal, HPA) ρυθμίζει την έκκριση κορτιζόλης, η οποία με τη σειρά της, μπορεί (i) να επηρεάσει τα ανοσοκύτταρα,, (ii) να αλλάξει τη διαπερατότητα του εντέρου και τη λειτουργία του ως φραγμός και (iii) να μεταβάλει τη σύσταση των μικροβίων του εντέρου. Αντιστρόφως, το μικροβίωμα του έντερο και τα προβιοτικά επηρεάζουν τα επίπεδα κυτοκινών στην κυκλοφορία, επηρεάζοντας αξιοσημείωτα την εγκεφαλική λειτουργία. Παράλληλα, τα εντερικά βακτήρια έχουν την ικανότητα να παράγουν νευροδιαβιβαστές, όπως ακετυλοχολίνη, σεροτονίνη, κατεχολαμίνες, γ-αμινοβουτυρικό οξύ (GABA), γλουταμικό και χαμηλής αλυσίδας λιπαρά οξέα (short chain fatty acids, SCFA).[4]

Στη γαστρεντερική οδό, όπως και σε άλλα συ-

στήματα οργάνων, το μονοξείδιο του αζώτου (NO) διαδραματίζει έναν καθοριστικό ρόλο στη ρύθμιση πολλών λειτουργιών τόσο στις φυσιολογικές όσο και στις παθολογικές καταστάσεις. Το NO συμβάλλει στη διατήρηση της ακεραιότητας και της αιματικής κυκλοφορίας του βλεννογόνου του γαστρεντερικού, τη ρύθμιση της έκκρισης οξέος, τη λειτουργία των λείων μυών ή την εκδήλωση της φλεγμονής του βλεννογόνου,[12] ενώ παράγεται από τη νευρωνική συνθετάση του NO (Neuronal nitric oxide synthase, nNOS) και είναι ένας από τους κύριους ανασταλτικούς νευροδιαβιβαστές των λείων μυών του γαστρεντερικού. Παρότι mRNA και των τριών ενζύμων που την παράγουν έχουν βρεθεί στα εντερικά νεύρα, η αναλογία των νιτρεργικών νευρώνων βρίσκεται περιορισμένη στο υποβλεννογόνιο πλέγμα, ενώ σημαντικά ενισχυμένη είναι η αναλογία τους στο μυεντερικό τμήμα όπου έχουν ανασταλτικό ρόλο στην κινητικότητα.[13] Βρέθηκε ότι δίαιτα με υψηλή περιεκτικότητα σε λιπαρά οδηγεί σε απόπτωση των νιτρεργικών νευρώνων του μυεντερικού πλέγματος μέσω ενεργοποίησης της κασπάσης-3 και των ανάλογων των Toll υποδοχείς-4 (Toll-like receptor-4, TLR4) στο λεπτό και παχύ έντερο, οδηγώντας στην εκδήλωση μειωμένης εντερικής κινητικότητας και στην αύξηση της συγκέντρωσης λιποπολυσακχαριτών και φλεγμονής, δείχνοντας και τη διασύνδεση μεταξύ φλεγμονής, μεταβολικού συνδρόμου και ψυχικών νόσων.[14]

Η εμπλοκή της φλεγμονής και η ενεργοποίηση νευροφλεγμονωδών μονοπατιών αποτελεί αιτιολογικό παράγοντα των SMI. Καταθλιπτικά συμπτώματα συνοδεύονται από αυξημένα επίπεδα προγλεγμονώδων κυτοκινών όπως της ιντερλευκίνης 1 και 6 και περιορισμού των αντιφλεγμονωδών κυτοκινών όπως οι ιντερλευκίνες 4 και 10, πιθανόν μέσω πολυμορφισμού των γονιδίων που κωδικοποιούν τις πρωτεΐνες αυτές και συνακόλουθης γενετικής προδιάθεσης σε SMI.[15] Άλλωστε, οι υποδοχείς της ιντερλευκίνης-1 και η ίδια, βρίσκονται υπερεκφρασμένοι σε περιοχές το εγκεφάλου σχετιζόμενες με την ανταπόκριση στο στρες και η δράση της είναι ουσιαστική στη ρύθμιση των νευροχημικών και ενδοκρινικών εκδηλώσεων της προσαρμογής, ενώ χρόνια χορήγηση της ιντερλευκίνης ή χρόνιο στρες εμφανίζονται καταθλιπτικές συμπεριφορές.[16] Επίσης, η κασπάση-1 και η πρωτεΐνη ενεργοποίησης της, η NLRP3, βρίσκονται υπερεκφρασμένες στα μονοκύτταρα καταθλιπτικών ασθενών υποδηλώνοντας τη σημασία του λεγόμενου «φλεγμονοσώματος» στην εκδήλωση της κατάθλιψης.[17] Ως αποτέλεσμα αυτών των ευρημάτων, η μινοκυκλίνη έδειξε να περιορίζει την απόπτωση, προσφέροντας νευροπροστατευτική και αντικαταθλιπτική δράση, μέσω αναστολής των κασπασών 1 και 3 και περιορισμού της μετατροπής των προφλεγμονώδων κυτοκινών στις τελικές μορφές τους.[18,19]

Ενδείξεις συνηγορούν στη διασύνδεση του ΑΝΣ και της χρόνιας χαμηλού βαθμού φλεγμονής στη δυσλειτουργία οργάνων. Στην περιφέρεια, το μικροβίωμα του εντέρου διαδραματίζει σημαντικό ρόλο στη διαμόρφωση ενός ισχυρού συστηματικού και εντερικού ανοσοποιητικού συστήματος.[20] Αναφέρεται συνήθως ως ένα «ουσιαστικό» έξωθεν τοποθετούμενο όργανο, αφού η σύνθεση και η κατανομή του τροποποιούνται συνεχώς ανάλογα με το περιβάλλον, τον ξενιστή, την ηλικία, τη διατροφή, τις τροποποιήσεις του τρόπου ζωής και τις ασθένειες.[7]

Σε ασθενείς με υπέρταση που λάμβαναν προβιοτικά για διάστημα τριών ή και περισσότερων μηνών, μειώθηκε σημαντικά τόσο η συστολική όσο και η διαστολική αρτηριακή πίεση,[21] υποδηλώνοντας ότι το μικροβιακό εντερικό σύστημα μπορεί να διαδραματίσει βασικό ρόλο στον έλεγχο της ομοιόστασης της αιματικής πίεσης και ροής. Μείωση του βακτηριακού πληθυσμού που παράγει βουτυρικό οξύ καθώς η τροποποίηση της ποικιλότητας του μικροβιώματος υπέρ ευκαιριακών παθογόνων μπορεί να παίξει ρόλο στην ενίσχυση ποικίλων ασθενειών και διαταραχών, όπως ΣΔ, παχυσαρκία και καρδιαγγειακή δυσλειτουργία, ενώ και φλεγμονώδεις παθήσεις του εντέρου δείχνουν να συνδέονται με αυτή τη δυσβίωση, παρά σε στοχευμένη τροποποίηση της σύνθεσης του μικροβιώματος. Παράλληλα, ενίσχυση των μηχανισμών που σχετίζονται με οξειδωτικό στρες έχουν βρεθεί αυξημένες σε τέτοιες καταστάσεις, θέτοντας και την αδυναμία αντιμετώπισης του οξειδωτικού φόρτου ως αποτέλεσμα αυτής της μικροβιακής ανισορροπίας.[22] Αυτή η ποικιλία στις ομάδες των μικροβίων του μικροβιώ-

ματος δείχνει να είναι σημαντική για την ενίσχυση αποικιών που παράγουν SCFA που είναι ωφέλιμα λόγω μείωσης της φλεγμονής, ενίσχυσης της ευαισθησίας των κυττάρων στην ινσουλίνη, ρύθμισης της διαφοροποίησης των επιθηλιακών κυττάρων, λειτουργίας ως φραγμού, ενεργοποίησης ρυθμιστικών κυττάρων και περιορισμού των νεοπλασιών μέσω της μειωμένης εξαλλαγής των κυττάρων.[23] Επίσης, προπιονικό και βουτυρικό οξύ έχουν δείξει να περιορίζουν την διάχυση των κυττάρων του ανοσοποιητικού και να μειώνουν την καταστροφή του γαστρεντερικού επιθηλίου λόγω ισχαιμίας, ενισχύοντας την αγγειοδιαστολή, ενώ οι αυξημένες συγκεντρώσεις των μικροβίων που παράγουν γαλακτικό οξύ έδειξαν να επιδρούν αρνητικά.[24] Επιπλέον, στοιχεία δείχνουν ότι ενίσχυση ανοτροποποιητικών μηχανισμών όπως ενεργοποίηση ιντερλευκίνης-4 (IL-4) και ρυθμιστικών μακροφάγων επέρχονται λόγω επιρροής επί του μικροβιώματος.[25] Αυτά τα χαμηλής αλυσίδας λιπαρά οξέα επεμβαίνουν στη λειτουργία του κυττάρου του ξενιστή και του παχέος εντέρου και δίνουν μια ποικιλία κυτταρικών αποκρίσεων, συμπεριλαμβανομένης της ρύθμισης της γονιδιακής έκφρασης.[26]

Επιπλέον, τα επίπεδα του γένους Bifidobacterium, ενός γένους που συσχετίζεται με την καταστολή της φλεγμονής μέσω της αναστολής της οδού του πυρηνικού παράγοντα-κΒ (Nf-KB), βρέθηκαν σημαντικά μειωμένα στα ζώα που υποβάλλονται σε περιορισμό της κίνησης, σε φαινόμενο δηλαδή εκδήλωσης βιολογικού στρες.[27,28] Τέτοια ευρήματα συμβαδίζουν με την άποψη ότι ο πυρηνικός παράγοντας-κΒ αυξάνεται ως απόκριση στο στρες και είναι ένας κρίσιμος μεσολαβητής της επαγόμενης από άγχος καταθλιπτικής συμπεριφοράς και της εξασθένησης της αναγέννησης των νευρώνων μέσω του στρες.[29,30] Επιπλέον, μικρόβια του γένους Allobaculum, το οποίο αντιπροσωπεύει ένα σημαντικό συστατικό της μικροχλωρίδας, απουσίαζαν σε αντίστοιχη περίπτωση περιορισμού της κίνησης και η ύπαρξη του συσχετίζεται αντιστρόφως με δείκτες φλεγμονής, συμπεριλαμβανομένων της λεπτίνης και των ιντερλευκινών.[31] Η χρόνια ακινητοποίηση οδήγησε επίσης σε αύξηση των συγκεντρώσεων του γένους Lactobacillus, τα μέλη του οποίου εμπλέκονται στην ενεργοποίηση της ιντερλευκίνης-1β (IL-1β) από τα μακροφάγα.[32]

Από κλινικής σκοπιάς, έχει δειχθεί ότι η σύνθεση της μικροβιακής χλωρίδας είναι σημαντικά τροποποιημένη σε ποντίκια με σοβαρή καταθλιπτική διαταραχή. Παράλληλα, η μεταφορά αυτής της χλωρίδας σε φυσιολογικά πειραματόζωα είχε ως αποτέλεσμα την εμφάνιση παρόμοιων καταθλιπτικών συμπτωμάτων.[33] Τροποποιήσεις στην έκφραση μικροβιακών γονιδίων καθώς και τροποποιημένοι μεταβολίτες εμφανίστηκαν στον ξενιστή, υποδεικνύοντας ότι η εμφάνιση των συμπτωμάτων κατάθλιψης προέρχεται εν τέλει από τον ίδιο τον ξενιστή υποδεικνύοντας ότι η συνεργασία μεταξύ μικροβιώματος-εντέρου-εγκεφάλου είναι αμφίδρομη. Αλλαγές της χλωρίδας επηρεάζουν τη συμπεριφορά ενώ και αλλαγές στη συμπεριφορά επηρεάζουν την εντερική χλωρίδα αντίστοιχα.[34]

3. Μικροβίωμα και ψυχικές διαταραχές

Ο άξονας μικροβιώματος-εντέρου-εγκεφάλου αποτελεί ένα περίπλοκο πολυ-οργανικό σύστημα αμφίδρομης σηματοδότησης μεταξύ της εντερικής χλωρίδας και του εγκεφάλου που παίζει βασικό ρόλο στη φυσιολογία του ξενιστή, την ομοιόσταση, την ανάπτυξη και το μεταβολισμό. Τα αναπτυσσόμενα στοιχεία δείχνουν επιδράσεις της σύστασης του μικροβιώματος στη συμπεριφορά ποντικών, υποστηρίζοντας το ρόλο τους στη διαμόρφωση αυτής.[35] Οι διαφορές στις συμπεριφορές που σχετίζονται με το στρες αναφέρονται συνήθως σε ποντίκια με τροποποιημένη εντερική χλωρίδα, που εμπλέκουν το ρόλο της στο άγχος και την κατάθλιψη. Από το μεγάλο φάσμα των νευροψυχιατρικών διαταραχών που εμπλέκονται με το μικροβίωμα, έχουν επιλεγεί να αναλυθούν οι συναισθηματικές διαταραχές, οι αγχώδεις διαταραχές και οι ψυχώσεις.

3.1. Ο ρόλος του μικροβιώματος στις συναισθηματικές διαταραχές

Σε ασθενείς με συναισθηματικές διαταραχές έχουν αναφερθεί τροποποιήσεις στη μικροβιακή σύσταση του εντέρου. Μελέτη, μετά από ανάλυση του μικρο-

βιώματος των κοπράνων ασθενών που είχαν διαγνωσθεί με καταθλιπτική διαταραχή σε σύγκριση με ομάδα ελέγχου, διαπίστωσε σημαντικές συσχετίσεις και κατέγραψε την ύπαρξη υπερβολικής και χαμηλής συγκέντρωσης των ειδών Bacteroidales και Lachnospiraceae, αντίστοιχα.[36] Το πιο έντονο αποτέλεσμα ήταν η γενική τροποποίηση των Bacteroidetes σε εκείνους που διαγνώστηκαν με κατάθλιψη. Πιο συγκεκριμένα, το Alistipes, ένα γένος των Bacteroidetes, βρέθηκε σε υψηλή συγκέντρωση, ενώ αντίστοιχη αύξηση παρατηρήθηκε και σε ποντίκια που εκτέθηκαν σε στρεσογόνους παράγοντες.[37] Επίσης, αυτό το είδος μικροβίων παρουσιάζεται υψηλό στο σύνδρομο χρόνιας κόπωσης και στο IBS, υποδηλώνοντας ένα πιθανό κοινό χαρακτηριστικό σε αρκετές διαταραχές που έχουν ως συνοδό χαρακτηριστικό κατάθλιψη και άγχος.[38] Έχει προταθεί ότι τα Alistipes ειδικότερα σχετίζονται με τη φλεγμονή και έτσι συνδέονται πιθανώς με την κατάθλιψη μέσω των φλεγμονωδών οδών. Σημειώνεται ότι τα επίπεδα του Alistipes και άλλων μικροβίων του εντέρου μπορούν να τροποποιηθούν μέσω διαιτητικής παρέμβασης.[39] Επίσης, δείγματα κοπράνων από ασθενείς με μείζων καταθλιπτική διαταραχή (Major depressive disorder, MDD) διαπίστωσαν ασθενή αρνητική συσχέτιση μεταξύ της σχετικής αφθονίας του Faecalibacterium και της σοβαρότητας των καταθλιπτικών συμπτωμάτων και ότι οι ασθενείς με MDD είχαν αυξημένα επίπεδα των Enterobacteriaceae και Alistipes.[40]

Μελέτη σε ποντικούς μοντέλα επί της κατάθλιψης έδειξε ότι τα βακτήρια του εντέρου σχετίζονται με μεταβολές στα επίπεδα νευροδιαβιβαστών και SCFAs.[41] Η σύνδεση μεταξύ του ισοβαλερικού οξέος, ενός συνήθους SCFA, και της κατάθλιψης είναι γνωστή,[42] όπως έχει δειχθεί και η σύνδεση των υποδοχέων αδενοσίνης με τον ύπνο και ορισμένες ψυχικές νόσους όπως συναισθηματικές και αγχώδεις διαταραχές.[43] Το βαλερικό οξύ, που περιέχεται σε εκχύλισμα βαλεριάνας, μπορεί να δράσει ως αντίστροφος αγωνιστής του υποδοχέα αδενοσίνης Α1 και να επηρεάσει την απελευθέρωση των νευροδιαβιβαστών στον εγκέφαλο.[44] Επιπλέον, τα επίπεδα σεροτονίνης καθώς και τρυπτοφάνης, πρόδρομη ένωση της σεροτονίνης, και 5-υδροξυινδολοοξικού οξέος, μεταβολίτης της σεροτονίνης, επηρεάζονται από την μικροβιακή χλωρίδα, συμβάλλοντας στην ανάπτυξη κατάθλιψης.[45] Παράλληλα, η χορήγηση προβιοτικών οδήγησε σε μείωση των δεικτών ιντερφερόνη-γ (IFN-γ), παράγοντα νέκρωσης όγκων α (TNF-α) και IL-6 καθώς και σε σημαντική αύξηση των συγκεντρώσεων τρυπτοφάνης και κινουρενικού οξέος στο πλάσμα πειραματοζώων.[46]

Σε άλλη διπλά τυφλή, ελεγχόμενη με εικονικό φάρμακο μελέτη, οι ασθενείς με MDD έλαβαν εικονικό φάρμακο ή συμπληρώματα προβιοτικών σε κάψουλες, που περιείχαν Lactobacillus acidophilus, Lactobacillus casei και Bifidobacterium bifidum, για οκτώ εβδομάδες. Στο τέλος της περιόδου μελέτης, οι ασθενείς που έλαβαν τις προβιοτικές κάψουλες εμφάνισαν σημαντικές βελτιώσεις στα συμπτώματα της κατάθλιψης, όπως εκτιμήθηκε από το ερωτηματολόγιο του Beck.[47] Αντίστοιχα, πρόσφατη πιλοτική μελέτη εξέτασε τα αποτελέσματα των συνδυασμένων συμπληρωμάτων προβιοτικών και οροτικού μαγνησίου σε μικρή ομάδα ασθενών με MDD, ανθεκτική σε εκλεκτικούς αναστολείς επαναπρόσληψης σεροτονίνης (Selective serotonin reuptake inhibitors, SSRI). Στο τέλος της περιόδου μελέτης οκτώ εβδομάδων, η πλειονότητα της ομάδας εμφάνισε σημαντικά βελτιωμένα τα συμπτώματα κατάθλιψης και ποιότητας ζωής. Ωστόσο, παρατηρήθηκε υποτροπή όταν οι ασθενείς σταμάτησαν να παίρνουν τα προβιοτικά συμπληρώματα και το οροτικό μαγνήσιο, παρότι ελάμβαναν αγωγή με SSRI.[48]

Τα προβιοτικά έχουν αποδειχθεί ότι βελτιώνουν τη διάθεση στον υγιή πληθυσμό. Μια διπλά τυφλή, ελεγχόμενη με εικονικό φάρμακο μελέτη ερεύνησε τα αποτελέσματα των προβιοτικών στη διάθεση.[49] Οι υγιείς εθελοντές έλαβαν ημερησίως γαλακτοκομικά που περιείχαν Lactobacillus casei ή εικονικό φάρμακο για τρεις εβδομάδες. Στο τέλος της περιόδου μελέτης, οι συμμετέχοντες που έλαβαν προβιοτικά, και είχαν αρχικά καταθλιπτική διάθεση, παρουσίασαν βελτίωση. Από την άλλη μεριά, μια τυχαιοποιημένη, διπλή τυφλή, ελεγχόμενη με εικονικό φάρμακο μελέτη, κατέληξε στο ότι η χορήγηση σκευάσματος προβιοτικών Lactobacillus και Bifidobacterium, δεν έδειξε στατιστικά σημαντική διαφορά με την ομάδα ελέγχου σε κανέναν ψυχολογικό δείκτη.[50]

Τα αντικαταθλιπτικά φάρμακα παρουσιάζουν μια σειρά αντιμικροβιακών δράσεων, με τους εκλεκτικούς αναστολείς επαναπρόσληψης σεροτονίνης (SSRI), όπως σερτραλίνη, φλουοξετίνη και παροξετίνη, να εμφανίζουν δραστικότητα έναντι θετικών και αρνητικών κατά Gram βακτηριδίων όπως σταφυλόκοκκους, εντερόκοκκους και ψευδομονάδες. Τα συγκεκριμένα SSRI, όπως η φλουοξετίνη, έχουν ακόμη συσχετιστεί με αυξημένο κίνδυνο ανάπτυξης λοίμωξης από Clostridium difficile.[51,52] Αξίζει να αναφερθεί ότι αν και ο μηχανισμός δράσης των SSRI για την κατάθλιψη δεν σχετίζεται με οποιαδήποτε αντιμικροβιακή δράση αυτών των φαρμάκων, οι πιθανές αλλαγές στο μικροβίωμα μπορεί να έχουν επίδραση σε άλλες φλεγμονώδεις ή φυσιολογικές παραμέτρους που συνδέονται με τη διάθεση, όπως αναφέρθηκε και παραπάνω.

Σε ασθενείς με οξεία μανία έχει προταθεί ότι η ανοσοποίηση που προκαλείται από το μικροβίωμα μπορεί να συνεισφέρει στη μείωση επανεισαγωγής στο νοσοκομείο μετά το εξιτήριο.[53] Αυτή η υπόθεση φαίνεται να προέρχεται από την παρατήρηση ότι ασθενείς με οξεία μανία εμφάνισαν υψηλότερα ποσοστά μικροβιακών λοιμώξεων, γεγονός που αποδείχθηκε μέσω πρόσφατης συνταγογράφησης αντιβιοτικών. Επιπλέον, η πρόληψη και αποτελεσματική θεραπεία βακτηριακών λοιμώξεων αποτελούν σημαντικές παρεμβάσεις για τη διαχείριση ασθενών με μανία.[54]

Από τότε, άρχισαν να φαίνονται τα στοιχεία που μαρτυρούν τη δράση της χλωρίδας στη διπολική διαταραχή. Το μικροβίωμα ασθενών με σοβαρά συμπτώματα διπολικής διαταραχής διαφέρει από την ομάδα ελέγχου.[55] Συγκεκριμένα, παρατηρήθηκε σημαντική μείωση σε δύο ξεχωριστά γένη των Firmicutes, όπως μείωση στα κύτταρα του είδους Faecalibacterium (η οποία παρατηρείται επίσης και στη μείζονα κατάθλιψη). Διακυμάνσεις και σε άλλα γένη, κυρίως μείωσης του αριθμού τους, φαίνεται να συσχετίζονται με τη σοβαρότητα των συμπτωμάτων. Το Faecalibacterium είναι ένας θετικός κατά gram μικροοργανισμός του εντέρου που εμφανίζει αντιφλεγμονώδεις ιδιότητες και χαμηλή παρουσία σε καταστάσεις όπως η φλεγμονώδης νόσος του εντέρου, η μη αλκοολική στεατοηπατίτιδα και σε ψυχικές διαταραχές. Επιπλέον, μια πρόσφατη πιλοτική μελέτη έδειξε ότι η συμπληρωματική χορήγηση προβιοτικών μειώνει τα ποσοστά επανανοσηλείας σε ασθενείς που έχουν πρόσφατα εξέλθει από νοσηλεία με μανία.[56]

Αντίστοιχα, σε πρόσφατη μελέτη[57] ανιχνεύθηκαν μειωμένα ποσοστά στην ποσότητα και την ποικιλομορφία των ειδών στα μικροβιακά δείγματα κοπράνων ατόμων με διάγνωση διπολικής διαταραχής, σε σύγκριση με την ομάδα ελέγχου. Επιπρόσθετα, οι συγγραφείς αναγνώρισαν σημαντική αύξηση στην αφθονία των οργανισμών που ταξινομήθηκαν στο φάσμα Actinobacteria και Coriobacteria.

