Poznan University of Medical Sciences
Poland

JMS Journal of Medical Science

previously Nowiny