Δεδομένου ότι δίνεται πλέον μεγαλύτερη προσοχή στη σχέση μικροβιώματος-ξενιστή, γίνεται αντιληπτό ότι τα μικρόβια του εντέρου είναι σημαντικά για την εξατομικευμένη ανταπόκριση στη φαρμακοθεραπεία. Οι αλληλεπιδράσεις μικροβίων-ξενιστών κυμαίνονται από την άμεση επίδραση στη φαρμακοκινητική του φαρμάκου μέχρι την έμμεση μεταβολή του μεταβολισμού του ξενιστή μέσω της τροποποίησης της ηπατικής ενζυμικής δραστηριότητας.[58,59] Τα μικρόβια του εντέρου είναι από τα πρώτα σημεία επαφής μεταξύ του σώματος και των φαρμάκων από το στόμα, επομένως η προσοχή πρόσφατα επεκτάθηκε στην εξέταση του μικροβιώματος επί στοχευμένων θεραπειών, ενώ ανομοιομορφία στη μικροβιολογική σύνθεση μπορεί να επιδρά στον τρόπο με τον οποίο τα άτομα ανταποκρίνονται στο φάρμακο, οδηγώντας σε υποθεραπεία.[60] Ένα μεγάλο ποσοστό φαρμάκων που κατευθύνονται από το ξενιστή (μη αντιμικροβιακά) παρουσιάζουν άμεση δράση εναντίον συνηθισμένων μικροβίων που μπορούν να μεταβάλλουν την κανονική λειτουργία των μικροβίων του εντέρου.[61]

Σε πρόσφατη μελέτη διερευνήθηκε η επίδραση φαρμάκων του ΚΝΣ στο μικροβίωμα αρουραίων.[62] Σε αυτή τη μελέτη, το λίθιο δεν παρουσίασε αντιμικροβιακή δράση έναντι του Escherichia coli ή του gram-θετικού οργανισμού Lactobacillus rhamnosus, ωστόσο, παρατηρήθηκε αύξηση της ποσότητας και της ποικιλομορφίας των διαφόρων μικροβιακών ειδών στο έντερο. Επίσης, το λίθιο, η αριπιπραζόλη και το βαλπροϊκό έδειξαν να προκαλούν σημαντικές αλλαγές στη σύσταση του εντερικού μικροβιώμα-

τος και των επιπέδων των χαμηλής αλυσίδας λιπαρών οξέων, ενώ η εσιταλοπράμη και η φλουοξετίνη έδωσαν διφορούμενα αποτελέσματα *in vitro* και *in vivo*, ενώ δείχνουν να τροποποιούν τη διαβατότητα του ειλεού μέσω αναστολής της δράσης του μεταφορέα της σεροτονίνης, με παρόμοιο μηχανισμό με το αυτόν της δράσης τους.

3.2. Ο ρόλος του μικροβιώματος στις αγχώδεις διαταραχές

Οι αγχώδεις διαταραχές, κυρίως ως κοινωνική αγχώδης διαταραχή (κοινωνική φοβία), διαταραχή πανικού, αγοραφοβία και γενικευμένη διαταραχή άγχους (Generalized anxiety disorder,GAD), αποτελούν σημαντική κατηγορία στο φάσμα των ψυχικών διαταραχών και το άγχος μπορεί να αποτελεί σύμπτωμα σε πολλές άλλες ψυχικές νόσους. Είναι γνωστό ότι το άγχος μπορεί να επηρεάσει τη σύσταση του μικροβιώματος του εντέρου. Παράλληλα, όλο και περισσότερες μελέτες συσχετίζουν το μικροβίωμα και τη δυσβίωση με το άγχος. Πρόσφατη μελέτη του 2021, σε σειρές ποντικιών μοντέλων για άγχος (Collaborative Cross mice), συμπέρανε ότι το μικροβίωμα εν μέρει μεσολαβεί και συντονίζει στις επιδράσεις της γενετικής πάνω στο άγχος.[63] Συστηματική ανασκόπηση παρουσίασε 21 τυχαιοποιημένες ελεγχόμενες μελέτες με συνολικό δείγμα πάνω από 1500 άτομα. Η ανασκόπηση κατέληξε ότι περισσότερες από τις μισές μελέτες που συμπεριελήφθησαν, έδειξαν ότι η ρύθμιση του εντερικού μικροβιώματος συνέβαλε θετικά στην αντιμετώπιση των συμπτωμάτων άγχους.[64]

Η σύσταση των μικροβίων του εντέρου μπορεί να μεταβληθεί με κατάλληλη διαιτητική παρέμβαση,[39] γεγονός που μπορεί να φανεί χρήσιμο και στις αγχώδεις διαταραχές. Για παράδειγμα, η χορήγηση του προβιοτικού, Lactobacillus casei Shirota, μείωσε το άγχος σε ασθενείς με σύνδρομο χρόνιας κόπωσης, ενώ σε ασθενείς με IBS με κλινικά σημαντικό άγχος, καθημερινή θεραπεία με ένα μίγμα προβιοτικών γαλακτοολιγοσακχαριτών για 4 εβδομάδες μείωσε τα ποσοστά άγχους και είχε σημαντική θετική επίδραση στην ποιότητα ζωής.[65] Να αναφερθεί επίσης, ότι υψηλότερος λόγος Firmicutes/Bacteroidetes σε ασθενείς με IBS, συσχετίστηκε με κλινικά σημαντικά επίπεδα άγχους και κατάθλιψης.[66] Ως μια γενικότερη έννοια, υψηλότερες βαθμολογίες άγχους καταγράφηκαν σε ασθενείς με IBS και συσχετίστηκαν με χαμηλότερη μικροβιακή ποικιλία των κοπράνων υποστηρίζοντας μια σχέση μεταξύ της μικροβιακής και της ψυχολογικής κατάστασης.

Τα προβιοτικά έχουν δείξει ότι βελτιώνουν τη διάθεση στον υγιή πληθυσμό. Σε μελέτη, σε ποντικούς, η χορήγηση σκευάσματος προβιοτικών με Lactobacillus και Bifidobacterium περιόρισε την αγχώδη συμπεριφορά και βελτίωσε τη διάθεση.[67] Συστηματική ανασκόπηση μελέτησε προκλινικά και κλινικά δεδομένα, σχετικά με την αγχολυτική δράση των προβιοτικών. Στη μελέτη αυτή προέκυψε η διαφορά ότι ενώ οι μελέτες σε πειραματόζωα έδειξαν πολύ θετικά αποτελέσματα στη μείωση του άγχους, αυτή η θετική επίδραση δεν έχει ακόμα μεταφερθεί και αποδειχθεί στα κλινικά δεδομένα, καταλήγοντας στην ανάγκη περαιτέρω μελέτης σε κλινικό επίπεδο και μάλιστα εστιάζοντας σε προβιοτικά με Lactobacillus rhamnosus.[68]

Αναφορικά με τη GAD, πρόσφατη μελέτη εντόπισε τη μικροβιακή δυσβίωση του εντέρου σε ασθενείς με GAD, προτείνοντας μάλιστα τη στόχευση του μικροβιώματος ως έναν χρήσιμο θεραπευτικά και προληπτικά στόχο για την εν λόγω διαταραχή.[69] Συγκεκριμένα, τα Firmicutes και Tenericutes είχαν χαμηλότερα επίπεδα σε ασθενείς με GAD ενώ βρέθηκαν και αρκετές διαφορές στη σύσταση των μικροβίων σε σχέση με την ομάδα ελέγχου. Τα Eubacterium coprostanoligenes, Ruminococcaceae και Prevotella συσχετίστηκαν αρνητικά με τη σοβαρότητα του άγχους και θετικά με τη μείωση του άγχους, ενώ τα Bacteroides και Escherichia-Shigella συσχετίστηκαν θετικά με τη σοβαρότητα του άγχους.[70]

Η διαταραχή μετατραυματικού στρες (Posttraumatic stress disorder, PTSD), διαφέρει από τις άλλες κύριες αγχώδεις διαταραχές και χαρακτηρίζεται από χαμηλές αποκρίσεις κορτιζόλης, γεγονός που μπορεί να συμβάλλει στην πρόβλεψη ατόμων με προδιάθεση ανάπτυξης PTSD μετά από ένα τραυματικό συμβάν. Πρόσφατα πειραματικά και κλινικά δεδομένα συγκλίνουν στην υπόθεση

ότι η διαταραχές στο μικροβίωμα του εντέρου, ειδικά στην πρώιμη ζωή, μπορεί να έχει μακροχρόνιες ανοσοποιητικές και άλλες φυσιολογικές επιδράσεις κάνοντας τα άτομα πιο ευαίσθητα στην ανάπτυξη PTSD μετά από ένα τραυματικό συμβάν.[71] Μετά από διερεύνηση της σύστασης του μικροβιώματος ασθενών με PTSD σε σύγκριση με ασθενείς που υπέστη τραύμα, αλλά χωρίς να αναπτύξουν PTSD, οι σχετικές αναλογίες των Actinobacteria, Lentisphaerae και Verrucomicrobia μειώθηκαν σε άτομα με PTSD σε βαθμό που μπορούσαν να διακριθούν οι ασθενείς αυτοί με υψηλό βαθμό ακρίβειας.[72]

3.3. Ο ρόλος του μικροβιώματος στις ψυχώσεις

Επιδημιολογικές μελέτες έχουν δείξει ότι η προγεννητική μικροβιακή μόλυνση είχε 10 έως 20 φορές αυξημένο κίνδυνο εμφάνισης σχιζοφρένειας[73] και έχουν μελετηθεί πιθανές συσχετίσεις μεταξύ σχιζοφρένειας και ενός διαταραγμένου εντερικού μικροβιώματος.[74] Επιπλέον, η σχιζοφρένεια συχνά συνυπάρχει σε διαταραχές του γαστρεντερικού συστήματος οι οποίες χαρακτηρίζονται από αλλοιώσεις των μικροβιακών ομάδων του εντέρου. Μελέτες σε πειραματόζωα έδειξαν ότι το μικροβίωμα του εντέρου είναι κρίσιμο για τη μεταγεννητική ανάπτυξη και την ωρίμανση των νευρικών, ανοσολογικών και ενδοκρινικών συστημάτων και αυτές οι συμπεριφορικές και φυσιολογικές διεργασίες συχνά είναι εξασθενημένες σε ασθενείς με σχιζοφρένεια, δείχνοντας και την αμφίδρομη επιρροή της ασθένειας με τη χλωρίδα.[45,75]

Το εντερικό μικροβίωμα ρυθμίζει μια ποικιλία νευροτροφινών και πρωτεϊνών, όπως ο νευροτροφικός παράγοντας που προέρχεται από τον εγκέφαλο (Brain-derived neurotrophic factor, BDNF), που έχει δειχθεί ότι εμπλέκονται στην ανάπτυξη του εγκεφάλου και στην πλαστικότητα του. Μείωση του BDNF και της έκφρασης της υπομονάδας 2α των υποδοχέων N-μεθυλ-d-ασπαρτικού (NMDA) βρέθηκε στον φλοιό και τον ιππόκαμπο ζώων με πλήρη απουσία εντερικών μικροβίων σε σύγκριση με την ομάδα ελέγχου.[76,77] Η υπολειτουργία του υποδοχέα NMDA πιστεύεται ότι είναι κεντρική στην παθοφυσιολογία της σχιζοφρένειας, καθώς οι ανταγωνιστές των υποδοχέων NMDA παράγουν συμπτώματα παρόμοια με τη σχιζοφρένεια, ενώ παράγοντες που ενισχύουν τη λειτουργία του υποδοχέα NMDA μειώνουν τα συμπτώματα και βελτιώνουν τη γνωστική λειτουργία.[78]

Η παραλλαγή στην έκφραση του BDNF έχει παρατηρηθεί ότι παίζει ρόλο στον μοριακό μηχανισμό που υποκρύπτει τη γνωστική δυσλειτουργία στη σχιζοφρένεια, ενώ μελέτες έδειξαν αυξημένα επίπεδα προ-φλεγμονωδών κυτοκινών ορού σε ασθενείς με σχιζοφρένεια σε σύγκριση με την ομάδα ελέγχου καθώς και συσχέτιση των επιπέδων των φλεγμονωδών δεικτών με τη σοβαρότητα των κλινικών συμπτωμάτων.[79] Επιπλέον, αυξημένος λόγος των συστημάτων των Τ-βοηθητικών κυττάρων (T helper cell, Th1/Th2) αναφέρεται σε ασθενείς με ψύχωση στην οξεία φάση, γεγονός που υποδηλώνει αυξημένη φλεγμονώδη απόκριση, ενώ η μετατόπιση αυτής της αναλογίας σε φυσιολογικές τιμές έχει συσχετιστεί με θετική απόκριση στη θεραπεία.[80] Αυτά τα στοιχεία μπορεί να επιβαρύνουν αρνητικά το μεταβολικό σύνδρομο, καθώς σημαντικές τροποποιήσεις του μικροβιώματος συμβαίνουν σε ΣΔ και παχυσαρκία, με την εντερική χλωρίδα να δείχνει να ελέγχει τη διακίνηση και την αποθήκευση των αποθεμάτων ενέργειας, μέσω ρύθμισης της ανοχής στην ινσουλίνη και την αρνητική επίδραση επί του συστήματος μέσω παραγωγής ενδοτοξινών. Τα ανωτέρω υποδεικνύουν τη συσχέτιση και ρίχνουν επιπλέον φως στους πιθανούς μηχανισμούς που εμπλέκουν τη σχιζοφρένεια με την εκδήλωση καρδιαγγειακών νόσων, αλλά και το αντίστροφο.81 Επίσης, όσον αφορά στη σύσταση της χλωρίδας και τη χρήση αντιψυχωσικής αγωγής, έχει δειχθεί ότι σε ασθενείς που λάμβαναν ολανζαπίνη παρατηρήθηκαν σημαντικές αλλαγές κυρίως στην αύξηση του λόγου Firmicutes/Bacteroidetes και στην αύξηση συστηματικών δεικτών όπως TNF-a, IL-1 και IL-6.[82] Επίσης, σε μελέτη πραγματοποιήθηκε μεταμόσχευση μικροβιώματος από μοντέλα ποντικών επί της σχιζοφρένειας σε ποντίκια ελεύθερα μικροβίων, οπότε παρατηρήθηκε περιορισμός της ποικιλότητας των μικροβίων, ανάπτυξη συγκεκριμένων κατηγοριών μικροβίων και επακόλουθα σχιζοφρενικά συμπτώματα και υπολειτουργία του γλουταμινερ-

γικού συστήματος.[83]

Εκτός των άλλων, μέσω τροποποίησης της διάβασης του τοιχώματος του γαστρεντερικού, παθογόνοι παράγοντες μπορούν να διέλθουν προκαλώντας ενεργοποίηση του ανοσοποιητικού και δημιουργία ακόμα και αυτοάνοσων αποκρίσεων, εμφανίζοντας μεγαλύτερη πιθανότητα να διαθέτουν αυτοαντισώματα κυρίως έναντι της αμυγδαλής, του ιππόκαμπου και του μετωπιαίου φλοιού.[84,85] Επίσης, αυξημένα αντιγόνα έναντι τροφών, γλουτένης και καζεΐνης, ειδικά βοδινής, βρέθηκαν σε ασθενείς με σχιζοφρένεια υποδηλώνοντας τη σημασία της αντίδρασης του ίδιου του οργανισμού σε σχέση με τον παράγοντα έκθεσης.[86]

Ο Dickerson και οι συνεργάτες του, σε μια τυχαιοποιημένη, ελεγχόμενη με εικονικό φάρμακο κλινική μελέτη διερεύνησαν τις επιδράσεις των συμπληρωμάτων προβιοτικών στα συμπτώματα της σχιζοφρένειας και της γαστρεντερικής λειτουργίας.[87] Ασθενείς με ήπια έως σοβαρά συμπτώματα ψύχωσης έλαβαν προβιοτικά (με Lactobacillus και Bifidobacterium) ή εικονικό φάρμακο για 14 εβδομάδες. Η μελέτη διαπίστωσε ότι, παρόλο που δεν προσέφερε βελτίωση στα συμπτώματα σχιζοφρένειας, η χορήγηση των προβιοτικών συσχετίστηκε με βελτιωμένη γαστρεντερική λειτουργία, πιθανόν λόγω ρύθμισης της εντερικής ανοσοαπόκρισης. Σε μετέπειτα μελέτη του ίδιου πληθυσμού εδείχθη ότι τα επίπεδα των IgG έναντι του Candida albicans σε άνδρες ασθενείς μειώθηκε σημαντικά, γεγονός που συσχετίστηκε με βελτιώσεις στις γαστρεντερικές ενοχλήσεις τους.[88]

Ασθενείς με σχιζοφρένεια εμφανίζουν συχνά γαστρεντερικά προβλήματα,[89] γεγονός που ενδεχομένως σχετίζεται με την προτεινόμενη ανοσολογική προέλευση της διαταραχής και παρέχει μια θεωρητική βάση για τη διερεύνηση του ρόλου του μικροβιώματος στη σχιζοφρένεια. Σε μελέτη ασθενών με ψύχωση πρώτου επεισοδίου εντοπίστηκαν διαφορές στη σύνθεση της εντερικής χλωρίδας, συμπεριλαμβανομένου του μειωμένου επιπολασμού των ειδών Lactobacillus και Bifidobacteria σε σύγκριση με υγιείς ασθενείς.[90] Είναι σημαντικό ότι οι διαφορές στην μικροβιακή σύσταση συσχετίστηκαν με τη σοβαρότητα των συμπτωμάτων και τον κίνδυνο ύφεσης κατά την παρακολούθηση 12 μηνών, αλλά δεν συσχετίστηκαν με τη διάρκεια της θεραπείας με αντιψυχωσικά φάρμακα.

Τα αντιψυχωσικά φάρμακα έχουν συσχετιστεί με επιβάρυνση της καρδιαγγειακής λειτουργίας και του μεταβολικού συνδρόμου, ενώ αντίστοιχα πολλά δεδομένα, που παρουσιάστηκαν και ανωτέρω, συνδέουν το τροποποιημένο μικροβίωμα με παχυσαρκία και μεταβολικό σύνδρομο. Ο Maier και οι συνεργάτες του[91] έδειξαν ότι υπάρχει άμεση επίδραση των αντιψυχωσικών επί συνηθισμένων μικροβίων που σχετίζονται με τη βελτίωση της μεταβολικής υγείας και περιορισμός τους, ενώ η ύπαρξη συγκεκριμένων κατηγοριών μικροβίων έδειξε να αποτελεί σημαντικό αιτιολογικό παράγοντα στην αύξηση βάρους, ενώ επηρέασε και σειρά φλεγμονωδών, φυσιολογικών και μικροβιακών δεικτών.[92] Επίσης, αξίζει να τονιστεί το γεγονός ότι αυτές οι αλλαγές ήταν εντονότερες σε αρσενικά ζώα και βελτιώθηκαν με τη χρήση αντιβιοτικών.[93] Αντίστοιχα, η χρήση της δεύτερης γενιάς αντιψυχωσικής ρισπεριδόνης και η δευτερογενής αύξηση βάρους έχουν συσχετιστεί με τροποποιημένο μικροβίωμα σε έφηβους αρσενικού φύλου, ενώ συσχετίστηκε και με περιορισμό της βιοποικιλότητας.[94] Λόγω αυτής της τεκμηριωμένης αλληλεπίδρασης των αντιψυχωσικών και της χλωρίδας του εντέρου, λαμβάνοντας υπόψη την επίδραση των συνταγογραφούμενων φαρμάκων στα μικρόβια, θα μπορούσαν να είναι μια σημαντική μεταβλητή όταν εξετάζονται οι αλληλεπιδράσεις μεταξύ ξενιστή και φαρμάκου σε μελλοντικές μελέτες.

4. Συμπεράσματα

Τα προβιοτικά χορηγούνται μέσα από τροφές όπως γαλακτοκομικά προϊόντα, αλλά και με τη μορφή φακελίσκων ή καψουλών. Αν και οι προκαταρκτικές μελέτες είναι σαφώς υποσχόμενες, απαιτούνται ελεγχόμενες με εικονικό φάρμακο τυχαιοποιημένες διπλά-τυφλές κλινικές δοκιμές για να διευκρινιστεί ο συμπληρωματικός ρόλος των προβιοτικών στη θεραπεία των ψυχικών νόσων. Η επιλογή των προβιοτικών βακτηρίων, η βέλτιστη δόση, ο τρόπος χορήγησης και η διάρκεια της θεραπείας μένει να καθοριστούν επακριβώς. Ο λεπτομερής χαρα-

κτηρισμός του στελέχους απαιτείται επίσης για όλα τα πιθανά προβιοτικά, δεδομένου ότι οι ενδείξεις συσσωρεύονται και υποδηλώνουν μια αλλοίωση της αναλογίας των βακτηριδίων του εντέρου. Επομένως, φαίνεται λογικό μια παρέμβαση προς την κατεύθυνση της διαμόρφωσης της εντερικής χλωρίδας να είναι θεμιτή. Όλο και περισσότερο, οι έρευνες υποδεικνύουν ότι τα προβιοτικά μπορεί να προσφέρουν εναλλακτική ή συμπληρωματική προσέγγιση της συμβατικής θεραπείας, μεταβάλλοντας την εντερική μικροχλωρίδα και τροποποιώντας το ανοσοποιητικό σύστημα του ξενιστή.

Η συνεχιζόμενη εξερεύνηση του ανθρώπινου μικροβιώματος υπόσχεται να φέρει τη σύνδεση μεταξύ του εντέρου και του εγκεφάλου σε σαφέστερη εστίαση, δείχνοντας τελικά τον αντίκτυπο στην κατάσταση του νου μας που μπορεί να επιφέρει η σύσταση της εντερικής χλωρίδας. Η σύνδεση εντέρου-εγκεφάλου φαίνεται να είναι αμφίδρομη, ο εγκέφαλος δρα επί γαστρεντερικών και ανοσοποιητικών λειτουργιών που συμβάλλουν στη διαμόρφωση της μικροβιακής σύνθεσης του εντέρου και τα μικρόβια του εντέρου δημιουργούν νευροδραστικές ενώσεις, συμπεριλαμβανομένων των νευροδιαβιβαστών και μεταβολιτών, που δρουν επίσης στον εγκέφαλο. Τα μικρόβια του εντέρου βοηθούν στον έλεγχο της διαπερατότητας τόσο μέσω του εντερικού επιθηλίου όσο και μέσω του αιματοεγκεφαλικού φραγμού, ο οποίος προστατεύει τον εγκέφαλο από δυνητικά επιβλαβείς παράγοντες.

Θα μπορούσε να προταθεί ότι ένα τροποποιημένο μικροβίωμα μπορεί να συμβάλλει σε ανωμαλίες στην ανάπτυξη του εγκεφάλου, σε ανοσολογική απορύθμιση, μεταβολική δυσλειτουργία και σε ψυχικές διαταραχές. Ωστόσο, η οριοθέτηση μιας σχέσης μεταξύ μίας συγκεκριμένης μικροχλωρίδας και μιας συγκεκριμένης νευροψυχιατρικής διαταραχής είναι πολύπλοκη, και ακόμα και αν υπάρχει διασύνδεση, μπορεί να είναι μη πλήρως συγκεκριμένη, ενώ οι συσχετισμοί μεταξύ ειδικής νευροψυχιατρικής νόσου και συγκεκριμένου περιβαλλοντικού παράγοντα ή ανοσολογικού δείκτη ή μικροβιακού προφίλ είναι συχνά ασαφείς.

Κατά την ολιστική προσέγγιση στη μελέτη της διατήρησης της ψυχικής υγείας και των παραγόντων των ψυχικών διαταραχών, τονίζεται η σημασία του μικροβιώματος του εντέρου το οποίο κατέχει πλέον έναν σημαντικό ρόλο. □

The gut Microbiome and its Association with Mental Disorders

Charalampos Triantis[1*], Panagiotis Theososis-Nobelos[1], Evanthia Asimakopoulou[2], Athanasios Spathis[1,3]

[1]Pharmacy Department, Frederick University, Nicosia, Cyprus
[2]Nursing Department, Frederick University, Nicosia, Cyprus
[3]Synoesis Therapeutics LTD, Nicosia, Cyprus

KEYWORDS:
Microbiome; brain-gut-microbiome axis; inflammation, mood disorder, anxiety disorders; psychosis

ABSTRACT

The microbiome is a multivariate system in species and quantity of microbes that it is involved in various physiological functions. Disruption, reduction or imbalance of microbial species appears consist of a factor in various pathophysiological disorders, such

as diabetes, obesity and cardiovascular disease. Inflammation and oxidative stress appear to be major interconnected mechanisms that lead to these imbalances via microbial imbalance. A major role in these processes play the microbiome-intestinal-brain axis, a complex multi-organ, bidirectional, signalling system between the intestinal flora and the brain, influencing host physiology, homeostasis, growth and metabolism and modifying the systemic and intestinal immune system. The microbiome seems to be related to the occurrence, promotion and development of serious psychiatric diseases. The microbiome and inflammatory markers are modified in these diseases, and potentially these are partly implicated to the occurrence of other complications of those diseases in the peripheral system. Furthermore, the medication against these diseases could modify the intestinal flora involved in the synthesis of neuroactive, inflammatory, and various others substances. Metabolic complications of various drugs also appears to be associated partly with a modified microbiome. Based on the above, it seems that microbiome and probiotics could play an important role in the development, regulation and function of the brain as well as in its deregulation. However, it is difficult to establish a perfectly clear demarcation and absolute correlation between microbiome and psychiatric disorders due to the complexity and variety of the involved systems and further studies are needed.

*** CORRESPONDING AUTHOR:**
Charalampos Triantis
E-mail: hsc.tc@frederick.ac.cy
Tel: +357 22394394
7 Y. Frederickou Str., Nicosia
1036 Cyprus

REFERENCES

1. Clemente J., Ursell L., Parfrey L., Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–70, 2012.
2. Arumugam M., Raes J., Pelletier E., Le Paslier D., Yamada T., Mende D. et al. Enterotypes of the human gut microbiome. *Nature* 473, 174–80, 2011.
3. Mariat D., Firmesse O., Levenez F., Guimarães Vd., Sokol H., Doré J. et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* 9, 123, 2009.
4. Cryan J., O'Riordan K., Cowan C., Sandhu K., Bastiaanssen T., Boehme M. et al. The microbiota-gut-brain axis. *Physiol. Rev.* 99, 1877–2013, 2019.
5. Nicholson J., Holmes E., Kinross J., Burcelin R., Gibson G., Jia W. et al. Host-gut microbiota metabolic interactions. *Science* 336, 1262–7, 2012.
6. Sanz Y., Moya-Perez A. Microbiota, inflammation and obesity. *Adv. Exp. Med. Biol*. 817, 291–317, 2014.
7. Sekirov I., Russell S., Antunes L., Finlay B. Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904, 2010.
8. Fung T., Olson C., Hsiao E. Interactions between themicrobiota, immune and nervous systems in health and disease. *Nat. Neurosci*. 20, 145–55, 2017.
9. Madan A., Thompson D., Fowler J.C., Ajami N.J., Salas R., Frueh B.C., et al. The gut microbiota is associated with psychiatric symptom severity and treatment outcome among individuals with serious mental illness. J. *Affect. Disord*.

264, 98-106, 2020.

10. NIH Human Microbiome Portfolio Analysis Team. A review of 10 years of human microbiome research activities at the US National Institutes of Health, Fiscal Years 2007–2016. *Microbiome* 7, 31, 2019.
11. Yoo B., Mazmanian S. The enteric network: interactions between the immune and nervous systems of the gut. *Immunity* 46, 910–26, 2017.
12. Forstermann U., Sessa W. Nitric oxide synthases: Regulation and function. *Eur. Heart J.* 33, 829–837, 2012.
13. Noorian A., Taylor G., Annerino D., Greene J. Neurochemical phenotypes of myenteric neurons in the rhesus monkey. *J. Comp. Neurol*. 519, 3387–3401, 2011.
14. Nyavor Y., Balemba O. Diet-induced dysmotility and neuropathy in the gut precedes endotoxaemia and metabolic syndrome: the chicken and the egg revisited. *J. Physiol.* 595, 1441–1442, 2017.
15. Wong M., Dong C., Maestre-Mesa J., Licinio J. Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and antidepressant response. *Mol. Psychiatry* 13, 800–812, 2008.
16. Goshen I., Kreisel T., Ben-Menachem-Zidon O., Licht T., Weidenfeld J., Ben-Hur T. et al. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol. Psychiatry* 13, 717–728, 2008.
17. Alcocer-Gomez, E. de Miguel M., Casas-Barquero N., Nunez-Vasco J., Sanchez-Alcazar J.A., Fernandez-Rodriguez A. et al. NLRP3 inflammasome is activated in mononuclear blood cells from patients with major depressive disorder. *Brain Behav. Immun*. 36, 111–117, 2014.
18. Chen M., Ona V.O., Li M., Ferrante R.J., Fink K.B., Zhu S. et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat. Med*. 6, 797–801, 2000.
19. Molina-Hernandez M., Tellez-Alcantara N., Perez-Garcia J., Olivera-Lopez J., Jaramillo-Jaimes M. Antidepressant-like actions of minocycline combined with several glutamate antagonists. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 380–386, 2008.
20. Chow J., Lee S.M., Shen Y., Khosravi A., Mazmanian S.K. Host-bacterial symbiosis in health and disease. *Adv. Imm*unol. 107, 243–274, 2010.
21. Khalesi S., Sun J., Buys N., Jayasinghe R. Effect of probiotics on blood pressure: A systematic review and meta-analysis of randomized, controlled trials. *Hypertension* 64, 897–903, 2014.
22. Qin J., Li Y., Cai Z., Li S., Zhu J., Zhang F., et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, 490, 55-60, 2012.
23. Yang T., Santisteban M., Rodriguez V., Li E., Ahmari N., Carvajal J. et al. Gut dysbiosis is linked to hypertension. *Hypertension* 65, 1331–1340, 2015.
24. Vieira E., Leonel A., Sad A., Beltrao N., Costa T., Ferreira T. et al. Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. *J. Nutr. Biochem.* 23, 430–436, 2012.
25. Felger J. Role of Inflammation in Depression and Treatment Implications. In: Macaluso M., Preskorn S. (eds) Antidepressants. Handbook of Experimental Pharmacology, vol 250. Springer, Cham, 2018.
26. Natarajan N., Pluznick J. From microbe to man: the role of microbial short chain fatty acid metabolites in host cell biology. *Am. J. Physiol. Cell Physiol.* 307, C979–C985, 2014.
27. Riedel C., Foata F., Philippe D., Adolfsson O., Eikmanns B., Blum S. Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-kappaB activation. *World J. Gastroenterol.* 12, 3729–3735, 2006.
28. Tsiakitzis K., Papagiouvannis G., Theodosis-Nobelos P., Tziona P., Kourounakis P., Rekka E. Synthesis, antioxidant and anti-inflammatoy effects of antioxidant acid amides with

GABA and n-acyl-pyrrolidin-2-ones. *Current Chem. Biol.* 11, 127-139, 2017.
29. Koo J., Russo S., Ferguson D., Nestler E., Duman R. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc. Natl. Acad. Sci. USA* 107, 2669–2674, 2010.
30. Theodosis-Nobelos P., Papagiouvannis G., Kourounakis P., Rekka E. Active anti-inflammatory and hypolipidemic derivatives of lorazepam. *Molecules* 9, 24, 3277, 2019.
31. Zenewicz L., Yin X., Wang G., Elinav E., Hao L., Zhao L. et al. IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. *J. Immunol.* 190, 5306–5312, 2013.
32. Miettinen M., Pietila T., Kekkonen R., Kankainen M., Latvala S., Pirhonen J. et al. Nonpathogenic Lactobacillus rhamnosus activates the inflammasome and antiviral responses in human macrophages. *Gut Microbes* 3, 510–522, 2012.
33. Zheng P., Zeng B., Zhou C., Liu M., Xu X., Zeng L. et al. Altered gut microbiome induces depression-like behaviors in a pathway that is mediated through the host's metabolism. *Mol. Psychiatry* 21, 786-96, 2016.
34. Wong M., Inserra A., Lewis M., Mastronardi C., Leong L., Choo J. et al. Inflammasome signaling affects anxiety- and depressive-like behavior and gut microbiome composition. *Mol. Psychiatry* 21, 797–805, 2016.
35. Sommer F., Backhed F. The gut microbiota—masters of host development and physiology. *Nat. Rev. Microbiol.* 11, 227–238, 2013.
36. Naseribafrouei A., Hestad K., Avershina E., Sekelja M., Linløkken A., Wilson R. et al. Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil.* 26, 1155–1162, 2014.
37. Bendtsen K.M.B., Krych L., Sørensen D.B., Pang W., Nielsen D.S., Josefsen K., et al. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. *PLoS One* 7, e46231, 2012.
38. Zhou L., Foster J. Psychobiotics and the gut-brain axis: in the pursuit of happiness. *Neuropsychiatr. Dis. Treat.* 11, 715–723, 2015.
39. David L.A., Maurice C.F., Carmody R.N., Gootenberg D.B., Button J.E., Wolfe B.E., et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559-563, 2014.
40. Jiang H., Ling Z., Zhang Y., Mao H., Ma Z., Yin Y. et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav. Immun.* 48, 186–94, 2015.
41. Wu M., Tian T., Mao Q., Zou T., Zhou C.-J., Xie J. et al. Associations between disordered gut microbiota and changes of neurotransmitters and short-chain fatty acids in depressed mice. *Transl. Psychiatry* 10, 350, 2020.
42. Szczesniak O., Hestad K.A., Hanssen J.F., Rudi K. Isovaleric acid in stool correlates with human depression. *Nutr. Neurosci*. 19, 279-283, 2016.
43. van Calker D., Biber K., Domschke K., Serchov T. The role of adenosine receptors in mood and anxiety disorders. *J. Neurochem*. 151, 11-27, 2019.
44. Lacher S., Mayer R., Sichardt K., Nieber K., Muller C. Interaction of valerian extracts of different polarity with adenosine receptors: identification of isovaltrate as an inverse agonist at A1 receptors. *Biochem. Pharmacol.* 73, 248–58, 2007.
45. Clarke G., Grenham S., Scully P., Fitzgerald P., Moloney R. D., Shanahan F., et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatry* 18, 666–673, 2013.
46. Desbonnet L., Garrett L., Clarke G., Bienenstock J., Dinan T.G. The probiotic Bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. *J. Psychiatr. Res*. 43, 164–174, 2008.
47. Akkasheh G., Kashani-Poor Z., Tajabadi-Ebrahimi M., Jafari P., Akbari H., Taghizadeh M.et al. Clinical and metabolic response to probiotic administration in patients with major depressive disorder: A randomized, double-blind,

placebo-controlled trial. *Nutrition* 32, 315–20, 2016.

48. Bambling M., Edwards S.C., Hall S., Vitetta L. A combination of probiotics and magnesium orotate attenuate depression in a small SSRI resistant cohort: an intestinal anti-inflammatory response is suggested. *Inflammopharmacology* 25, 271-274, 2017.
49. Benton D., Williams C., Brown A. Impact of consuming a milk drink containing a probiotic on mood and cognition. *Eur. J. Clin. Nutr*. 61, 355-361, 2007.
50. Romijn A., Rucklidge J., Kuijer R., Frampton C. A double-blind, randomized, placebo-controlled trial of Lactobacillus helveticus and Bifidobacterium longum for the symptoms of depression. *Aust. N. Z. J. Psychiatry* 51, 810-821, 2017.
51. Flowers S., Ward K., Clark C. The Gut Microbiome in Bipolar Disorder and Pharmacotherapy Management. *Neuropsychobiology* 79, 43-49, 2020.
52. Rogers M., Greene M., Young V., Saint S., Langa K., Kao J. et al. Depression, antidepressant medications, and risk of Clostridium difficile infection. *BMC Med*. 11, 121, 2013.
53. Dickerson F., Severance E., Yolken R. The microbiome, immunity, and schizophrenia and bipolar disorder. *Brain Behav. Immun*. 62, 46–52, 2017.
54. Yolken R., Adamos M., Katsafanas E., Khushalani S., Origoni A., Savage C. et al. Individuals hospitalized with acute mania have increased exposure to antimicrobial medications. *Bipolar Disord.* 18, 404–409, 2016.
55. Evans S.J., Bassis C.M., Hein R., Assari S., Flowers S.A., Kelly M.B., et al The gut microbiome composition associates with bipolar disorder and illness severity. *J. Psychiatr. Res.* 87, 23-29, 2017.
56. Dickerson F., Adamos M., Katsafanas E., Khushalani S., Origoni A., Savage C., et al. Adjunctive probiotic microorganisms to prevent rehospitalization in patients with acute mania: a randomized controlled trial. *Bipolar. Disord*. 20, 614-621, 2018.
57. Painold A., Mörkl S., Kashofer K., Halwachs B., Dalkner N, Bengesser S. et al. A step ahead: exploring the gut microbiota in inpatients with bipolar disorder during a depressive episode. *Bipolar Disord*. 21, 40–9, 2019.
58. ElRakaiby M., Dutilh B.E., Rizkallah M.R., Boleij A., Cole J.N., Aziz R.K. Pharmacomicrobiomics: the impact of human microbiome variations on systems pharmacology and personalized therapeutics. *OMICS* 2014, 18, 402-414.
59. Selwyn F., Cheng S., Klaassen C., Cui J. Regulation of Hepatic Drug-Metabolizing Enzymes in Germ-Free Mice by Conventionalization and Probiotics. *Drug Metab. Dispos.* 44, 262–74, 2016.
60. Kuntz T., Gilbert J. Introducing the Microbiome into Precision Medicine. *Trends Pharmacol. Sci*. 38, 81–91, 2017.
61. Maier L., Pruteanu M., Kuhn M., Zeller G., Telzerow A., Anderson E. et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 555, 623–8, 2018.
62. Cussotto S., Strain C.R., Fouhy F., Strain R.G., Peterson V.L., Clarke G., et al. Differential effects of psychotropic drugs on microbiome composition and gastrointestinal function. *Psychopharmacology (Berl)*. 236, 1671-1685, 2019.
63. Jin X., Zhang Y., Celniker S.E., Xia Y., Mao J.-H., Snijders A.M. el al. Gut microbiome partially mediates and coordinates the effects of genetics on anxiety-like behavior in Collaborative Cross mice. *Sci. Rep*. 11, 270, 2021.
64. Yang B., Wei J., Wei J., Ju P., Chen J. Effects of regulating intestinal microbiota on anxiety symptoms: A systematic review. *Gen. Psychiatr*. 32, e100056, 2019.
65. Silk D., Davis A., Vulevic J., Tzortzis G., Gibson G. Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome. *Aliment Pharmacol. Ther*. 29, 508–518, 2009.
66. Jeffery I., O'Toole P., Öhman L., Claesson M., Deane J., Quigley E. et al. An irritable bowel

syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 61, 997–1006, 2012.
67. Messaoudi M., Lalonde R., Violle N., Javelot H., Desor D., Nejdi A. et al. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *Br. J. Nutr*. 105, 755–64, 2011.
68. Reis D., Ilardi S., Punt S. The anxiolytic effect of probiotics: A systematic review and meta-analysis of the clinical and preclinical literature. *PLoS One* 20, 13, e0199041. 2018.
69. Jiang H.-Y., Zhang X., Yu Z.-H., Zhang Z., Deng M., Zhao J.-H., et al. Altered gut microbiota profile in patients with generalized anxiety disorder. *J. Psychiatr. Res*.104, 130-136, 2018.
70. Chen Y.H., Bai J., Wu D., Yu S.-F., Qiang X.-L., Bai H., et al. Association between fecal microbiota and generalized anxiety disorder: severity and early treatment response. *J. Affect. Disord*. 259, 56–66, 2019.
71. Leclercq S., Forsythe P., Bienenstock J. Posttraumatic Stress Disorder: Does the Gut Microbiome Hold the Key? *Can. J. Psychiatry* 61, 204-213, 2016.
72. Hemmings S.M.J., Malan-Muller S., van den Heuvel L.L., Demmitt B.A., Stanislawski M.A., Smith D.G, et al. The microbiome in posttraumatic stress disorder and traumaexposed controls: an exploratory study. *Psychosom. Med*. 79, 936-946, 2017.
73. Babulas V., Factor-Litvak P., Goetz R., Schaefer C. A., Brown A. S., Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. *Am. J. Psychiatry* 163, 927–929, 2006.
74. Lv F., Chen S., Wang L., Jiang R., Tian H., Li J. et al. The role of microbiota in the pathogenesis of schizophrenia and major depressive disorder and the possibility of targeting microbiota as a treatment option. *Oncotarget* 8, 100899–100907, 2017.
75. Fadgyas-Stanculete M., Buga A-M., Popa-Wagner A., Dumitrascu D. The relationship between irritable bowel syndrome and psychiatric disorders: From molecular changes to clinical manifestations. *J Mol Psychiatry* 2, 4, 2014.
76. Douglas-Escobar M., Elliott E., Neu J. Effect of intestinal microbial ecology on the developing brain. *JAMA Pediatr*, 167, 374-379, 2013.
77. Sudo N., Chida Y., Aiba Y., Sonoda J., Oyama N., Yu X.-N. et al. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. Physiol.* 558, 263-275, 2004.
78. Coyle J.T. NMDA receptor and schizophrenia: a brief history. *Schizophr. Bull* 38, 920-926, 2012.
79. Song X., Fan X., Song X., Zhang J., Zhang W., Li X. et al. Elevated levels of adiponectin and other cytokines in drug naïve, first episode schizophrenia patients with normal weight. *Schizophr. Res.* 150, 269-273, 2013.
80. Kim Y., Myint A., Lee B., Han C., Lee H., Kim D. et al.Th1, Th2 and Th3 cytokine alteration in schizophrenia. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 28, 1129-1134, 2004.
81. Theodosis-Nobelos P., Asimakopoulou E., Madianos M. Pathophysiological mechanisms of major mental disorders related to cardiovascular diseases. *Psychiatriki*, in press.
82. Davey K.J., O'Mahony S.M., Schellekens H., O'Sullivan O., Bienenstock J., Cotter P.D., *et al.* Gender-dependent consequences of chronic olanzapine in the rat: effects on body weight, inflammatory, metabolic and microbiota parameters. *Psychopharmacology* 221, 155-169, 2012.
83. Zheng P., Zeng B., Liu M., Chen J., Pan J., Han Y. et al. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. *Sci. Adv.* 6, 5, eaau8317, 2019.
84. Strous R., Shoenfeld Y. Schizophrenia, autoimmunity and immune system dysregulation: a comprehensive model updated and revisited. *J. Autoimmun.* 27, 71-80, 2006.

85. McGeachy M., McSorley S. Microbial-induced Th17: superhero or supervillain? *J Immunol.* 189, 3285-3291, 2012.
86. Severance E., Alaedini A., Yang S., Halling M., Gressitt K., Stallings C. et al. Gastrointestinal inflammation and associated immune activation in schizophrenia. *Schizophr. Res.* 138, 48-53, 2012.
87. Dickerson F.B., Stallings C., Origoni A., Katsafanas E., Savage C.L., Schweinfurth L.A., et al. Effect of probiotic supplementation on schizophrenia symptoms and association with gastrointestinal functioning: a randomized, placebo-controlled trial. *Prim. Care Companion CNS Disord*. 16, 1, 2014.
88. Severance E., Gressitt K., Stallings C., Katsafanas E., Schweinfurth L., Savage C. et al. Probiotic normalization of Candida albicans in schizophrenia: A randomized, placebo-controlled, longitudinal pilot study. *Brain Behav Immun* 62, 41–5, 2017.
89. Severance E., Prandovszky E., Castiglione J., Yolken R. Gastroenterology issues in schizophrenia: why the gut matters. *Curr Psychiatry Rep* 17, 27, 2015.
90. Schwarz E., Maukonen J., Hyytiäinen T., Kieseppä T., Orešič M., Sabunciyan S. et al. Analysis of microbiota in first episode psychosis identifies preliminary associations with symptom severity and treatment response. *Schizophr Res* 192, 398–403, 2018.
91. Maier L., Pruteanu M., Kuhn M., Zeller G., Telzerow A., Anderson E. et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 555, 623–8, 2018.
92. Morgan A., Crowley J., Nonneman R., Quackenbush C., Miller C., Ryan A. et al. The antipsychotic olanzapine interacts with the gut microbiome to cause weight gain in mouse. *PLoS One* 9, e115225, 2014.
93. Davey K.J., Cotter P.D., O'Sullivan O., Crispie F., Dinan T.G., Cryan J.F., et al. Antipsychotics and the gut microbiome: olanzapine-induced metabolic dysfunction is attenuated by antibiotic administration in the rat. *Transl. Psychiatry* 3, e309, 2013.
94. Bahr S.M., Tyler B.C., Wooldridge N., Butcher B.D., Burns T.L., Teesch L.M., et al. Use of the second-generation antipsychotic, risperidone, and secondary weight gain are associated with an altered gut microbiota in children. *Transl. Psychiatry* 5, e652, 2015.

Promising Targets for Neuroregenerative Drugs Among Intracellular Signaling Molecules of Nerve Tissue Progenitors

Gleb N. Zyuz'kov[1]*, Larisa A. Miroshnichenko[1], Tatyana Yu. Polyakova[1], Larisa A. Stavrova[1], Elena V. Simanina[1]

[1]Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russia

KEY WORDS:
neurodegenerative diseases; neuronal-committed progenitors; neural stem cells; neuroregeneration; signaling transduction

ABSTRACT

A promising direction of the development of new approaches to improving the effectiveness of neurodegenerative disease therapy is the stimulation of neurogenesis by activating the functions of progenitor cells of nerve tissue. In vitro, the role of NF-κB-, cAMP/PKA-, JAKs/STAT3-, ERK1/2-, p38-, JNK- и p53-mediated signaling pathways in realizing the growth potential of neuronal-committed progenitors (NCP, PSA-NCAM + clonogenic cells) was studied. To do this, the method of the pharmacological blockade with the use of selective inhibitors of individual signaling molecules was used. The features of intracellular signaling in the neuronal-committed progenitors have been revealed. The functional value of individual signaling molecules in the precursors of neurons and multipotent neural stem cells (NSC) was compared. Some of the fundamental differences in participation and the role of certain signaling molecules in determining the proliferation and differentiation of progenitors of different types have been revealed. It has been possible to develop tools with neuroregenerative activity based on STAT3 inhibitors or JNK activators. It is shown that the blockade of STAT3 leads to the stimulation of the combined predecessors against the background of the absence of a "negative" effect on the functioning of multipotent progenitor cells. The possibility of isolated induction of the progression of the NSC cell cycle by activation of JNK has been revealed. The possibility of induction of the progression of the NSC cell cycle with the help of JNK activation has been detected.

***CORRESPONDING AUTHOR:**
Gleb N. Zyuz'kov
Email: zgn@ pharmso.ru

1. Introduction

Neurodegeneration, including the dysfunction of the systems of cellular renewal of nerve tissue, is accompanied by a pronounced reorganization of the central nervous system and the formation of a qualitatively new pathological, pattern of brain structures[1-3]. The current concept of pharmacotherapy of these states, based on the principle of modulation of the functions of mature cells of nerve tissue preserved in the conditions of pathology, is completely untenable. Available drugs are often not able to restore

the morphofunctional state of the central nervous system as well as to prevent the progression of diseases[4, 5]. In this regard, it seems relevant to develop new drugs for the treatment of neurodegenerative diseases with fundamentally different mechanisms of action.

The development of approaches for stimulating neurogenesis by activating the functions of progenitor cells of the nervous tissue — multipotent neural stem cells (NSC) and neuronal-committed progenitors (committed precursors of neurons) looks promising[6, 7].

Information obtained in recent years on the properties and patterns of stem cell (SC) functioning has led to the development of the «Pharmacological strategy of regenerative medicine», which is based on the principle of imitation of the activity of natural regulatory systems for the functioning of endogenous progenitor cells using drugs - analogs of endogenous regulators of SC functions[3]. At the same time, the modern trend in the development of drugs is the creation of highly selective (targeted) drugs. By selectivity, in this case, it should be understood selective action, to a specific organ or tissue and the effect on specific cellular and/or subcellular structures[5, 8, 9]. In this aspect receptors to growth factors could be considered. However, genetically engineered growth factors do not fully comply with the requirements for drugs, in terms of selectivity and safety criteria. Almost all growth factors, to one degree or another, are pleiotropic and multifunctional regulators, and their protein nature, initially determines relatively high immunogenicity and toxicity. Several pharmacokinetic characteristics of cytokine analogs are also unacceptable[3]. At the same time, it is known that the implementation of external signals occurs through an intracellular signaling system, represented by cascades of signaling molecules sequentially activated by each other[8, 10]. Our information on the particularities of intracellular signaling in epigenetically and functionally different progenitor cells[5] served as the basis for creating a new direction of targeted therapy in regenerative medicine - "Strategy of the pharmacological regulation of intracellular signal transduction in regeneration-competent cells"[3, 9]. This approach involves the use of key components of intracellular signal transduction as targets that determine the realization of the growth potential of various progenitor cells and the functioning of tissue microenvironment elements that indirectly determine the course of reparative processes in tissues in various progenitors. In this case, the identification of the specific role of individual signaling molecules (potential targets) in the regulation of the cell cycle of various types of parent cells is a basic stage in the development of this direction. For the development of agents with neuroregenerative activity, signaling molecules that do not possess the opposite importance for the functioning of NSC and neuronal-committed progenitors (NCP)- eg. the stimulating effect on one type of progenitors does not inhibit at the same time the functioning of another type of progenitors)- and which also do not possess a "negative" effect on the vital functions of mature neurons and the neurotrophic function of glial cells (neurotrophic function), can be considered a promising approach[11]. Previously, we studied the features of intracellular signaling in the NSC[5].

The work aimed to study the role of NF-κB-, cAMP/PKA-, JAKs/STAT3-, ERK1/2-, p38-, JNK- и p53-mediated pathways in the implementation of the functions of the neuronal-committed progenitors in vitro.

2. Materials and methods

2.1 Chemicals and Drugs

Serum-free MACS Neuro Medium; anti-PSA-NCAM MicroBeads (Miltenyi Biotec, Germany); The following inhibitors of signaling molecules have been used (in brackets the concentration of in vitro is indicated). For NF-κB : aurothiomalate (50 μM), for ERK1/2 : PD98059 (100 μM); for p38: SB203580 (10 μM); for adenylate cyclase (AC): 2',5'-dideoxyadenosine (30 μM), for PKA: KT3761 (10 μM); for STAT3: STAT3 Inhibitor XIV, LLL12» (5 μM) (all manufactured by Calbiochem, USA); for JAKs : Pan JAK Inhibitor Ruxolitinib (200 nM), for JNK: SP600125» (10 μM) (all manufactured by InvivoGen, USA); for p53 «Pifithrin-α, Cyclic» (5 μM) (Santa Cruz Biotechnology, Inc., USA).

The working concentrations of inhibitors of signaling molecules were determined following the instructions of the developers of these reagents and confirmed as optimal in preliminary experiments on test crops. Hydroxyurea was from Calbiochem, USA; plastic plates for cultural studies were from Costar, USA).

2.2 Animals and Experimental Design

All animal experiments were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The study was approved by the Institute`s local Ethics Committee (Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences). Experiments were carried out on C57B1/6 mice (n=69) at the age of 2-2.5 months, weighing 20-22 g (7 mice in each experimental group (with each signaling molecule inhibitor) and 6 mice in the control group (culture without signaling molecule inhibitors)). Animals of the 1st category (conventional linear mice) were obtained from the Experimental Biological Models Department of Goldberg Research Institute of Pharmacology and Regenerative Medicine (Tomsk, Russia) (certificate available). Before the beginning of experiments (during 10 days) and over the study period, animals were contained in the vivarium (air temperature 20–220C, humidity 50-60 %) in plastic cages (10-15 mice) on a normal diet (solid diet pellets (Limited Liability Company «Assortiment Firm», Sergiev Posad city, Russia), water ad libitum. To exclude seasonal fluctuations of studied parameters, all the experiments were performed in the autumn-winter period. The animals were removed from the experiment (sacrificed) using CO2 cameras.

Using the cultural methods, we studied the direct effect of the signaling molecule inhibitors on the realization of the growth potential of neural tissue precursor cells in vitro.

2.3 Study of functional activity of neuronal-committed progenitors

To study neuronal-committed progenitors (NCP) from cells of the subventricular zone (SVZ) of the brain an immunomagnetic separator "MIniMACS Cell Separator" (Miltenyi Biotec, Germany), PSA-NCAM (CD56 +) was used. Cells were obtained by positive selection[12] (using appropriate antibody kits according to the methodological manufacturer's instructions). The PSA-NCAM + cells at a concentration of 105 / ml were incubated in MACS Neuro Medium (Miltenyi Biotec, Germany) for 5 days in a CO2 incubator at 37°C, 5% CO2, and 100% air humidity with signaling molecules inhibitors. After incubation, the content of clonogenic cells, their mitotic activity, and intensity of specialization was calculated.

The number of NCP was determined by the yield in the respective cultures of colony-forming units (CFU, CFU-NPSA-NCAM+, colonies containing more than 100 cells). The proliferative activity of the progenitor cells was assessed by the method of cell suicide using hydroxyurea (1 μM). The pool of CFU in the S-phase of the cell cycle was determined according to the formula: N = [(a-b)/a] × 100%, where a is the average for the group the number of CFU from cells not treated with hydroxyurea; b - the average for the group the number of CFU from cells treated with hydroxyurea. The intensity of the processes of specialization (differentiation/maturation) of progenitor elements was determined by calculating the ratio of the corresponding cluster-forming units (ClFU, neurospheres of 30 - 100 cells) to CFU (differentiation index)[13]. The control was the NCAM + cells culture without signaling molecules inhibitors.

2.4 Statistical Analysis

The results were analyzed with one-way ANOVA followed by Dunnett's test, Wilcoxon's test for dependent samples, and Mann–Whitney test for independent samples. The data are expressed as arithmetic means. The significance level was $p < 0.05$[14].

3. Results

3.1 Role of cAMP/PKA-signaling in the regulation of the functions of the neuronal-committed progenitors

There has been an increase in the output of CFU-NPSA-

NCAM+ (Figure 1), their proliferation activity (Figure 2), and the rate of differentiation (Figure 3) during the blockade of adenylate cyclase against the background in the absence of significant changes in the functioning of the selective inactivation of PKA. The different effects of the adenylate cyclase and PKA inhibitors on the functional state of CFU-NPSA-NCAM+ are likely to indicate the potential bivalent nature of cAMP-mediated signaling for the functioning of the committed precursors of neurons[5]. The described phenomenon is the absence of PKA/CREB-signaling effect on their mitotic activity, while the decrease in intracellular concentration of cAMP (blockade of adenylate cyclase) stimulates the progression of the cell cycle by interacting with alternative secondary messengers (without the participation of PKA), possibly by activating the Ca2+/calmodulin-dependent protein kinase and MAPK-pathways[15]. It is important that the increase in intracellular cAMP

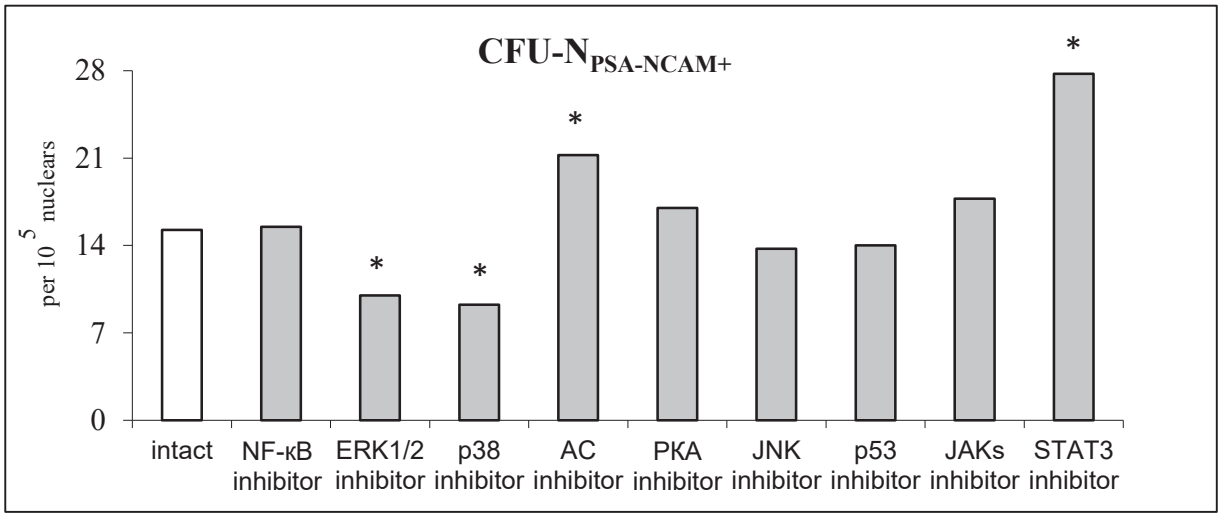


Figure 1. *Number of CFU-NPSA-NCAM+ in the cell culture.*
*Here and in figures 1-3: cell culture without inhibitors alcohol (intact) and when adding in vitro inhibitors of the: NF-κB, ERK1 / 2, p38, adenylate cyclase (AC), PKA, JNK, p53, JAKs, STAT3. * - the significance of differences in indicators with intact at p < 0.05.*

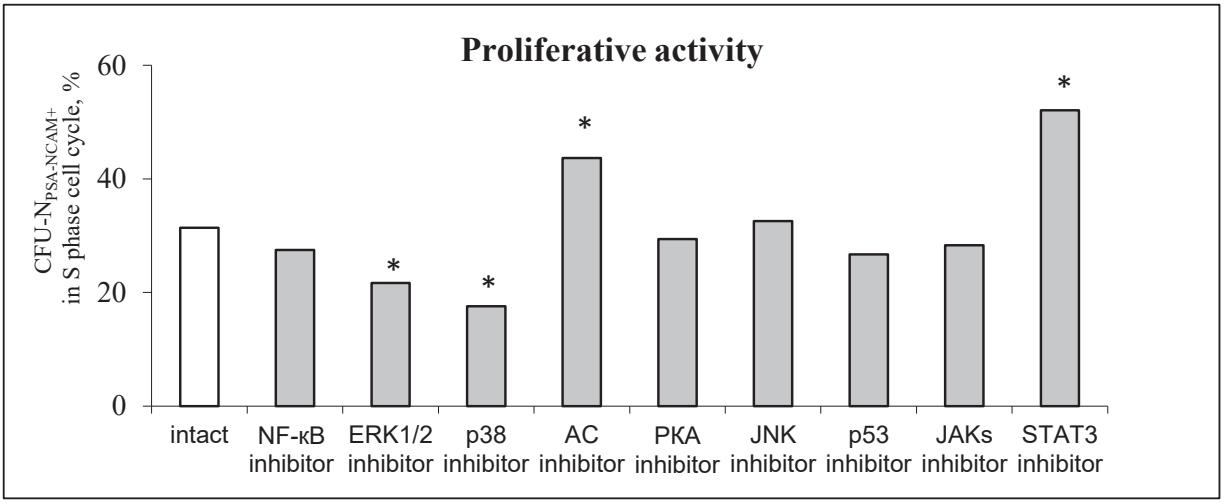


Figure 2. *Proliferative activity of neuronal-committed progenitors.*

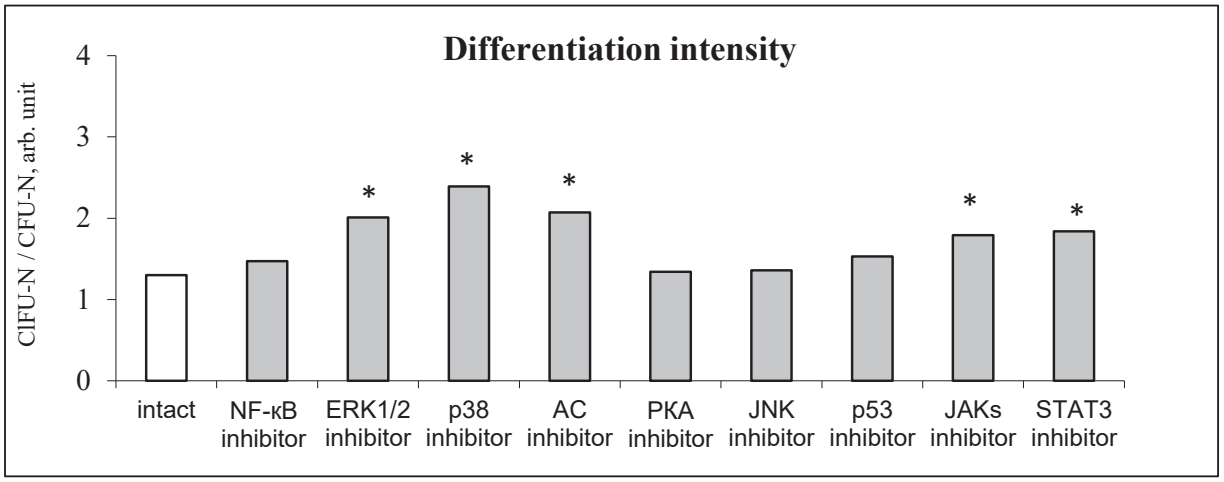


Figure 3. *Differentiation intensity (ClFU-NPSA-NCAM+ / CFU-NPSA-NCAM+) (D) of neuronal-committed progenitors.*

concentrations in their most postnatal cells, inhibits on the contrary mitotic activity[8, 16].

3.2 Role JAKs/STAT3-signaling in the regulation of the functions of the neuronal-committed progenitors

The stimulation of colony formation of PSA-NCAM+ cells and their proliferative activity was observed in violation of STAT3 phosphorylation (additions to the cultural environment of the STAT3 inhibitor) (Figure 1, 2). The identified phenomenon is of particular interest in the development of pharmacological approaches to stimulation of neurogenesis, as it is known that in some cases plays a key role in carcinogenesis. On this basis, the development of drugs based on blockers of this protein (STAT3) meets the requirements of carcinogenic safety, which is especially important in the creation of drugs for regenerative medicine (to minimize the risk of tumor transformation).

At the same time, the inactivation of JAKs, phosphorylating of the STAT proteins family[13, 17], caused a change only in the rate of differentiation of CFU-NPSA-NCAM+ (Figure 3). This fact was related to the selectivity of the Ruxolitinib used as a Pan-JAKs inhibitor. The Ruxolitinib has a pronounced blocking effect against JAK1, JAK2, and, to a lesser extent, JAK3, against TYK2 (the fourth representative of JAKs)[18]. Thus, JAK3 and/or TYK2 are likely to play a major role in activating STAT3 in the combined neuronal precursors, and the influence of Ruxolitinib on the intensity of the specialization of CFU-NPSA-NCAM+ is determined by the phosphorylation of JAK1 and JAK2 by other representatives of STAT proteins.

3.3 Role ERK1/2 and p38-signaling in the regulation of the functions of the neuronal-committed progenitors

Unambiguous results were obtained in the study of the participation of individual MAPK (mitogen-activated protein kinase) in the realization of the growth potential of the neuronal-committed progenitors. The ERK1/2 inhibitor and the p38 inhibitor reduced colony formation of PSA-NCAM+ cells (Figure 1) and CFU-NPSA-NCAM+ proliferative activity (Figure 2) against the backdrop of increasing maturation rate (Figure 3).

3.4 Role JNK/p53-signaling in the regulation of the functions of the neuronal-committed progenitors

The pharmacological blockade of JNK did not affect the output of CFU-NPSA-NCAM+, as well as the num-

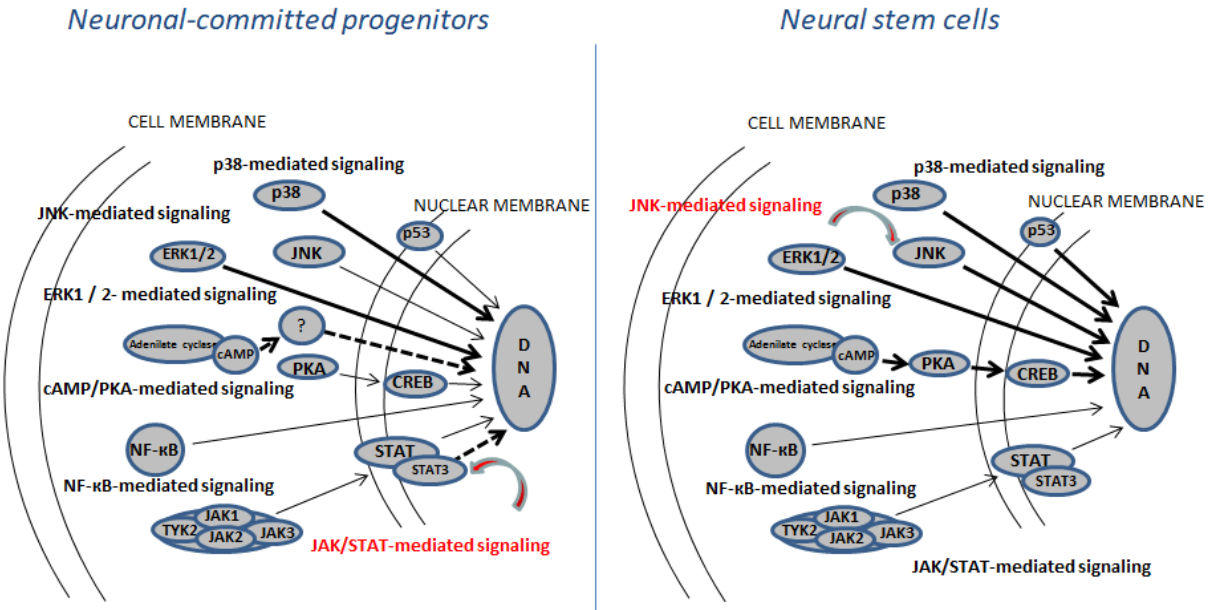


Figure 4. *The role of individual intracellular signaling pathways in the regulation of proliferative activity of neuronal-committed progenitors and neural stem cells.*
Arrows marked by a simple line indicate not involvement of the proteins in the regulation; arrows marked by a thick line indicate stimulation of proliferative activity; ***a****rrows marked by a dashed line indicate target inhibition of proliferative activity; curly (red) arrows show on prospective targets for drugs with neuroregenerative activity.*

ber of their mitotic-active cells and the intensity of differentiation (Figure 1, 2, 3).

Similar results were obtained in the inactivation of p53. There was no progression of the cell cycle. This circumstance undoubtedly has a significant scientific and theoretical interest and requires further study. The pro-apoptotic function of this protein and its role in preventing spontaneous carcinogenesis [19], for drug safety, make the study of the possibility of using p53 inhibitors for neuroregeneration unpromising.

3.5 Role NF-κB-signaling in the regulation of the functions of the neuronal-committed progenitors

The study of the participation of the nuclear transcription factor in the regulation of the functions of PSA-NCAM+ clonogenic cells did not reveal the effect of the NF-κB inhibitor on the parameters under study (Figure 1, 2, 3). The levels of colony formation, the amount of CFU-NPSA-NCAM+ in the S-phase of the cell cycle, and the intensity of cell differentiation were not different from those in the environment without the addition of the NF-κB inhibitor.

4. Discussion

Comparative analysis of the identified role of individual signaling molecules in the functioning of neuronal-committed progenitors with their role in multipotent neural SC, studied previously by our group[5, 6] (Figure 4), confirms the findings on the presence of specific features of intracellular signaling transduction in the regulation of different types of progenitors[3]. The results of the experiments indicate the existence of some fundamental differences in the par-

ticipation and role of individual signaling molecules in determining the proliferative and differential status of the neuronal-committed progenitors and NSC.

Previous studies have shown the same role as ERK1/2 and p38 in controlling the proliferative activity of NSC as that found in this study for neuronal-committed progenitor functions[5] (Figure 4). At the same time, the distinctive feature of the molecular mechanisms of the regulation of NSC functions in comparison with those in NCP is the lack of participation of STAT3 in their functions. Besides, cAMP/PKA/CREB-pathways have the opposite value in realizing the growth potential of NSC. The cAMP synthesis in NSC is accompanied by an increase in their functional activity[3, 16].

An important difference in the regulation of NSC functions is the stimulating role of JNK and p53 concerning the progression of the cell cycle. However, in the neuronal-committed progenitors, such a fact was not detected. At the same time, it is known that the main value of p53 in cells is to ensure the stability of their genome[19]. Therefore, from the point of view of drug safety, p53 in principle can not be considered as drug target for the therapy of neurodegenerative diseases.

5. Conclusion

Thus, the comparison of the role of individual signaling molecules in different types of progenitors of the nervous tissue demonstrates the prospect of developing novel neuroregenerative drugs, based on STAT3 inhibitors and JNK activators. These modifiers of signaling molecule activity can stimulate the realization of the growth potential of the neuronal-committed progenitors and NSC respectively. At the same time, the blockade of STAT3 does not have a "negative" effect on the functioning of multipotent progenitor cells, and the increase in the content of phosphorylated forms of JNK in NCP does not inhibit their proliferation and differentiation. Also, to date, it is believed that the blockage of STAT3 or activation of JNK is not able to reduce the neurotrophic function of neuroglia cells[9], the importance of which is difficult to overestimate for neuroregeneration[20]. □

Acknowledgments
The study was carried out with the financial support of the RFBR in the framework of scientific project No. 18-015-00013.

Conflict of interest
The authors declare no conflict of interest.

Abbreviations
NF-κB - nuclear factor kappaB, nuclear factor κ-light-chain-enhancer of activated B cells); JAK – Janus kinases; STAT – signal transducer and activator of transcription; MAPK - mitogen-activated protein kinases; ERK ½ - extracellular signal-regulated kinase; p38 - p38 mitogen-activated protein kinase; cAMP - cyclic adenosine monophosphate; PKA – protein kinase A; CREB – cAMP response element-binding protein; JNK - c-Jun N-terminal kinase; p53 –p53 tumor suppressor; CFU-NPSA-NCAM+ - colony-forming units of clonogenic PSA-NCAM+ cells; ClFU-NPSA-NCAM+ - cluster-forming units of clonogenic PSA-NCAM+ cells.

REFERENCES

1. Neal M, Richardson JR. Epigenetic regulation of astrocyte function in neuroinflammation and neurodegeneration. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 432-43, 2018.
2. Jellinger KA. Neuropathology and pathogenesis of extrapyramidal movement disorders: a critical update. II. Hyperkinetic disorders. *J. Neural. Transm. (Vienna)* 126, 997-1027, 2019.
3. Zyuz'kov G.N. Targeted Regulation of Intracellular Signal Transduction in Regeneration-Competent Cells: A new Direction for Therapy in Regenerative Medicine. *Biointerface Res. Appl. Chem.* 11, 12238-51, 2021.
4. Brick RM, Sun AX, Tuan RS. Neurotrophically Induced Mesenchymal Progenitor Cells Derived from Induced Pluripotent Stem Cells Enhance Neuritogenesis via Neurotrophin and Cytokine Production. *Stem Cells Transl. Med.* 7, 45-58, 2018.
5. Zyuz`kov GN, Zhdanov VV, Udut EV, Miroshnichenko LA, Polyakova TYu, Stavrova LA, Udut VV. Strategy of Pharmacological Regulation of Intracellular Signal Transduction in Regeneration-Competent Cells. *Bull. Exp. Biol. Med.* 166, 448-55, 2019.
6. Zyuz'kov GN, Zhdanov VV, Udut EV, Miroshnichenko LA, Polyakova TYu, Stavrova LA, Chaikovskii AV, Simanina EV, Minakova MY, Udu, VV. Peculiarities of Intracellular Signal Transduction in the Regulation of Functions of Mesenchymal, Neural, and Hematopoietic Progenitor Cells. *Bull. Exp. Biol. Med.* 167, 201-6, 2019.
7. Zyuz'kov GN, Miroshnichenko LA, Simanina EV, Polyakova TYu. Prospects for using mitogen-activated protein kinases ERK1/2 and p38 of nerve tissue progenitors as pharmacological targets for the treatment of neurodegeneration caused by alcohol. *Bull. Pharm. Sci.* 43, 217-24, 2020.
8. Mavers M, Ruderman EM, Perlman H. Intracellular signal pathways: potential for therapies. *Curr. Rheumatol. Rep*. 11, 378–85, 2009.
9. Zyuz'kov GN, Suslov NI, Miroshnichenko LA, Simanina EV, Polyakova TYu, Stavrova LA, Zhdanov VV, Minakova MYu, Udut EV, Udut VV. Halogenated (Cl-ion) songorine is a new original agonist of fibroblast growth factor receptors of neuronal-committed progenitors possessing neuroregenerative effect after cerebral ischemia and hypoxia in experimental animals. *Biointerface Res. Appl. Chem.* 9, 4317-26, 2019.
10. Kenakin T. Signaling bias in drug discovery. *Expert Opin. Drug Discov.* 12, 321-33, 2017.
11. Zyuz'kov GN, Stavrova LA, Miroshnichenko LA, Polyakova TYu, Simanina EV. Prospects for the Use of NF-κb Inhibitors to Stimulate the Functions of Regeneration-Competent Cells of Nerve Tissue and Neuroregeneration in Ethanol-Induced Neurodegeneration. *Biointerface Res. Appl. Chem.* 11, 8065 –74, 2021.
12. Seki T. Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. *J. Neurosci. Res*. 70, 327-34, 2002.
13. Zyuz'kov GN, Udut EV, Miroshnichenko LA, Polyakova TY, Simanina EV, Stavrova LA, Chaikovskii AV, Agafonov VI, Borodulina EV, Timofeev MS, Zyuz'kova YuN, Danilets MG, Zhdanov VV, Udut VV. Particular Role of JAK/STAT3 Signaling in Functional Control of Mesenchymal Progenitor Cells. *Bull. Exp. Biol. Med.* 164, 316-19, 2018.
14. Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA. Experimental design and analysis and theirreporting: new guidance for publication in BJP. *Br. J. Pharmacol.* 172, 3461–71, 2015.
15. Jauregui E, Du L, Gleason C, Poovaiah BW. Autophosphorylation of calcium/calmodulin-dependent protein kinase (CCaMK) at S343 or S344 generates an intramolecular

interaction blocking the CaM-binding. *Plant Signal. Behav.* 12, e1343779, 2017.
16. Mu Y, Lee SW, Gage FH. Signaling in adult neurogenesis. *Curr. Opin. Neurobiol*. 20, 416-23, 2010.
17. Zgheib A, Pelletier-Bonnier É, Levros LC Jr, Annabi B. Selective JAK/STAT3 signalling regulates transcription of colony stimulating factor-2 and -3 in Concanavalin-A-activated mesenchymal stromal cells. *Cytokine* 63, 187-93, 2013.
18. Heine A, Held SA, Daecke SN, Wallner S, Yajnanarayana SP, Kurts C, Wolf D, Brossart P. The JAK-inhibitor ruxolitinib impairs dendritic cell function in vitro and in vivo. *Blood* 122, 1192-202, 2013.
19. Karakostis K, Fåhraeus R. Shaping the regulation of the p53 mRNA tumour suppressor: the co-evolution of genetic signatures. *BMC Cancer* 19, 915, 2019.
20. Dzamba D, Harantova L, Butenko O, Anderova M. Glial Cells - The Key Elements of Alzheimer´s Disease. *Curr. Alzheimer. Res*. 13, 894-911, 2016.

Neuroprotective and Anti-apoptotic Activity of the IL-1 Antagonist RAIL-gel in Rats after Ketamine Anesthesia

Igor F. Belenichev[1], Bogdan S. Burlaka[2], Olga I. Ryzhenko[3], Victor P. Ryzhenko [4], Olena G. Aliyeva[5], Lyudmyla V. Makyeyeva*[5]

[1]Department of Pharmacology and Medical Formulation with Course of Normal Physiology, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

[2]Department of Technology of Drugs, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

[3]Department of Children Diseases, Faculty of Post-graduate Education, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

[4]Department of Medical and Pharmaceutical Informatics and Advanced Technologies, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

[5]Department of Histology, Cytology and Embryology, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

KEY WORDS:
IL-1 antagonist, Citicoline, Piracetam, Neuroprotective agents, c bcl-2 proteins, c fos proteins, neurons.

ABSTRACT

Introduction: The choice of medicines for total intravenous anesthesia remains a relevant issue in practical anesthesiology. Ketamine is a well-known drug that has been widely used in the world, however its' effect on the CNS is debatable. It is reasonable to question the use of neuroprotective agents to protect against the negative effects of general anesthesia. Some studies have shown the neuroprotective activity of the RAIL. A new dosage form of RAIL - a gel for intranasal administration has been developed. This study was designed to evaluate the neuroprotective and anti-apoptotic activity of RAIL-gel in comparison with Citicoline and Piracetam during ketamine anesthesia

Methods: In this study, 50 white nonlinear rats were randomly assigned to 5 groups: intact, ketamine anesthesia group, ketamine anesthesia + Piracetam (500 mg/kg, intraperitoneally) group, ketamine anesthesia + Citicoline (500 mg/kg, intraperitoneally) group, ketamine anesthesia + RAIL-gel (1 mg / kg intranasally) group. Expression of c-fos in the CA1 zone of the hippocampus and concentration of bcl-2 protein in the cytoplasmic fraction of the brain were determined by indirect immunofluorescence and Western blot analysis respectively.

Results: Our research demonstrated the neurodegradative effect of ketamine anesthesia. The use of neuroprotective agents (Piracetam, Citicoline, RAIL-gel) in rats after general anesthesia led to a decrease in the neurodegradative effect of ketamine. The neuroprotective effect of RAIL-gel was significantly higher compared to reference drugs ($p < 0.05$).

Conclusion: The neuroprotective effect of RAIL-gel is an experimental justification for further study of IL-1β RAIL antagonist as a potential neuroprotective agent.

***CORRESPONDING AUTHOR:**
Lyudmyla V. Makyeyeva
e-mail:
lyudmylamakyeyeva@gmail.com

Introduction

The choice of medicines for total intravenous anesthesia remains a relevant issue in practical anesthesiology for many years. Requirements for drugs that cause anesthesia are numerous and varied - rapid onset of anesthesia, high efficiency, controllability, safety and more, but in recent years, these criteria have been supplemented by the absence or minimal negative impact on the central nervous system (CNS)[1].

Ketamine is a well-known drug that has been widely used in the world since the 1970s. Its mechanism of action is associated with non-competitive N-methyl-D-aspartate receptors (NMDA receptor) antagonism, leading to functional disorganization of nonspecific midbrain and thalamic ligaments, causing dissociative anesthesia[2, 3]. Ketamine was developed as a safe alternative to phencyclidine, the neurotoxicity of which has been known and experimentally proven[4-10]. For many years, scientific debate about ketamine effect on the CNS is going on. Some authors in their articles report data on the neuroprotective properties of this drug, suggesting its use in order to reduce brain damage in acute CNS lesions[50], or discuss its neuroprotection at the cellular level[6]. Others have experimentally demonstrated that it causes neuroapoptosis and prolonged behavioral disorders in rodents of different age groups, including newborns[7,11]. The neurotoxic effect of ketamine is due to the expression of c-fos early responsive genes[8, 12]. Furthermore ketamine reduces the total antioxidant capacity and causes excessive formation of reactive oxygen species (ROS) and malondialdehyde[13], and apoptosis levels have been shown to correlate with the level of 3-methyladenine autophagy inhibitor[8].

NMDA receptors' hyperstimulation is known to cause excess Ca2+ influx, leading to free radical formation, activation of proteolytic processes and, ultimately, neuron necrosis. At the same time, moderate activation of NMDA receptors leads to oxidative stress and neuroapoptosis[14]. Therefore, ketamine, as a non-competitive antagonist of NMDA receptors, should not cause excitotoxicity, but rather cause a neuroprotective effect. However, it has been experimentally demonstrated that ketamine anesthesia causes pervasive developmental disorder in the form of anxiety and cognitive disorders associated with neuroapoptosis[9]. There is a strong evidence for dose-dependent apoptotic neurodegeneration caused by ketamine in the immature brain of mice[15]. The mechanism of neuroapoptosis is associated with the ketamine induced manifested expression of c-fos in the posterior cingulate gyrus and in the retrospinal cortex. Ketamine-induced expression is mediated not only through NMDA receptors but also through sigma-receptors[12]. Apoptosis is a programmed cell death with a complex pathogenesis. The regulation of apoptosis in the nervous system is carried out by numerous signaling systems. One way to implement this process is through the direct activation of early immediate response genes (c-jun, c-fos). C-fos protein acts as a regulator of transcription of a number of inducible genes and plays a significant role in cellular growth and differentiation processes and appears a recognized marker of neuronal cell activation[16]. One of the defense mechanisms is the production of anti-apoptotic modulators, one of which is the bcl-2 protein, which action is associated with the normalization of mitochondrial function. There is a mechanism of caspase1-dependent apoptosis of CA1 hippocampal neurons after ketamine anesthesia, associated with the exit of proapoptotic proteins from mitochondria. It is established that the active caspase-3 and -9 proteins, which are responsible for the release of cytochrome C and the mitochondrial translocation of p53, which is associated with mitochondrial apoptosis, are found to be significantly activated after a single administration of ketamine. Also, the introduction of ketamine increases the levels of pyroptosis-related proteins, including caspase-1 and -11, the NOD-like receptor family, the pyrin domain containing 3 (NLRP3), and IL-1β[17]. Anesthesia with ketamine or thiopental is also known to increase IL-1 and TNFα levels in the hippocampus 2 hours after anesthesia[18].

According to modern ideas, the nature of the immune response and the development of pathophysiological changes in neurodegradation, both ischemic and non-ischemic, depends on the predominant

activation of subpopulations of T-lymphocytes, their synthesis of various types of cytokines and the formation of a "cytokine cascade," namely, the ratio of pro-inflammatory and anti-inflammatory cytokines[19]. In case of brain tissue damage, activated microglia begin to produce pro-inflammatory cytokines, primarily IL-1β, which contributes to an increase in cerebral edema and increases the adherence to endothelium[20,21]. IL-1β can increase the expression of iNOS and stimulate the NO-mechanisms of neurodegradation. This induces neuroapoptosis, leads to delayed neuronal losses and a decrease in cognitive and mnestic functions of the CNS[9,17,19].

Many scientific papers and researches are devoted to the medico-social problem of cognitive impairment after anesthesia and surgery known as postoperative cognitive dysfunction (POCD). POCD is a cognitive disorder that develops in the postoperative period (may be delayed in time) and is associated with impaired higher cortical function, more commonly manifested as impairment of memory, speech, decreased attention spans, and more[22-24]. The most accurate, attention-grabbing study of the occurrence of POCD is the international randomized controlled trial of the ISPOCD1 study, published in 1998. According to the results of this study, cognitive dysfunction was diagnosed in 8% of patients 7 days after surgery and 9.9% 99 days after surgery[8], which indicates the prevalence of this problem in the world and the relevance of its study. The etiology and pathogenesis of POCD are complex and multifactorial. Risk factors include genetic and some somatic diseases as well as preoperative stress, but general anesthesia has been conclusively proven to be the major etiologic factor in the ISPOCD1 study[25]. In connection with these data, it is reasonable to question the use in everyday practice of effective and safe neuroprotective agents to face the negative effects of general anesthesia. These agents should act on the same systems that are suppressed by general anesthesia and interrupt the cascades of pathological reactions. According to the definition of The World Health Organization, nootropics are pharmacological agents that activate learning, improve memory and mental activity, while they increase the brain's resistance to aggressive hypoxia, trauma and intoxication. It may be justified to use the metabolite-tropic and endothelial protective drug Piracetam, which has a neuroprotective effect, as it has been proven in many studies[26,27]. Also, the drug Citicoline can be considered as a cerebroprotector, which inhibits the activation of phospholipases A1, A2, C and D, reduces the formation of free radicals, prevents the destruction of membrane systems, preserves antioxidant protective systems and inhibits apoptosis. Recently, studies have appeared on the cytokine mechanisms of the pathogenesis of anesthesia-induced neurodegradation[20,28]. Cytokine imbalance - the overproduction of IL-1β and the relative deficiency of its receptor antagonist IL-2 - has a great importance in brain damage. This allows us to consider IL-1 receptors as a promising target for neuroprotection in case of anesthesia-induced brain damage. Several studies have shown the neuroprotective activity of the interleukin-1 receptor antagonist (RAIL - IL-1 blocker) in experimental acute cerebrovascular accident, traumatic brain injury, and diabetic encephalopathy[19-21,28]. Recombinant RAIL-1 is a non-glycosylated analogue of an interleukin 1 which differs by one amino acid sequence in the N-terminal part from its native form. A new dosage form of RAIL - a gel for intranasal administration has been developed.

In this study, we first made attempts to interrupt the reaction of initiation of neuroapoptosis after ketamine, by the use of IL-1b antagonists - the active pharmacological agent RAIL in the form of an intranasal gel. The aim of the study was to evaluate the neuroprotective and anti-apoptotic activity of the IL-1 antagonist RAIL-gel in comparison with reference drugs Citicoline and Piracetam during ketamine anesthesia.

Materials and methods

Materials

Ketamine and Piracetam were purchased by PJSC FARMAK, Ukraine and Citicoline byTakeda, Japan. The substance RAIL was obtained from the Federal State Unitary Enterprise "State Scientific Research Institute of Highly Pure Biological Preparations" (Russia,

S-Petersburg, LSR-007452 / 1-0300710). Recombinant substances and RAIL in particular were obtained biotechnologically from microorganisms, protozoa and cereal. The test sample (RAIL) was obtained biotechnologically from E. coli TG1 (pTAC-hlL-1ra) and consists of 153 amino acids. Its molecular weight is 17, 906 kDa. RAIL-gel (5 mg / 1 ml) was developed at the Department of Technology of Drug of ZSMU. We also used paraplast (MkCormick, USA), bovine serum albumin solution (Sigma, USA, Cat. No. A2153), primary antibodies to the protein c-fos (Sigma Chemical, USA), secondary antibodies (fluorescent conjugated goat IgG - Sigma Chemical, USA), primary antibodies to bcl2 (Santa Cruz Biotechnology), solution of secondary antibodies (1: 1000) (biotinylated anti-mouse IgG, Sigma, USA, cat. No. 051M4885), ExtrAvidin-peroxidase solution (Sigma, USA, Cat. No. 051M4885), solution of AEC (1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. No. a6926), solution of NaCl 0.9% (Ukraine), 30% H2O2 (Ukraine).

Animal experiments

The study used 50 white nonlinear rats at the age of 6 months from the nursery of the GA «Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine». Weight of rats was 180-200 g. The acclimatization period was 14 days for all animals. The experimental studies were carried out in accordance with the "Regulations on the Use of Animals in Biomedical Research" and with the European Convention on the Protection of Animals Used for Scientific and Other Purposes. The experiment was approved by the Bioethics Committee of Zaporizhzhia State Medical University. Animals were kept under standard vivarium conditions with a change in the light cycle with an ambient temperature of 220 C. The manipulations were performed under ethanol-sodium anesthesia. Anesthesia was caused by intraperitoneal injection (IP injection) of ketamine at a dose of 100 mg/kg. Immediately after rats' emergence from anesthesia they were administered drugs at the following doses: Piracetam - 500 mg/kg, intraperitoneally, Citicoline - 500 mg/kg, intraperitoneally, RAIL-gel - 1 mg/kg intranasally using the dispenser pipette. The animals were divided into 5 groups (10 rats in each group): the first group - intact (control), the second - animals with experimental ketamine anesthesia. The third group - rats with ketamine anesthesia, which were administered Piracetam (PJSC FARMAK, Ukraine, 200 mg/ml) The fourth group received Citicoline (Takeda, Japan, 1000 mg / 4ml) after anesthesia. The fifth group received RAIL-gel after ketamine anesthesia. The intact group was injected intraperitoneally one time with a solution of NaCl 0.9% at the rate of 1 ml/0.1 kg, and the control group was administered the same dose of saline solution after ketamine anesthesia. The slaughter of rats was performed after 3 hours.

Instrumentation

Instruments used were the following: Buen clamp, rotary microtome Microm-325 (Microm Corp., Germany), refrigerated centrifuge Sigma 3-30k (Germany), fluorescence microscope Axioscop (Ziess, Germany), a high-sensitivity COHU4922 video camera (COCHU Inc., USA), HistoStar paraffin filling station (Thermo Fisher Scientific, USA), an automated PT-module (Thermo Scientific, USA), Red Line thermostats (Binder, Nimechchina), pH meter MP-220 (Mettler Toledo, Switzerland), installation for immunoblotting Mini Trans-Blot, Bio-Rad Laboratories (USA), thermostat TDB-120, torsion scales, set of batchers Bio HIT (Finland), Axiovision digital image analysis system (Carl Zeiss, Germany) with analysis programs and software development environment AZxioVision and Zen (Carl Zeiss, Germany).

Western blotting

The blood was quickly removed from the brain, separated from the meninges, and the test pieces were placed in liquid nitrogen. Then it was ground in liquid nitrogen to a powdery state and homogenized in a 10-fold volume of a medium at (2° C) containing (in mmol): sucrose - 250, Tris-HCl buffer - 20, EDTA -1 (pH 7.4). The mitochondrial fraction was isolated by differential centrifugation at a temperature +4° C. To purify the mitochondrial fraction from large cell

fragments, centrifugation was preliminarily carried out for 7 minutes at 1000g, and then the supernatant was re-centrifuged for 20 minutes at 17000g. The supernatant was decanted and stored at -80° C. The concentration in the cytoplasmic fraction of the brain bcl-2 was determined by Western blot analysis. Proteins were separated on a 10% polyacrylamide gel (PAGE). Separation of protein fractions was carried out by electrophoresis at a voltage of 100 V (for gel compaction), when the samples reached the interface between the gels - at a voltage of 200 V, until the samples reached the end of the gel. Proteins from the gel were transferred to a nitrocellulose membrane at a voltage of 100 V and a current of 0.35 A for 1 h. After transfer, the membrane was placed in a blocking buffer containing 1% bovine serum albumin solution for 20 hours. Washed on a shaker for 5 min in a solution of 0.1 M phosphate buffer (pH 7.4), the membrane was placed in a solution of primary antibodies against bcl-2 and incubated for 2 h at room temperature. Washed on a shaker 4 times for 5 minutes in 0.1 M phosphate buffer (pH 7.4). The membrane was placed in a solution of secondary antibodies (1: 1000), incubated for 2 hours. Washed on a shaker 4 times for 5 minutes in a solution of 0.1 M phosphate buffer. The membrane was placed in a solution of ExtrAvidin-peroxidase in 1% bovine serum albumin solution (1: 1000). Incubated for 1 hour and washed. For visualization, the membrane was treated with AEK solution: 1 tablet of 3-amino-9-ethylcarbazole dissolved in 2.5 ml of DMF containing 47.5 ml of 0.05M acetate buffer, pH 5.0, 25 μl 30% H2O2. The membrane was incubated in the substrate mixture for 5-10 min. A red insoluble precipitate characterizes the antigen-antibody complex in the blot. The membrane was washed in distilled water several times. The strips were dried between sheets of filter paper under a flow of cold air. Detection of bcl-2 was carried out using densitometry in the Adobe Photoshop program.

Immunohistochemical studies

To detect the expression of c-fos in the CA1 zone of the hippocampus, an immunohistochemical method of indirect immunofluorescence was used. For histochemical studies, the brain was fixed in Carnoy's fluid for 24 hours and then embedded in paraplast according to the standard scheme. On a rotary microtome Microm-325 (Microm Corp., Germany), 15-micron sections of the CA1 hippocampus were made, which were dewaxed according to a standard technique. Histological sections of the brain were isolated from the paraplast and rehydrated, washed three times for 5 minutes with phosphate buffer (pH = 7.4) and incubated with 2n hydrochloric acid (T = 37° C) for 30 minutes. Then, each was washed twice for 5 minutes with phosphate buffer (pH = 7.4), twice for 5 minutes with borate buffer according to Holmes (pH = 8.4) and four times for 5 minutes with phosphate buffer (pH = 7.4), ~~after which incubated~~ followed by incubation for 30 minutes with 0.1% trypsin solution in phosphate buffer (T = 37° C). After incubation, the sections were washed four times for 5 minutes with phosphate buffer (pH = 7.4). First, primary antibodies to the c-fos protein were applied to the sections and incubated at +40° C for 24 hours. After incubation, the sections were washed three times with 0.1 M phosphate buffer. Then, secondary antibodies (fluorescent conjugated goat IgG) were applied to the samples and incubated at room temperature for 60 min. After incubation, the sections were washed with 0.1 M phosphate buffer (pH = 7.4). After the final four-fold washing with phosphate buffer, the sections were embedded in a mixture of glycerol-phosphate buffer (9:1). Fos-immunopositive neurons were examined using a fluorescence microscope. The image of fos-immunopositive neurons of the CA1-zone of the hippocampus, obtained on a microscope, using a highly sensitive video camera was introduced into the computer hardware and software system for digital image analysis VIDAS.

Statistical analysis

Statistical analysis was performed using the standard statistical package "STATISTICA® for Windows 6.0" (StatSoftInc., No. AXXR712D833214FAN5), "SPSS 16.0" and "Microsoft Office Excel 2003". The normality of distribution was evaluated by the Shapiro-Wilk criterion. When the results were consistent with the

Table 1: Density of c-fos-positive cells in the brain of rats with ketamine anesthesia under the influence of Piracetam, Citicoline and RAIL-gel (M ± m, n = 10)

Groups of animals	Density of c-fos-positive cells
Intact animals (n=10)	12.9 ± 2.0
Control – animals with ketamine anesthesia(n=10)	127.4 ± 5.0*
Animals with ketamine anesthesia +Piracetam(n=10)	116.4 ± 5.0*
Animals with ketamine anesthesia +Citicoline(n=10)	109.6 ± 7.6* [1]
Animals with ketamine anesthesia +RAIL-gel(n=10)	91.1 ± 4.6* [1,2]

**- Differences are significant at p < 0.05 compared to intact group; 1- Differences are significant at p < 0.05 compared to control group; 2- Differences are significant at p < 0.05 compared to group treated with Piracetam; n – number of animals in the group.*

Table 2: Concentration of bcl-2 protein in the brain of rats with ketamine anesthesia under the influence of Piracetam, Citicoline and RAIL-gel (M ± m, n = 10)

Groups of animals	Concentration of bcl-2 protein
Intact animals(n=10)	45.2 ± 2.3
Control – animals with ketamine anesthesia(n=10)	23.2 ± 2.3*
Animals with ketamine anesthesia + Piracetam(n=10)	24.4 ± 1.6*
Animals with ketamine anesthesia + Citicoline(n=10)	32.6 ± 4.8* [1,2]
Animals with ketamine anesthesia + RAIL-gel(n=10)	38.2 ± 3.9* [1,2]

**- Differences are significant at p < 0.05 compared to intact group; 1- Differences are significant at p < 0.05 compared to control group; 2- Differences are significant at p < 0.05 compared to group treated with Piracetam; n – number of animals in the group.*

law of normal distribution of the trait, the reliability was estimated by the Student's t-test. In the case of a distribution other than the normal one, the U Mann-Whitney criterion was used. For comparison of independent variables in more than two samples, ANOVA for normal distribution or Kruskal-Wallis criterion for non-normal distribution was used. P-values less than 0.05 (*p < 0.05) were considered statistically significant for all types of analysis.

Results and Discussion

As a result of the study, it was found that ketamine anesthesia is accompanied by the onset of signs of apoptosis of CA1 hippocampus neurons. These findings confirm the apoptotic effect of ketamine anesthesia by the increased expression of c-fos proteins (**Table 1**). The c-fos expression in the CA1 zone of the brain hippocampus increased by almost 10 times (p <0.01) in the control group of rats, treated with ketamine anesthesia compared with the intact group, indicating the activation of neuroapoptosis. In addition Ketamine significantly reduces the body's anti-apoptotic protection by inhibiting the bcl-2 protein in the brain (**Table 2**) and in combination with the above mentioned, increase in the expression of the c-fos rapid response protein in the CA1 area of the hippocampus, leads to programmed neuronal death. The use of neuroprotective drugs is considered to prevent the occurrence of cascades of such pathological reactions, reducing the expression of early response genes and activating anti-apoptotic protective mechanisms. Indeed, there was a decrease in the number of c-fos-positive neurons in rats treated with

piracetam as a neuroprotective agent after ketamine anesthesia by 8.6% (p <0.05), compared to the control group. There was a 14% (p <0.05) decrease in the amount of c-fos compared to controls in the group of animals with ketamine anesthesia treated with Citicoline, and a 25.5% (p <0.05) decrease in animals treated with RAIL-gel compared to the control group. Thus, the best stabilizing effect on the c-fos protein level in the CA1 zone of the rat brain hippocampus was demonstrated by RAIL-gel, which exceeded the anti-apoptotic effect of Citicoline by 11.5% and Piracetam by 16.9%. The effect of these neuroprotective agents on the level of anti-apoptotic protein bcl-2 is shown in Table 2. After ketamine anesthesia a decrease in bcl-2 level was observed in the control group by 48.7% (p <0.01), compared with the intact group. In rats treated with neuroprotective agents after ketamine anesthesia, the following results were observed:

The amount of bcl-2 protein decreased by 46% in the rat brain upon the use of Piracetam compared to the intact group, while it was 5% higher compared to the control group.

The decrease in amount of bcl-2 protein was 27.9% in the group of rats treated with Citicoline compared to the intact group, while its concentration was 28.8 higher compared to the control group.

- animals receiving RAIL-gel after ketamine showed a 15,4% decrease in the amount of bcl-2 in the homogenate compared to intact, and a 39.2% increase in its concentration compared to the control group.

The RAIL-gel showed the best results, both in reducing the density of c-fos-positive neurons and in the storage of anti-apoptotic protein bcl-2 under conditions of ketamine anesthesia compared to the nootropics piracetam and citicoline. These findings provide experimental justification for further in-depth study of RAIL as a potential neuroprotective agent in order to prevent the negative consequences of general ketamine anesthesia.

As shown in the study the anti-apoptotic and neuroprotective effect of RAIL-gel in ketamine-induced neurodegradation is associated with its ability to interrupt IL-1β-dependent mechanisms of neuroapoptosis. Namely, the RAIL-gel inhibits IL-1-dependent expression of iNOS in glial cells and reduces NO overproduction, inhibits the exit of mitochondria proapoptotic proteins. RAIL-gel can inhibit the expression of redox-sensitive apoptosis genes of early response, mainly JunD and c-fos, due to a decrease of NO levels [21,28]. Citicoline can have an anti-apoptotic and neuroprotective effect in case of anesthesia-induced brain damage due to the presence of a pronounced mitoprotective effect, which may underlie its anti-apoptotic activity. Apparently, Citicoline modulates the activity of cyclosporin A-dependent mitochondrial pore during ischemia and terminates the release of proapoptotic factors through it.

It has been shown that Citicoline can maintain the integrity of the inner mitochondrial membrane. A similar mechanism is associated with the restoration of cardiolipin levels in the inner mitochondrial membrane. In addition, it was found that citicoline indirectly, by increasing the activity of glutathione-linked enzymes (glutathione reductase and glutathione transferase), regulates the level of reduced glutathione and, thus, can reduce the level of cytoxic derivatives of NO[19,20,29-31]. Citicoline's ability to inhibit apoptosis by reducing the expression of procaspase is also known[31]. Piracetam did not show significant anti-apoptotic and neuroprotective effects in ketamine-induced neurodegradation. The mechanism of action of this nootropic is associated with the activation of anaerobic pathways of ATP synthesis in the brain, an increase in the level of acetylcholine and the concentration of n-cholinergic receptors. The membrane stabilizing and stress-protective properties of Piracetam are known. It is proven that Piracetam is ineffective in extreme conditions of the central nervous system, it can enhance lactic acidosis during cerebral ischemia. We found that Piracetam enhances anxiety and does not affect cognitive-mnestic disorders after ketamine anesthesia[9,20,26-29,38].

Conclusions:

The neurodegradative effect of ketamine anesthesia (100 mg / kg) was demonstrated in the reproduced experiment by activating apoptosis processes (in-

creased density of c-fos positive cells in the CA1 area of the hippocampus) and inhibiting the organism protective capacity (decreased amount of bcl-2 protein in the brain).

The use of the neuroprotective agents such as Piracetam, Citicoline, and RAIL-gel in rats treated with general anesthesia was found to lead to a decrease of the neurodegradative effect of ketamine, as reflected in a decrease in the concentration of c-fos protein and an increase in the expression of bcl-2 protein.

The best neuroprotective effect was demonstrated by the RAIL-gel. It had the most significant effect on the expression of c-fos and bcl-2 proteins, reducing the first one and activating the second one, compared to other drugs. The second most effective drug is Citicoline, followed by Piracetam. □

Ethical considerations

All investigations conformed to the ethical of research and were approved by the Bioethics Committee of Zaporizhzhia State Medical University (prot. No. 2 of 04/14/2015) and the authors of this manuscript observed ethical issues. Animals were handled according to the International Guidelines for Care and Handling of Experimental Animals.

Funding/Support
This research was financially supported by Zaporizhzhia State Medical University (Grant No. 2301020).

ORCID and contributionship:
Igor F. Belenichev: 0000-0003-1273-5314[A, E, F]
Bogdan S. Burlaka: 0000-0003-4539-7331[B, C, D]
Victor P. Ryzhenko: 0000-0003-3466-7148[E, F]
Olena G. Aliyeva: 0000-0003-1287-674X[B, D]
Lyudmyla V. Makyeyeva: 0000-0002-3188-2638[B, D]
Olga I.Ryzhenko:https://scholar.google.com.ua/citations?user=yV9ItZ8AAAAJ&hl=ruC, [D]
[A] - Work concept and design,
[B] – Data collection and analysis,
[C] – Responsibility for statistical analysis,
[D] – Writing the article,
[E] – Critical review,
[F] – Final approval of the article

Conflict of interest:
The Authors declare no conflict of interest.

REFERENCES

1. Anderson B.J., Bagshaw O. Practicalities of total intravenous anesthesia and target-controlled infusion in children. *Anesthesiology* 131, 164–185, 2019. doi:https://doi.org/10.1097/ALN.0000000000002657
2. Liu F, Patterson T.A., Sadovova N., et al. Ketamine-induced neuronal damage and altered N-methyl-D-aspartate receptor function in rat primary forebrain culture. *Toxicol Sci.* 131(2), 548-57, 2013. doi: 10.1093/toxsci/kfs296
3. Zhang Z., Liu W., Shen M., et al. Protective effect of GM1 attenuates hippocampus and cortex apoptosis after ketamine exposure in neonatal rat via PI3K/AKT/GSK3β Pathway. *Mol Neurobiol.* 2021 doi: 10.1007/s12035-021-02346-5.
4. Olney J.W, Labruyere J., Price M.T. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* 244(4910), 1360-62, 1989. doi: 10.1126/science.2660263
5. Bell J.D. In Vogue: Ketamine for Neuroprotection in Acute Neurologic Injury. *Anesth. Analg.*124(4),1237-43, 2017. doi: 10.1213/ANE.0000000000001856
6. Pfenninger E., Himmelseher S. Neuroprotection by ketamine at the cellular level. *Anaesthesist* 46, 47-54, 1997. doi: 10.1007/pl00002465.
7. Perouansky M. Neurotoxicity of general anes-

thetics. *Anesthesiology* 111(6), 1365-71, 2009. doi: 10.1097/ALN.0b013e3181bf1d61

8. Li X., Li Y., Zhao J., et al. Administration of Ketamine Causes Autophagy and Apoptosis in the Rat Fetal Hippocampus and in PC12 Cells. *Front Cell Neurosci.* 12, 21, 2018. doi: 10.3389/fncel.2018.00021
9. Belenichev I., Burlaka B., Puzyrenko A., et al Management of amnestic and behavioral disorders after ketamine anesthesia. *Georgian Med News* 294, 141-5, 2019
10. Orhurhu V.J., Vashisht R., Claus L.E., Cohen S.P. (2020)Ketamine Toxicity. In: StatPearls. Treasure Island (FL): StatPearls Publishing,
11. Wang Q., Shen F.Y., Zou R., et al. Ketamine-induced apoptosis in the mouse cerebral cortex follows similar characteristic of physiological apoptosis and can be regulated by neuronal activity. *Front Cell Neurosci.* 12, 21, 2018. doi: 10.3389/fncel.2018.00021
12. Nakao S., Miyamoto E., Masuzawa M., et al. Ketamine-induced c-Fos expression in the mouse posterior cingulate and retrosplenial cortices is mediated not only via NMDA receptors but also via sigma receptors. *Brain Res.* 926(1-2), 191-6, 2002. doi: 10.1016/s0006-8993(01)03338-8
13. Abdel-Salam O.M.E., Youness E.R., Mohammed N.A. et al Effect of ketamine on oxidative stress following lipopolysaccharide administration. *Comp.Clin. Pathol*. 24, 53–63, 2015. doi: 10.1007/s00580-013-1854-x
14. Lipton S.A. Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat.Rev.* 5, 161-70, 2006. doi: 10.1038/nrd1958
15. Young C., Jevtovic-Todorovic V., Qin Y.Q., et al. Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. *Br.J.Pharmacol.* 146, 189–97, 2005. doi: 10.1038/sj.bjp.0706301
16. Heuer K.H., Mackay J.P., Podzebenko P., et al. Development of a sensitive peptide-based immunoassay: application to detection of the Jun and Fos oncoproteins. *Biochemistry* 35(28), 9069-75, 1996. doi: 10.1021/bi952817o
17. Ye Z., Li Q., Guo Q., et al. Ketamine induces hippocampal apoptosis through a mechanism associated with the caspase-1 dependent pyroptosis. *Neuropharmacology* 128, 63-75, 2018. doi: 10.1016/j.neuropharm.2017.09.035
18. Li Y., Shen R., Wen G., et al. Effects of Ketamine on Levels of Inflammatory Cytokines IL-6, IL-1β, and TNF-α in the Hippocampus of Mice Following Acute or Chronic Administration. *Front. Pharmacol.* 20(8), 139-47, 2017. doi: 10.3389/fphar.2017.00139
19. Belenichev I.F., Cherniy V.I., Buhtiyarova N.V. (2009) Rachional'naia neyiroprotekchiya [Rational neuroprotection]. *Donets'k: Zasslavkiy* 260p. (in Russian)
20. Belenichev I.F., Nagornaya E.A., Buhtiyarova N.V. (2015) Neyroptotekchiya I neiroplastichnost' [Neuroprotection and neuroplasticity]. *Kiev: Logos* 512 p. (in Russian)
21. Belenichev I.F., Suprun E.V., Gromov L.A. IL-1Ra stabilises the thiol–disulfide system in the brain tissues of rats with experimental diabetes and cerebral ischemia. *27Th European ConGRESS (ECNP) Basic and clinical neuroscience – Neuropharmacology* 236, 2014
22. Martins S., Fernandes L. Delirium in Elderly People *Front.Neurol.* 3, 101, 2012. doi: 10.3389/fneur.2012.00101
23. Ntalouka MP, Arnaoutoglou E, Tzimas P Postoperative cognitive disorders: an update *Hippokratia* 22(4), 147–154, 2018
24. Kotekar N., Shenkar A., Nagaraj R. Postoperative cognitive dysfunction – current preventive strategies. *Clin.Interv.Aging*. 13, 2267-2273, 2018. https://doi.org/10.2147/CIA.S133896
25. Moller J.T., Cluitmans P., Rasmussen L.S., et al. Long-term postoperative cognitive dysfunction in the elderly. *The Lancet* 351, 1742, 1998. doi: 10.1016/s0140-6736(97)07382-0
26. Mazur I.A., Chekman I.S., Belenichev I.F. (2007) Metabolitotropnye preparaty [Metabolite drugs]. *Zaporizhzhia* 309 p. (in Russian)
27. Belenichev I.F., Gorbacheva S.V., Bukhtiyarova

N.V. The Thiol-Disulfide Balance and the Nitric Oxide System in the Brain Tissue of Rats Subjected to Experimental Acute Impairment of Cerebral Blood Flow: The Therapeutic Effects of Nootropic Drugs. *Neurochem.J.* 8(1), 24-7, 2014. doi: 10.1134/S181971241401005X

28. Suprun E.V. (2011) Cytokine mechanisms of pathogenesis and experimental therapy of acute brain blood circulation. *Dissertation for achieving a scientific degree of Doctor of Medical Sciences Kiev* 348p.
29. Citicoline (Monograph) *Alternative Medicine Review* 13(1), 50-7, 2008
30. Adibhatla R.M., Hatcher J.F., Dempsey R.J. Citicoline: neuroprotective mechanisms in cerebral ischemia. *J. Neurochem.* 80(1), 12-23, 2002. doi: 10.1046/j.0022-3042.2001.00697.x
31. Alvarez-Sabín J., Román G.C. The Role of Citicoline in Neuroprotection and Neurorepair in Ischemic Stroke. *Brain Sci.* 3, 1395-414, 2013. doi: 10.3390/brainsci3031395
32. Burchinskiy S.G. Piracetam: novye otkritiya – novie vozmozhnosti v klinicheskoy praktike [Piracetam: new discoveries - new opportunities in clinical practice]. *Nevrologiya* 1, 42-4, 2013 (in Russian)

Lc-MS/MS Method Development for the Quantitative Determination of Valsartan from Caco-2 Cell Monolayers: Application to Permeability Assay

Kateryna Peleshok, Olha Poliak, Nadiya Zarivna, Oleksandra Oleshchuk, Uliana Mudryk, Vitaliy Hlushok, Andriy Sverstiuk, Olga Svan, Nataliia Terenda, Andriy Makhnitskyy, Olha Yaremchuk, Liliya Logoyda

I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ:
LC-MS/MS; Valsartan; Caco-2 cells; Recovery; Permeability

***CORRESPONDING AUTHOR:**
Liliya Logoyda -
I. Horbachevsky Ternopil National Medical University, Ukraine,
Ternopil, Ruska, 36
email: logojda@tdmu.edu.ua

ABSTRACT

Introduction Valsartan is widely used in the treatment of hypertension and shows different Biopharmaceutics Classification System (BCS) in the literature (BCS class II or III). LC-MS/MS method development is very important role in procedure for determining permeability in Caco-2 cell monolayers and plays a key role in the reliability of the results. **The aim of the study** was to develop an efficient LC-MS/MS method for determination of valsartan from Caco-2 cell monolayers. **Research methods.** Chromatography was achieved on Phenomenex Luna, 50 × 2.0 mm, 5 µm column. Samples were chromatographed in a gradient mode. Eluent A: acetonitrile – water, 5 : 95, v/v, eluent B: acetonitrile. Formic acid 0.1 v/v was added in both eluent A and eluent B. The initial content of eluent B is 15%, which linearly increases to 100% in 0.9 min and up to 1.2 min is 100% from 1.21 min returns to the original 15%. The mobile phase was delivered at a flow rate of 0.400 mL/min into the mass spectrometer Electron Spray Ionization (ESI) chamber. The sample volume was 3 µl. **Results and discussion.** Under the conditions used, the peak of valsartan is eluted for about 1.23 min. The total chromatographic run time is 1.5 min, so the developed analytical method for the determination of valsartan to study intestinal permeability in the model of the Caso-2 test is rapid. Valsartan showed low permeability and efflux by p-glycoprotein (P-gp). A decrease in the efflux coefficient in the presence of verapamil indicates that valsartan is a substrate of the P-gp transporter. Recovery value of valsartan is 94 % and this indicates that the results of the experiment are reliable. **Conclusions.** A rapid, simple and low cost LC-MS/MS method was developed for determination of valsartan from Caco-2 cell monolayers. Statistical analysis proves that the method is reproducible and selective for the estimation of valsartan.

1. Introduction

The procedure «biowaver» based on Biopharmaceutics Classification System (BCS) is designed to confirm the bioequivalence between generic and reference drugs. BCS is an important tool which uses *in vitro* results for comparison with bioavailability *in vivo* (biowaiver)[1]. These tests are used to establish bioequivalence in applications for generic drugs, for changes that require re-registration of innovative drugs, for changes that may be during the validity of the registration certificate, requiring bioequivalence studies, as well as between drugs used in clinical trials, and drugs that will be produced on an industrial scale and are on the pharmaceutical market. The two parameters that are analyzed for API for the possibility of the procedure «biowaver» are solubility and permeability . Methods that make it possible to assess the degree of permeability of API include: *in situ* studies (in the small intestine of rats), *in vitro* models (on monolayers of different cell lines), *in silico* models (using calculated partition coefficient log P or distribution coefficients logD)[2]. The results obtained by *in situ* studies are highly reliable, but these studies are quite expensive and therefore can not be used for routine or screening purposes. Among the *in vitro* models (on monolayers of different cell lines), the most widely used is the culture of adenocarcinoma of the large intestine - Caco-2[3-11]. The generalized procedure for determining the permeability of Caco-2 cell monolayers is presented in Fig. 1.

Assays of compounds have been replaced in most laboratories by the use of liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS/MS). It suppose reduces the amount of compounds but permits the simultaneous measurement of multiple compounds. The measurement of multiple compounds per assay reduces the number of incubations that need to be carried out, thus increasing the throughput of the experiments. Furthermore, LC-MS and LC-MS-MS add another dimension to Caco-2 assays by enabling the investigation of the metabolism of compounds by Caco-2 cells. LC-MS/MS method development is very important role in procedure for determining permeability in Caco-2 cell monolayers and plays a key role in the reliability of the results.

Pgp is a transmembrane protein that belongs to the superfamily of ABC transporters and acts as an energy-dependent efflux pump to transport substances across membranes[12, 13].

Valsartan is widely used in the treatment of hypertension and shows different BCS classification in the literature (BCS class II or III)[14]. Therefore permeability studies for this drug are still necessary.

The aim of this study was to develop an efficient rapid and low cost LC-MS/MS method for determination of valsartan from Caco-2 cell monolayers. The effect of verapamil, which is a well known inhibitor of p-glycoprotein (P-gp) was investigated.

2. Methods

Chemicals and reagents

Valsartan (purity 99.9%) was purchased from Sigma-Aldrich (Switzerland).

The following materials were used in the experiments: Trypsin. EDTA (10x) 0.5% / 0.2% in DPBS (PAA, UK; Cat L11-003), HEPES, High Purity Grade (Helicon, Am-0485), Dulbecco's PBS (1x) without Ca & Mg (PAA, UK; Cat H15-002), Hanks' BSS (1x) without Ca & Mg without Phenol Red (PAA, UK; Cat H15-009), DMSO Chromasolv Plus, HPLC grade, ≥99.7% (Sigma-Aldrich, USA; Cat 34869), DMEM (4.5g/l) liquid without L-Glutamine (PAA, UK; Cat E15-009), L-Glutamine (200 mM) (PAA, UK; Cat M11-004), Fetal Bovine Serum «GOLD» EU approved (PAA, UK; Cat A15-151), Penicillin/Streptomycin (100x) (PAA, UK; Cat P11-010), Acetonitrile Chromasolv gradient grade for HLC (>99.9 %) (Sigma-Aldrich, USA; Cat 34851), Formic acid for mass sectrometry 98 % (Fluka, USA; Cat 94318),

Propranolol hydrochloride ≥99% (TLC) (Sigma-Aldrich, USA), quinidine anhydrous ≥99% (TLC) (Sigma-Aldrich, USA), verapamil hydrochloride ≥99% (TLC) (Sigma-Aldrich, USA) and atenolol, ana-

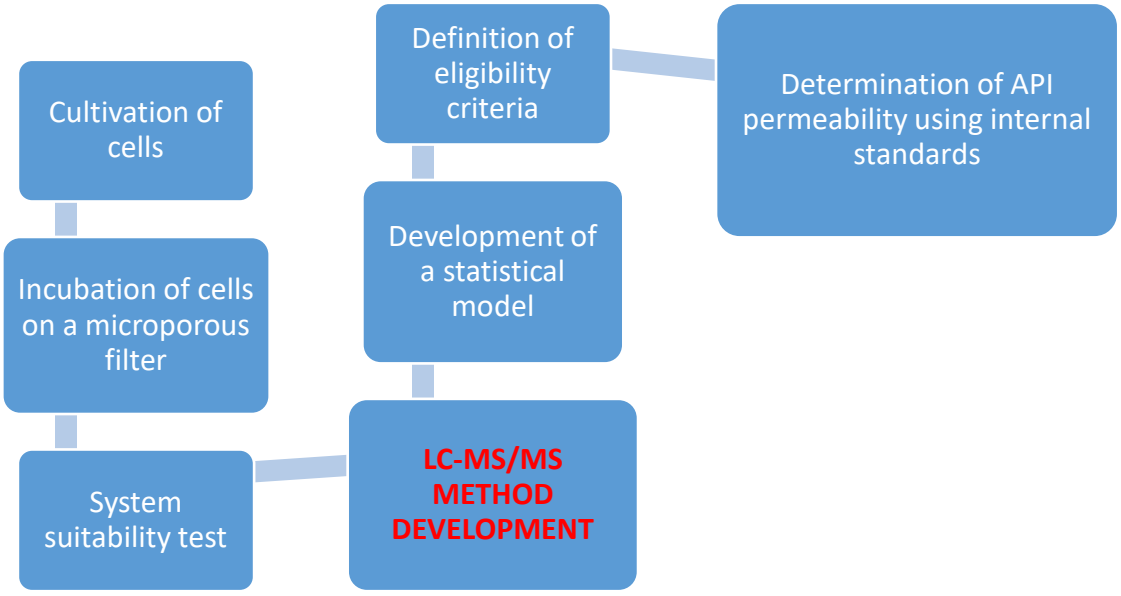


Figure 1: *Procedure for determining permeability in Caco-2 cell monolayers*

Table 1: Chromatographic conditions

Parameter	Chromatographic conditions
Equipment	Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-14A), binary pump (LC-20ADXR) along with auto-sampler (SIL-20ACXR)
Column	Phenomenex Luna, 50 × 2.0 mm, 5 µm
Mobile phase	Gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile–formic acid, 100 : 0.1 v/v)). The initial content of eluent B is 15%, which linearly increases to 100% in 0.9 min and up to 1.2 min is 100% from 1.21 min returns to the original 15%.
Flow rate	0.4 mL/min
Runtime	1,5 min
Column temperature	30° C
Volume of injection loop	3 µl

lytical reference material, ≥98.5% (HPLC) were used as reference compounds.

Instrumentation and chromatographic conditions

All measurements were performed using Shimadzu VP HPLC system including vacuum degasser, gradient pumps, column oven and autosampler. The LC system was coupled with tandem mass spectrometer API 3000 (PE Sciex). The TurboIonSpray ion source was used in both positive and negative ion modes. Parameters of electrospray ionizer and MRM

Table 2: Parameters of electrospray ionization

	Parameter	Value
1	Polarity	Positive
2	Nebulizer Gas (NEB, Gas 1)	15
3	Curtain Gas (CUR)	8
4	Collision Gas (HCD)	5
5	IonSpray Voltage (IS)	5000
6	Temperature (TEM)	500
7	Turbo IonSpray Gas	8
8	Horizontal Position	5.0
9	Lateral Position	5.0

parametrs are listed in Table 1. Acquisition and analysis of the data were performed using Analyst 1.5.2 software (PE Sciex).

Stationary phase was a reversed –phase column Phenomenex Luna, 50 × 2.0 mm, 5 μm. Samples were chromatographed in a gradient mode using the following conditions.

Eluent A: acetonitrile – water, 5 : 95, v/v, eluent B: acetonitrile. Formic acid 0.1 v/v was added in both eluent A and eluent B. The initial content of eluent B is 15%, which linearly increases to 100% in 0.9 min and up to 1.2 min is 100% from 1.21 min returns to the original 15%. The mobile phase was delivered at a flow rate of 0.400 mL/min into the mass spectrometer Electron Spray Ionization (ESI) chamber. The sample volume was 3 μl.

The chromatographic conditions are summarized in Table 1, ESI parameter in Table 2, multiple reaction monitoring (MRM) parameters of valsartan in Table 3.

n=3

Cultivation of Caco-2 cells

Caco-2 cells were cultivated in 75 cm^2 flasks to 70-80% of confluence according to the ATCC and Millipore recommendations in humidified atmosphere at 37°C and 5% CO_2[15]. Cells were detached with Trypsin/EDTA solution and resuspended in the cell culture medium to a final concentration of 2x105 cells/ml. 500 μl of the cell suspension was added to each well of HTS 24-Multiwell Insert System and 35 ml of prewarmed complete medium was added to the feeder tray. Caco-2 cells were incubated in Multiwell Insert System for 21 days before the transport experiments. The medium in filter plate and feeder tray was changed every other day. After 21 days of cell growth, the integrity of the monolayer was verified by measuring the transepithelial electrical resistance (TEER) for every well using the Millicell-ERS system ohm meter. The final TEER values were within the range 150-600 Ω×cm2 as required for the assay conditions. 24-well insert plate was removed from its feeder plate and placed in a new sterile 24-well transport analysis plate. The medium was aspirated and inserts washed with PBS twice.

Procedure

To determine the rate of compounds transport in apical (A) to basolateral (B) direction, 300 μL of the test compound dissolved in transport buffer at 10 μM (HBSS, 10 mM HEPES, pH=7.4) was added into the filter wells; 1000μL of buffer (HBSS, 10 mM HEPES, pH=7.4) was added to transport analysis plate wells. The plates were incubated for 90 min at 37°C with shaking at 100 RPM. 75 μL aliquots were taken from the donor and receiver compartments for LC-MS/MS analysis. All samples were mixed with 2 volumes of acetonitrile with following protein sedimentation by centrifuging at 10000 rpm for 10 minutes. Supernatants were analyzed using the LC system coupled with tandem mass spectrometer. Propranolol (high permeability), atenolol (low permeability) and quinidine (moderate permeability) were used as reference compounds.

Permeability coefficient (Papp) was calculated for Caco-2 permeability assay using equation 1:

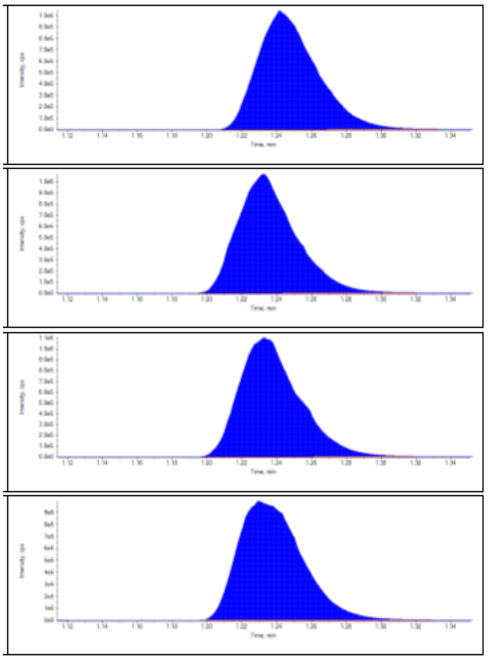


Figure 2: *Typical multiple reaction monitoring chromatograms of valsartan*

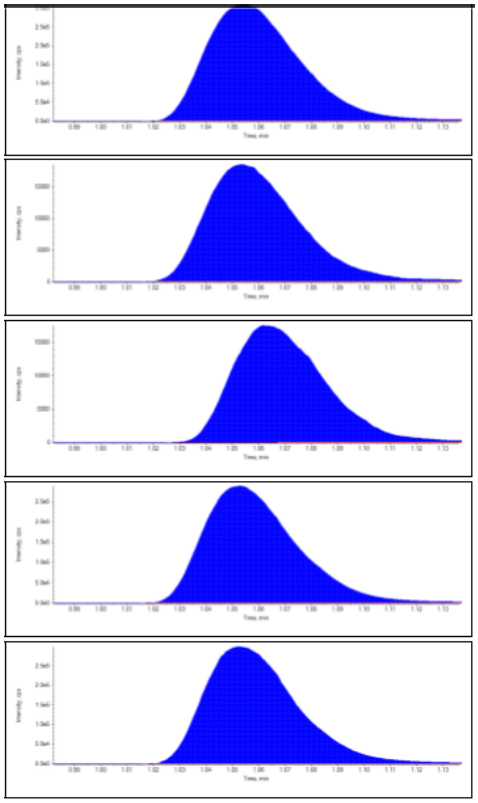


Figure 3: *Typical multiple reaction monitoring chromatograms of propranolol*

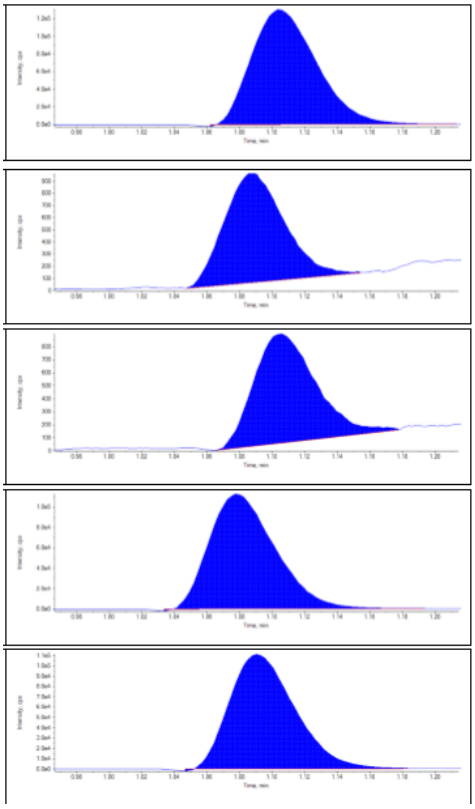


Figure 4: *Typical multiple reaction monitoring chromatograms of atenolol*

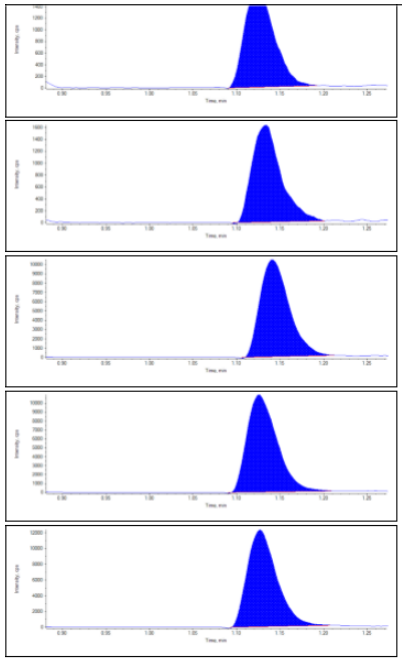


Figure 5: *Typical multiple reaction monitoring chromatograms of quinidine*

Table 3: Multiple reaction monitoring (MRM) parameters of valsartan

Analyte	Parent, m/z	Daughter, m/z	Time, ms	DP, V	EP, V	CE, V	CXP, V
Valsartan	436.1	235.2	120	36	10	27	10

** Abbreviations: DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential*

Table 4: Permeability coefficient (Papp) of valsartan through the monolayer of Caco-2 cells in the apical-basolateral and basolateral-apical directions

Analyte	Papp (AB), 10^{-6} cm/sec				Papp (BA), 10^{-6} cm/sec				Efflux coefficient
	1	2	M	m	1	2	M	m	
Propranolol	17.3	21.4	19.3	2.9	11.6	10.9	11.2	0.5	0.6
Atenolol	0.2	0.5	0.3	0.2	1.2	1.2	1.2	0.0	3.5
Quinidine	5.0	9.5	7.3	3.2	36.8	35.7	36.3	0.8	5.0
Valsartan	0.3	0.3	0.3	0.0	0.9	0.8	0.8	0.1	2.9

M is the arithmetic mean value of the permeability coefficient; m is the standard deviation.

Table 5: Permeability coefficient (Papp) of valsartan through the monolayer of Caco-2 cells in the presence of the P-gp inhibitor verapamil

Analyte	Papp (AB), 10^{-6} cm/sec				Papp (BA), 10^{-6} cm/sec				Efflux coefficient
	1	2	M	m	1	2	M	m	
Quinidine	25.1	23.6	24.4	1.1	27.6	26.2	26.9	1.0	1.1
Valsartan	0.7	0.6	0.6	0.1	0.6	0.6	0.6	0.0	0.9

M is the arithmetic mean value of the permeability coefficient; m is the standard deviation.

$$P_{app} = \frac{V_A}{Area \times time} \times \frac{[drug]_{acc}}{[drug]_{initial\ donor}} * 1000000 \quad (1)$$

V_A – volume of transport buffer in acceptor well,

Area – surface area of the insert (equals to effective growth area of the insert - 0.31 cm2),

Time – time of the assay,

$[drug]_{acc}$ – concentration of test compound in acceptor well,

$[drug]_{initial,d}$ – initial concentration of test compound in a donor well.

P_{app} is expressed in 10-6cm/sec.

n=3

Efflux coefficient, indicating the active excretion of the substance, was calculated using using the following equation:

Efflux coefficient = P_{app} (B-A)/ P_{app} (A-B)

The % recovery can be useful in interpreting the Caco-2 data. If the recovery is very low, this may indicate problems with poor solubility, binding of the compound to the test plate materials, metabolism by the Caco-2 cells or accumulation of the compound in the cell monolayer. The % recovery was calculated using equation 2:

$$\%\ recovery = \frac{C_{acc} \times V_{acc} + C_d \times V_d}{C_{initial,d} \times V_d} \times 100, \quad (2)$$

V_{acc} – volume of compound solution in acceptor well (cm2),

V_d – volume of compound solution in donor well (cm2),

C_{acc} – concentration of test compound in acceptor well (μM),

$C_{initial,d}$ – initial concentration of test compound in a donor well (μM).

n=3

3. Results and discussion

Due to the reliability of the correlation of the results, the Caco-2 test is recommended as «gold standard» for modeling intestinal absorption, determining bioavailability and bioequivalence. In our study, optimization and critical evaluation of mobile phase composition, flow rate, and analytical column were important to obtain good resolution of peaks, which in turn affect reproducibility of the method[16-22]. The resolution of peaks was achieved with Phenomenex Luna, 50 × 2.0 mm, 5 μm column. Phenomenex Luna has found a place as one of the World's top reversed phase columns because it provides a measurable improvement over many HPLC columns for two important chromatographic properties: resolution and peak shape[23].

Typical multiple reaction monitoring chromatograms of valsartan shown in Fig. 2. Under these conditions, the peak of valsartan is eluted for about 1.23 min (Fig. 2). Typical multiple reaction monitoring chromatograms of reference compounds propranolol, atrnolol and quinidine are shown in Fig. 3-5. The total chromatographic run time is 1.5 min, so the developed analytical method for the determination of valsartan to study intestinal permeability in the model of the Caso-2 test is rapid.

A-B and B-A permeability data and efflux coefficient for the test compound of valsartan and the three reference compounds are listed in the Table 4. Permeability coefficient (Papp) of valsartan through the monolayer of Caco-2 cells in the presence of the P-gp inhibitor verapamil are presented in the Table 5.

A-B and B-A permeability data for all the reference compounds correspond to the literature data[24-27], thus validating this study. According to the results presented in Table 3 and 4, valsartan showed low permeability and efflux by p-glycoprotein (P-gp). Indeed a decrease in the efflux coefficient in the presence of verapamil, which is a well known P-gp inhibitor, indicates that valsartan is a substrate of the P-gp transporter.

Recovery data of transport of valsartan and control substances through a monolayer of cells of the Caco-2 are listed in Table 5.

It should be noted that the recovery value (Table 5) of valsartan is 94 % and this indicates that the results of the experiment are reliable.

4. Conclusion

In conclusion, a rapid, simple LC-MS/MS method was developed for determination of valsartan from Caco-2 cell monolayers. Obtained result proves that the method is reproducible and selective for the estimation of valsartan. Valsartan showed low permeability and efflux by p-glycoprotein (P-gp). This is justified by the decrease in the efflux coefficient in the presence of verapamil indicating that valsartan is a substrate of the P-gp transporter. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for examination of valsartan from Caco-2 cell monolayers. □

Table 6: Recovery data of transport of valsartan and control substances through a monolayer of cells of the Caco-2

Analyte	% recovery		
	1	2	M
Propranolol	71	80	76
Atenolol	91	86	89
Quinidine	106	102	104
Valsartan	94	95	94

Acknowledgement

Authors are grateful to the Ministry of Health of Ukraine Fund for providing scholarship for studies related to solutions for development of original combinations of antihypertensive agents, their analysis and standardization (0120U104201 (№509 date 24.02.2020)).

Source of Support: Nil.
Conflict of Interest: None declared.

REFERENCES

1. https://www.fda.gov/media/70963/
2. Berben P., Bauer-Brandl A., Brandl M., Faller B., Flaten G.E, Jacobsen A.C., Brouwers J., Augustijns P., Drug permeability profiling using cell-free permeation tools: Overview and applications. *Eur.J. Pharm. Sci.* 119, 219-33, 2018.
3. Phillips J.E., Arena A., Optimization of Caco-2 cell growth and differentiation for drug transport assay studies using a 96 well MultiScreen Caco-2 Assay System. Millipore protocol note PC1060EN00P. rev. 08/2003.
4. Srinivasan B., Kolli A. R., Esch M. B., Abaci H. E., Shuler M. L., Hickman J. J. TEER measurement techniques for in vitro barrier model systems. *J. Lab. Autom*, 20, 107-26, 2015.
5. https://www.pheculturecollections.org.uk/media/51860/Permeability_values_and_efflux_ratios_C ACO-2.pdf
6. http://www.cyprotex.com/admepk/in-vitro-permeability/caco-2-permeability
7. http://www.bdbiosciences.com/documents/webinar_2009_02_invitro_transporter_testing.pdf
8. Teksin Z. S., Seo P. R., Poli J. E. Comparison of Drug Permeabilities and BCS Classification: Three Lipid-Component PAMPA System Method versus Caco-2 Monolayers, *AAPS J.,* 12, 238, 2010.
9. Fujikawa M., Ano R., Nakao K., Shimizu R., Akamatsu M, Relationships between structure and high-throughput screening permeability of diverse drugs with artificial membranes: application to prediction of Caco-2 cell permeability. *Bioorg. Med. Chem*. 13, 4721-32, 2005.
10. Gertz M., Harrison A., Houston J. B., Galetin A., Prediction of human intestinal first-pass metabolism of 25 CYP3A substrates from in vitro clearance and permeability data. *Drug Metab*. *Dispos.* 38, 1147-58, 2005.
11. Hou T. J., Zhang W., Xia K., Qiao X. B., Xu X. J. ADME evaluation in drug discovery. 5. Correlation of Caco-2 permeation with simple molecular properties. *J. Chem. Inf. Comput. Sci.* 44, 1585–1600, 2004.
12. Zhu T., Howieson C., Wojtkowski T., Garg J.P., Han D., Fisniku O., Keirns J. The Effect of Verapamil, a P-Glycoprotein Inhibitor, on the Pharmacokinetics of Peficitinib, an Orally Administered, Once-Daily JAK Inhibitor. *Clin. Pharmacol. Drug Dev.* 6, 548-555, 2017.
13. Rautio J., Humphreys J. E., Webster L. O., Balakrishnan A., Keogh J. P., Kunta J. R., Serabjit-Singh C. J., Polli J. W. In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. *Drug Metab. Dispos.*34, 786-92, 2006.
14. Hamed R., Alnadi S. H.= Transfer Behavior of the Weakly Acidic BCS Class II Drug Valsartan from the Stomach to the Small Intestine During Fasted and Fed States, *AAPS PharmSciTech*. 19, 2213-2225, 2018.
15. Pagliara A., Reist M., Geinoz S., Carrupt P.A., Testa B., Evaluation and prediction of drug permeation. *J. Pharm. Pharmacol.* 51, 1339–1357, 1999.
16. Piponski M., Peleshok K., Logoyda L., Kravchuk L., Piatnochka V., Zakharchuk U. Efficient Validated HPLC/UV method for determination of valsartan and atenolol in dosage form and in vitro dissolution studies. *Biointerface Res. Appl. Chem.* 10, 6669–6675, 2020.
17. Logoyda L., Herasymiuk M., Popovych D., Pid-

ruchna S., Hlushok V., Herasymiuk N., Zarivna N. HPLC MS/MS method development for the quantitative determination of verapamil hydrochloride from Caco-2 cell monolayers. *Pharmacia*, 67, 63–69, 2020.

18. Logoyda L. HPLC-MS/MS method development for the quantitative determination of nifedipine for Caco-2 permeability assay. *Pharmacia*, 67, 83–88, 2020.
19. Logoyda L., Korobko D. A HPLC MS/MS method development for the quantitative determination of bisoprolol from Caco-2 cell monolayers. *Asian J. Pharm. Clin. Res*. 11, 386–389, 2018.
20. Logoyda L. Quantitative determination of amlodipine from Caco-2 cell monolayers by HPLC MS/MS. *Asian J. Pharm. Clin. Res*. 11, 204–207, 2018.
21. Logoyda L. A High-performance liquid chromatography-mass spectrometry method development for the quantitative determination of enalapril maleate from Caco-2 cell monolayers. *Asian J. Pharm. Clin. Res*. 11, 89–92, 2018.
22. Polyauk O., Logoyda L. The investigation of conditions of API from group of calcium channel blockers extraction by organic solvents by using high-performance liquid chromatography as assay method. *Asian J. Pharm. Clin. Res*. 10, 354-6, 2017.
23. https://www.phenomenex.com
24. Siddiqui N., Husain A., Chaudhry L., Alam M. S., Mitra M., Bhasin P. S. Pharmacological and pharmaceutical profile of valsartan: A review. *J. Appl. Pharm. Sci.* 1, 12-9, 2011.
25. Flesch G., Müller P., Lloyd P. Absolute bioavailability and pharmacokinetics of valsartan, an angiotensin II receptor antagonist, in man. *Eur. J. Clin. Pharmacol*. 52, 115-20, 1997.
26. de Castro L.M.L., de Souza J., Caldeira T.G., de Carvalho Mapa B., Soares A.F.M., Pegorelli B.G., Della Croce C.C., Barcellos N.M.S. The Evaluation of Valsartan Biopharmaceutics Properties. *Curr. Drug Res. Rev.* 12, 1-9, 2020.
27. Amidon G. L., Lennernas H., Shah V. P., Crison J. R. A theoretical basis for a biopharmaceutic drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm. Res.* 12, 413–420, 1995.

Determination of Amino Acids Content in two Herbal Mixtures with Antidiabetic Activity by GC-MS

Alona Savych[1*], Sofia Nakonechna[2]

[1]*Department of Pharmacognosy with Medical Botany, I. Ya. Horbachevsky Ternopil National Medical University, Ukraine*

[2]*Department of Physiology, Bioethics and Biosafety, I. Ya. Horbachevsky Ternopil National Medical University, Ukraine*

KEY WORDS:
herbal mixtures; amino acids; gas chromatography-mass spectrometry; diabetes mellitus

***CORRESPONDING AUTHOR:**
Alona Savych, I. Ya. Horbachevsky Ternopil National Medical University, Ukraine, Ruska, 36
e-mail: alonasavych@gmail.com

ABSTRACT

Due to the wide range of biologically active substances, herbal mixtures can influence the development of diabetes mellitus and its complications. Amino acids attract lately particular attention, due to their ability to stimulate insulin secretion, reduce hyperglycemia and regulate metabolic processes in patients with diabetes. The aim of this study was to investigate the content of amino acids in the following herbal mixture samples: 1) *Urtica dioica* leaf, *Cichorium intybus* roots, *Rosa majalis* fruits, *Elymus repens* rhizome, *Taraxacum officinale* roots, 2) *Arctium lappa* roots, *Elymus repens* rhizome, *Zea mays* columns with stigmas, *Helichrysum arenarium* flowers, *Rosa majalis* fruits, which have shown antidiabetic activity in studies *in vivo*. A number of amino acids were separated by validated method of gas chromatography-mass spectrometry with pre-column derivatisation. Quantitative analyses of amino acids showed that the predominant components were *L*-proline in sample 1 and *L*-leucine and *L*-proline in sample 2 of the examined herbal mixtures.

1. Introduction

Diabetes mellitus is one of WHOs priorities matters, which requires immediate solutions, as the epidemiological situation is alarming – the number of patients is growing rapidly each year, leading to increased disability and mortality due to the development of macro- and microangiopathies [1, 2]. According to the official data of the International Diabetes Federation (2019), the incidence of diabetes in the world is expected to increase by 1.5 times in 2030, amounting to more than 500 thousand patients[3]. Therefore, the optimization of existing antidiabetic pharmacotherapy, search and study of new drugs for the prevention and treatment of this disease and its complications are currently very important issues in modern pharmacy and medicine.

One of the above areas uses phytomedicines in the form of monotherapy in the mild stages of the disease and for its prevention, and in combination with traditional therapy for more severe forms of the disease [4-6]. Phytotherapy is a promising and reasonable approach, as it offers a number of advantages e.g. relatively low toxicity, mild pharmacological effect, the ability to be used for a long time without significant side effects, and the ability to be combined well with synthetic drugs[7-9]. The combinations of different medicinal plants deserve

particular attention. Herbal mixtures are expected to contain several biologically active substances with a wide range of pharmacological actions and a variety of mechanisms for influencing the development of diabetes and diabetic angiopathies [10-14]. Therefore, in order to establish correlations between the phytochemical composition of the studied herbal mixtures and its antidiabetic activity, which was studied in previous studies[15-20], it is advisable to conduct phytochemical analysis, in particular, amino acids as important biologically active substances in the therapy of diabetes.

Amino acids, in addition to their main function as precursors of protein synthesis, play a key role in many metabolic processes, because they have a powerful secretolytic activity - stimulate the secretion of insulin, glucagon, cortisol and insulin-like growth factor-1 (IGF-1)[21]. Except this, literature sources indicate the regulatory role of amino acids in the transcription and translation of genes, as well as their important function in intracellular signaling[21, 22]. The effectiveness of amino acids in the treatment and prevention of diabetes is primarily due to their ability to stimulate insulin secretion in pancreatic β-cells, as well as increase blood glucose utilization and reduce alimentary hyperglycemia. The greatest insulinotropic effect is inherent in arginine, leucine, isoleucine, alanine and phenylalanine[22, 23]. In addition, amino acids have the ability to reduce muscle proteolysis and/or stimulate protein synthesis, which leads to improved protein balance in skeletal muscle and, as a result, increases the process of glucose utilization. This is an important component of antidiabetic therapy, because such patients often have a deficiency of skeletal muscle mass, which, in turn, contributes to the development of insulin resistance and progression of this disease[13]. Therefore, the aim of this study was to investigate the content of amino acids in some herbal mixtures, with observed antidiabetic activity *in vivo* [11, 12].

2. Material and Methods

2.1. Plant materials

The herbal raw materials were harvested from June to August 2019 in the Ternopil region (Ukraine). The raw materials were then dried, crushed and stored according to the general GACP requirements[24]. The plants were identified in the Department of Pharmacognosy with Medical Botany, I. Ya. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. The voucher specimens of herbal raw materials have been deposited in the departmental herbarium for future records. Two different herbal mixtures with reliable antidiabetic activity established during pharmacological studies *in vivo*[18-20] were used. The composition of the herbal mixtures is given in **Table 1.**

2.2. Chemicals and standards

All applied reagents were of analytical grade (≥ 99 % purity). Standard reagents including glycine, *L*-alanine, *L*-valine, *L*-leucine, *L*-serine, *L*-threonine, *L*-isoleucine, *L*-proline, *L*-asparagine, *L*-aspartic acid, *L*-glutamic acid, *L*-phenylalanine, *L*-glutamine, *L*-lysine, *L*-histidine, *L*-tyrosine, *L*-tryptophan were purchased from Sigma-Aldrich Chemical Co. (USA), as well as hydrochloric acid, sodium hydroxide, methanol, pyridine, methyl chloroformate, chloroform, sodium bicarbonate. Water used in the studies was produced by MilliQ Gradient water deionizaton system (USA).

2.3. *Extraction of amino acids*

For the extraction of free amino acids the samples of the herbal raw material were grinded into a powder by laboratory mill, then about 0.1 g (accurately weighed) was selected and placed into vial with 2.0 mL of 0.1 N aqueous solution of hydrochloric acid. The extractions were carried out in the ultrasonic water bath at 50 °C for 3 hours.

Extraction of bound amino acids was carried out by adding 2 mL of 6 N an aqueous solution of hydrochloric acid to 0.03 g of powdered herbal raw materials. Hydrolysis was carried out for 24 hours in a thermostat at 110 °C.

The resulting extracts were centrifuged at 3000 rpm and the supernatants were evaporated to dryness on a rotary evaporator washing three times with distilled water to remove hydrochloric acid.

2.4. *Pre-column derivatisation*

The dry samples of herbal mixtures were dissolved in 390 μL of 1 M sodium hydroxide, then 333 μL of methanol and 67 μL of pyridine were added and mixed thoroughly for 5 seconds. To the resulting mixtures 80 μL of methyl chloroformate was added, stirred thoroughly for 60 seconds. The amino acid derivatives were extracted with 400 μL of chloroform followed by the addition of 400 μL 50 mM sodium bicarbonate. The chloroform phase was used for future analysis[25].

2.5. *Instrumentation and conditions of gas chromatography-mass spectrometry*

The amino acids composition in the samples of the herbal raw materials was studied by gas chromatography-mass spectrometry (GC-MS) method using the Agilent Technologies (USA) system, model 6890N/5973inert (6890 gas chromatography with mass spectrometry detector 5973) and capillary column HP-5ms (30 m × 0.25 mm × 0.25 mm, Agilent Technologies)[26]. The evaporator temperature was at 250 °C, the interface temperature at 280 °C. The separation was performed in the mode of temperature programming – the oven temperature was initially set to 50 °C, held for 4 min, then ramped at the rate of 5 °C/min to 300 °C and finally held at this temperature for 5 min. Injections of 1 μL were made in the split mode 1:50. The carrier gas flow rate through the column was 1.0 mL/min.

2.6. *Identification and calculation by GC-MS*

Amino acid identification was performed by comparing retention times (t_R) of amino acid standards, and the presence of representative molecular and fragment ions (**Table 2**). The content of bound amino acids was determined by subtracting the content of free amino acids from their total content [18].

The method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ) and precision. Linearity was performed by injecting a series of standard solutions (0.1–10.0 mg/100g) with a threefold derivatization procedure and a single injection for each reference standard. The mean value and standard deviation, as well as regression analysis were calculated using Microsoft Excel software package 2016 (USA). The LOD and LOQ under the chromatographic conditions were calculated as 3 times and 10 times, respectively. Linearity testing was repeated with the same samples after a complete restart of the system with removement and re-installation of the column. Repeatability precision was determined by five-fold injection of the same sample in a row. For the resulting relative peak area of the quantifier ions the relative standard deviation (RSD) was calculated. To determine intra-day precision, five standard preparations of each reference standard with the same concentration were single injected and the resulting relative peak areas were used to calculate the RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting five standard sample of each reference standard preparations once each on all three days. The RSD of the samples on that day together with the previous samples were calculated as above[27].

3. Results and Discussion

The analytical procedure has been validated to confirm its reliability. All the peaks of reference standards showed good linearity (R2 > 0.98) in a wide concentration range (0.1–10.0 mg/100g). The results showed that the LODs and the LOQs of amino acids were in the range of 0.01–0.07 mg/100g and 0.02–0.20 mg/100g, respectively, indicating that the sensitivity of the method was satisfactory. The repeatability of the subsequent derivatization and GC-measurement of five standard samples of each reference standard with the same concentration resulted in precision values for the derivatization procedure. For intra- and inter-day precision, the RSD was in a range of 1.24 % to 8.10 %, which is acceptable (**Table 3.).** The results of qualitative and quantitative analyses of free and bound amino acids in the herbal mixtures are shown in Figures 1-4 and in Table 4.

Following GC-MS analysis, 5 amino acids in free form and 11 in bound form were identified in the sample 1 of the herbal mixture (**Figs. 1, 2**); 5 amino acids in free form and 14 in bound form in the sample 2 (**Figs. 3, 4**).

Table 1: Composition of the herbal mixtures

Herbal mixture	Herbal drug component	Portion in the mixture, %	Relative ratio
Sample 1	Urtica dioica leaf	26.32	5
	Cichorium intybus roots	26.32	5
	Rosa majalis fruits	21.05	4
	Elymus repens rhizome	15.79	3
	Taraxacum officinale roots	10.52	2
Sample 2	Arctium lappa roots	26.32	5
	Elymus repens rhizome	26.32	5
	Zea mays columns with stigmas	21.05	4
	Helichrysum arenarium flowers	15.79	3
	Rosa majalis fruits	10.52	2

Table 2: Conditions for chromatographic identification of amino acids

Amino acids	tR, min	Molecular ion, m/z	Main fragmentary ions, m/z
Glycine	14.83	147	88
L-alanine	14.83	161	102, 88
L-valine	18.57	189	146, 130, 115, 98
L-leucine	20.77	203	144, 115, 102, 88
L-serine	21.04	191	176, 144, 114, 100, 88
L-threonine	21.28	205	147, 115, 100, 88
L-isoleucine	21.87	203	144, 115, 101, 88
L-proline	21.97	187	128, 84
L-asparagine	22.09	262	146, 127, 95
L-aspartic acid	23.93	219	160, 128, 118, 101
L-glutamic acid	26.90	233	201, 174, 142, 114
L-phenylalanine	29.75	237	178, 162, 146, 131, 103, 91
L-glutamine	31.90	276	141, 109, 82
L-lysine	35.94	276	244, 212, 142, 88
L-histidine	37.08	285	254, 226, 210, 194, 140, 81
L-tyrosine	38.94	296	252, 236, 220, 192, 165, 146, 121
L-tryptophan	42.01	276	130

The predominant amino acids in free form were *L*-proline its content was 7.255 mg/g in the sample 1 and 17.829 mg/g in the sample 2 (**Table 4)**. Proline has significant hypoglycemic activity, due to a decrease in hepatic glucose production owing to inhibition of glycogenolysis, gluconeogenesis and glucose-6-phosphatase activity [12]. Another free amino acid with a high content in both studied herbal mixtures was is *L*-isoleucine its content was 4.657 mg/g and 8.498 mg/g, respectively. As for the amino acids in the bound form, predom-

Table 3: Sensitivity and linearity parameters obtained for individual amino acids after GC-MS analysis

Amino acids	Regression Curve	R^2	LOD, mg/100g	LOQ, mg/100g
Glycine	y = 95.25x + 4.308	0.992	0.01	0.03
L-alanine	y = 81.03x + 2.372	0.996	0.01	0.04
L-valine	y = 108.4x – 1.502	0.996	0.02	0.06
L-leucine	y = 44.24x + 2.285	0.984	0.01	0.03
L-serine	y = 110.90x - 0.241	0.998	0.01	0.03
L-threonine	y = 77.24x + 3.222	0.990	0.01	0.04
L-isoleucine	y = 44.24x + 2.285	0.984	0.01	0.03
L-proline	y = 124.50x + 0.359	0.998	0.01	0.02
L-asparagine	y = 80.84x + 2.885	0.990	0.01	0.03
L-aspartic acid	y = 154.4x + 2.375	0.999	0.01	0.03
L-glutamic acid	y = 65.30x + 3.934	0.992	0.06	0.20
L-phenylalanine	y = 149.5x + 9.568	0.990	0.01	0.04
L-glutamine	y = 44.24x + 2.285	0.984	0.06	0.20
L-lysine	y = 127.80x + 5.598	0.984	0.07	0.20
L-histidine	y = 69.28x + 1.579	0.992	0.03	0.10
L-tyrosine	y = 124.90x + 2.897	0.995	0.01	0.05
L-tryptophan	y = 189.40x + 2.673	0.994	0.01	0.04

inant was *L*-proline in both samples 1 and 2 at content 11.751 mg/g and 10.273 mg/g respectively. It was further found that sample 2 contains the largest amount of bound amino acid *L*-leucine – 10.375 mg/g (**Table 4)**. *L*-leucine is a branched-chain amino acid that plays an important role in controlling protein synthesis and regulating cell metabolism. One of the most important functions of leucine in diabetes is that it has the ability to stimulate insulin secretion in **β-cells of the pancreas, and also acts as a source of energy for metabolic processes and an** allosteric activator of glutamate dehydrogenase to enhance glutaminolysis 15. Isoleucine, an isomer of leucine, which was also found in both samples 1 and 2 in free and bound form does not have itself the ability to stimulate insulin synthesis, but in combination with leucine, their secretolytic activity increases significantly, causing a more pronounced hypoglycemic effect[21, 23]. *L*-aspartic acid was found in both free and in bound form in sample 1 at a content of 4.070 mg/g and 5.735 mg/g respectively, while in sample 2 the free form is at low content (0.321mg/g) compared to the bound form (6.787 mg/g). In addition, high levels of *L*-phenylalanine in bound form were found in both samples of the herbal mixtures at contents 3.096 mg/g and 5.489 mg/g, respectively (**Table 4).** Phenylalanine, an aromatic amino acid, that has a direct effect on the course of diabetes, in particular due to its ability to regulate carbohydrate metabolism by stimulating the release of glucan-like peptide-1 (GLP-1), which in turn enhances secretion of insulin, stimulates proliferation and neogenesis of β-cells of the pancreas, reduces insulin resistance[22].

Consequently, the studied herbal mixtures due to sufficient quantities of plant amino acids could play an important role as an additional aid in diabetes treatment.

4. Conclusion

The results of the present study indicate a sufficient content of essential amino acids in the investigated herbal mixtures, which have the ability to stimulate insulin secretion, reduce hyperglycemia and regulate

Table 4: Content of amino acid composition in the samples of the herbal mixtures.

Number of peak on chromato-gram	Name of amino acid	tR (min)	Content of amino acids, mg/g			
			Sample 1		Sample 2	
			Free	Bound	Free	Bound
1.	Glycine	14.77	n/d	n/d	n/d	6.871±0.12
2.	L-alanine	14.85	n/d	0.103±0.01	n/d	n/d
3.	L-valine*	18.56	0.163±0.01	0.392±0.02	0.160±0.01	5.672±0.11
4.	Nor-valine	19.57	Internal standart			
5.	L-leucine*	20.77	n/d	0.748±0.02	n/d	10.375±0.16
6.	L-serine	21.11	n/d	2.174±0.07	n/d	4.153±0.08
7.	L-threonine*	21.31	n/d	n/d	n/d	0.799±0.03
8.	L-isoleucine*	21.87	4.657±0.09	2.811±0.04	8.498±0.08	5.918±0.12
9.	L-proline*	21.97	7.255±0.12	11.751±0.09	17.829±0.19	10.273±0.16
10.	L-asparagine	23.90	0.731±0.03	1.532±0.05	0.198±0.02	6.215±0.07
11.	L-aspartic acid	24.02	4.070±0.05	5.735±0.06	0.334±0.03	6.787±0.11
12.	L-glutamic acid	26.86	n/d	0.321±0.03	n/d	3.810±0.08
13.	L-phenylalanine*	29.74	n/d	3.096±0.08	n/d	5.489±0.09
14.	L-glutamine	31.90	n/d	n/d	n/d	n/d
15.	L-lysine*	35.91	n/d	0.400±0.03	n/d	6.483±0.09
16.	L-histidine*	37.24	n/d	n/d	n/d	0.128±0.01
17.	L-tyrosine	38.91	n/d	n/d	n/d	5.217±0.11
18.	L-tryptophan	42.01	n/d	n/d	n/d	n/d

Note:

*1. * – essential amino acid;*

2. n/d – not detected;

3. Values are expressed as mean ± SD (n=5).

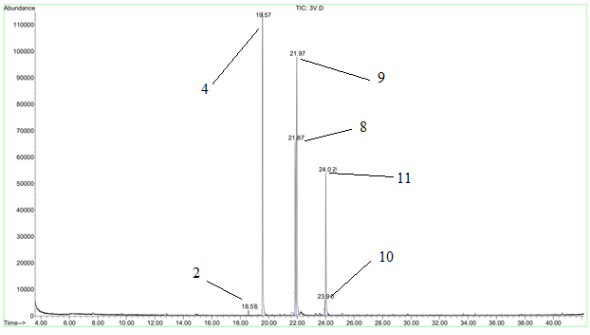


Figure 1. *GC-MS chromatogram of free amino acids in the sample 1 of the herbal mixture.*

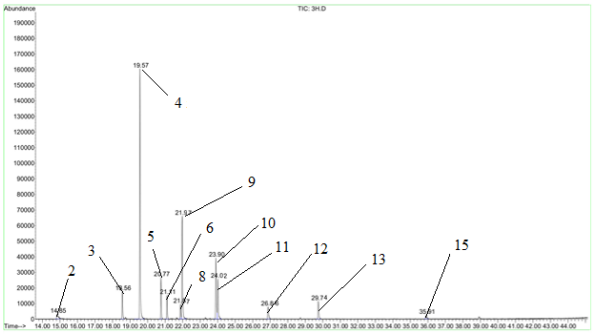


Figure 2. *GC-MS chromatogram of amino acids after hydrolysis in the sample 1 of the herbal mixture.*

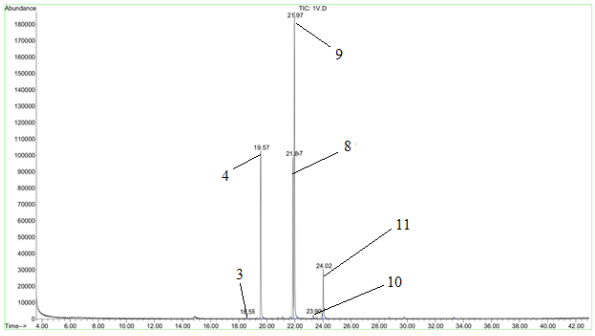


Figure 3. *GC-MS chromatogram of free amino acids in the sample 2 of the herbal mixture.*

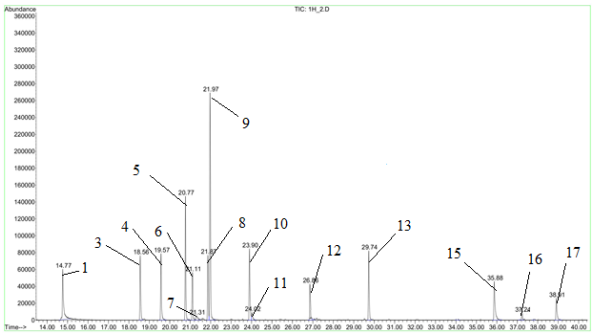


Figure 4. *GC-MS chromatogram of amino acids after hydrolysis in the sample 2 of the herbal mixture.*

metabolic processes in patients with diabetes. The predominant amino acids L-leucine in free form and L-proline in free and bound form in sample 2 and L-proline in free form and bound form in sample 1 are the amino acids with the most pronounced insulin secretolytic activity. The obtained data testify to the expediency of using the studied herbal mixtures as an additional aid antidiabetic pharmacotherapy. □

Conflict of Interests

The authors declare that they have no conflict of interests to disclose.

REFERENCES

1. Sakamoto M. Type 2 diabetes and glycemic variability: various parameters in clinical practice, *J. Clin. Med. Res*. 10, 737-742, 2018, doi: 10.14740/jocmr3556w.
2. American Diabetes Association (2017). Standards of medical care in diabetes. *Diabetes care*, 40 (1), pp. 142.
3. International Diabetes Federation (2019) Diabetes Atlas., 9th edn. *IDF,* Brussels, Belgium, http://www.diabetesatlas.org
4. Gothai S., Ganesan P., Park S., Fakurazi S., Choi D., Arulselvan P. Natural phyto-bioactive compounds for the treatment of type 2 diabetes: inflammation as a target, *Nutrients.* 8, 461, 2016, doi: 10.3390/nu8080461.
5. Slobodianiuk L., Budniak L., Marchyshyn S., Basaraba R. Investigation of the hepatoprotective effect of the common cat's foot herb dry extract. *PhOL.* 3, 310-318, 2020.
6. Budniak L., Slobodianiuk L., Marchyshyn S., Demydiak O. Determination of *Arnica foliosa* Nutt. fatty acids content by GC/MS method. *ScienceRise: Pharmaceutical Science.* 6 (28), 14-18, 2020, doi: 10.15587/2519-4852.2020.216474
7. Marchyshyn S., Slobodianiuk L., Budniak L., Skrynchuk O. Analysis of carboxylic acids of *Crambe cordifolia* Steven. *Pharmacia.* 68(1), 15–21, 2021, doi: 10.3897/pharmacia.68.e56715
8. Marchyshyn S., Budniak L., Slobodianiuk L., Ivasiuk I. Determination of carbohydrates and fructans content in *Cyperus esculentus* L. *Pharmacia.* 68(1), 211-216, 2021, doi: 10.3897/pharmacia.68.e54762
9. Budniak L., Slobodianiuk L., Marchyshyn S., Klepach P., Honcharuk Ya. Determination of carbohydrates content in *Gentiana cruciata* L. by GC/MS method. *Int. J. App. Pharm.* 13(1), 124-128, 2021.
10. Marchyshyn S., Polonets O., Savych A., Nakonechna S. Determination of carbohydrates of *Chrysanthemum morifolium L.* leaves and flowers by GC-MS. *Pharmakeftiki*. 32(4), 202-212, 2020.
11. Savych A., Marchyshyn S., Basaraba R. Determina-

tion of fatty acid composition content in the herbal antidiabetic collections. *Pharmacia*. 67(3), 153-159, 2020, doi: 10.3897/pharmacia.67.e51812.

12. Savych A., Marchyshyn S., Milian I. Determination of carbohydrates in the herbal antidiabetic mixtures by GC-MC. *Acta Pharm.* 71(3), 429-443, 2021, doi: 10.2478/acph-2021-0026.
13. Savych A., Marchyshyn S., Kozyr H., Yarema N. Determination of inulin in the herbal mixtures by GC-MS method. *Pharmacia*. 68(1), 181-187, 2021, doi: 10.3897/pharmacia.68.e55051
14. Savych A., Marchyshyn S., Harnyk M., Kudria V., Ocheretniuk A. Determination of amino acids content in two samples of the plant mixtures by GC-MS. *Pharmacia.* 68(1), 283-289, 2021, doi: 10.3897/pharmacia.68.e63453
15. Savych A., Marchyshyn S., Basaraba R., Kryskiw L. Determination of carboxylic acids content in the herbal mixtures by HPLC. *ScienceRise: Pharmaceutical Science.* 2(30), 33-39, 2021, doi: 10.15587/2519-4852.2021.229132
16. Savych A., Basaraba R., Muzyka N., Ilashchuk P. Analysis of fatty acid composition content in the plant components of antidiabetic herbal mixture by GC-MS. *Pharmacia*. 68(2), 433-439, 2021, doi: 10.3897/pharmacia.68.e66693
17. Savych A., Marchyshyn S., Kyryliv M., Bekus I. Cinnamic acid and its derivatives in the herbal mixtures and their antidiabetic activity. *Farmacia*. 69(3), 595-601, 2021, doi: 10.31925/farmacia.2021.3.23
18. Savych A., Marchyshyn S., Basaraba R., Lukanyuk M. Antihyperglycemic, hypolipidemic and antioxidant properties of the herbal mixtures in dexamethasone-induced insulin resistant rats. *PhOL*. 2, 73-82, 2020.
19. Savych A., Marchyshyn S., Basaraba R. Screening study of hypoglycemic activity of the herbal mixtures (Message 1). *ScienceRise: Pharmaceutical Science.* 4(26), 40-46, 2020, doi: 10.15587/2519-4852.2020.210734.
20. Savych A., Marchyshyn S., Nakonechna S. Influence of some herbal mixtures on insulin resistance and glucose tolerance in rats. *PhOL*. 1, 356-364, 2021.
21. Comerford K. B., Pasin G. Emerging evidence for the importance of dietary protein source on glucoregulatory markers and type 2 diabetes: different effects of dairy, meat, fish, egg, and plant protein foods. *Nutrients*. 8(8), 446, 2016, doi: 10.3390/nu8080446.
22. Owei I., Umekwe N., Stentz F., Wan J., Dagogo-Jack S. Amino acid signature predictive of incident prediabetes: A case-control study nested within the longitudinal pathobiology of prediabetes in a biracial cohort. *Metabolism*. 98, 76-83, 2019, doi: 10.1016/j.metabol.2019.06.011
23. Birech Z., Mwangi P. W., Bukachi F., Mandela K. M. Application of Raman spectroscopy in type 2 diabetes screening in blood using leucine and isoleucine amino-acids as biomarkers and in comparative anti-diabetic drugs efficacy studies. *PloS one*. 12(9), e0185130, 2017, doi: 10.1371/journal.pone.0185130.
24. World Health Organization (2003), WHO Guidelines on Good Agricultural and Mixture Practices (GACP) for Medicinal Plants. *World Health Organization*, Geneva, pp. 72.
25. Vancompernolle B., Croes K., Angenon G. Optimization of a gas chromatography–mass spectrometry method with methyl chloroformate derivatization for quantification of amino acids in plant tissue. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1017, 241-249, 2016, doi: 10.1016/j.jchromb.2016.02.020.
26. Chen W. P., Yang X. Y., Hegeman A. D., Gray W. M., Cohen J. D. Microscale analysis of amino acids using gas chromatography-mass spectrometry after methyl chloroformate derivatization. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 878(24), 2199-2208, 2010, doi: 10.1016/j.jchromb.2010.06.027.
27. Jing Wang, Liu S. M., Long J., Lei D. A., Gao F. Derivatization method for the determination of amino acids in tobacco by gas chromatography–mass spectrometry. *J. Anal. Chem.* 75, 1046–1053, 2020, doi: 10.1134/S1061934820080171.

ΕΚΔΗΛΩΣΕΙΣ - MEETINGS

Η εξάπλωση του δεύτερου κύματοςτης πανδημίας COVID-19 που δοκιμάζει τις κοινωνίες σε παγκόσμιο επίπεδο έχει στερήσει τις επιστημονικές εταιρείες από τη δυνατότητα να πραγματοποιούν επιστημονικές εκδηλώσεις και συνέδρια, με φυσική παρουσία.. Ως εκ τούτου τα συνέδρια που είχαν προγραμματιστεί μέχρι και την άνοιξη του 2021 έχουν αναβληθεί ή θα πραγματοποιηθούν on line εικονικά (virtually). Μένουμε ασφαλείς!

The spread of the second wave of COVID-19 pandemia does not allow scientific activi¬ties to take place with physical presence Thus , all scientific events scheduled till spring 2021 have been cancelled, or will be conducted virtually. while all face-to-face activities have been replaced with online meetings. Stay safe!

• APRIL 14 - 15, 2021 | VIRTUAL EVENT | KINASE 2021: 9TH RSC / SCI SYMPOSIUM ON KINASE INHIBITOR DESIGN
https://www.rsc.org/events/detail/40755/kinase-2021-9th-rsc-sci-symposium-on-kinase-inhibitor-design

• MAY 26 - 28, 2021 | VIRTUAL MEETING
EUROPEAN CHEMICAL BIOLOGY SYMPOSIUM (ECBS2021)
https://ecbs2021.eu/

• JUNE 27 - JULY 1, 2021 | VIRTUAL MEETING
40TH EDITION OF THE EUROPEAN SCHOOL OF MEDICINAL CHEMISTRY (ESMEC)
https://eventi.uniurb.it/esmec/

• AUGUST 29 - SEPTEMBER 2, 2021 - BASEL, SWITZERLAND
XXVI EFMC INTERNATIONAL SYMPOSIUM ON MEDICINAL CHEMISTRY (EFMC-ISMC 2021) EFMC SYMPOSIUM
https://www.efmc-ismc.org/

• SEPTEMBER 2 - 3, 2021, BASEL, SWITZERLAND | 8TH EFMC YOUNG MEDICINAL CHEMISTS' SYMPOSIUM
(EFMC-YMCS 2021) EFMC Symposium
https://www.efmc-ymcs.org/

• SEPTEMBER 19-23, 2021, BARCELONA, SPAIN
23RD EUROPEAN SYMPOSIUM ON QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP (23RD EUROQSAR)
EFMC Sponsored Event
https://www.euroqsar2020.org/

• SEPTEMBER 20-23, 2021, VIRTUAL EVENT
12TH INTERNATIONAL CONFERENCE ON "INSTRUMENTAL METHODS OF ANALYSIS" (IMA-2021)
www.ima2021.gr

• SEPTEMBER 22-24, 2021 | VIRTUAL MEETING | SUMMER SCHOOL IN PHARMACEUTICAL ANALYSIS (SSPA2021)
http://www.sspaweb.com

• APRIL 18-20, 2022, TOKYO, JAPAN
INTERNATIONAL MEET ON PHARMACEUTICS AND DRUG DELIVERY SYSTEMS (PHARMAMEET2022)
https://www.albedomeetings.com/pharmameet/index.php

ZITA GROUP

since 1982

ZITA CONGRESS ZITA MEDICAL MANAGEMENT

ΠΕΡΙΟΔΙΚΟ ΔΥΟ ΔΙΑΤΡΟΦΗ ΥΓΕΙΑ ΟΜΟΡΦΙΑ

ΔΙΑΤΡΟΦΗ | ΥΓΕΙΑ | ΟΜΟΡΦΙΑ ΔΥΟ FORUM

Στηρίζουμε κάθε σας δημιουργική σκέψη & προσπάθεια διάχυσης επιστημονικής γνώσης

- Διοργάνωση συνεδρίων, εκθέσεων, πολιτιστικών εκδηλώσεων και ταξιδίων κινήτρων
- Διαχείριση ιατρικών εταιρειών και οργανισμών
- Website και Ηλεκτρονικό Marketing
- Επιστημονικές Εκδόσεις Περιοδικών
- Χορηγίες
- Γραφιστικό - Δημιουργικό
- Γραμματειακή Υποστήριξη
- Τουρισμός Υγείας
- Νοσοκομειακό Marketing
- Γραφείο Τύπου

LETRINA S.A. CB ISO 9001

LETRINA S.A. CB ISO 14001

www.zita-group.com

Ομήρου 29, Πέτα Σαρωνικού, 190 01, Αττική, Ελλάδα

Τηλ: +30 22994 40962

k.ge@zita-congress.gr, info@zita-congress.gr, info@zita-management.com

Follow us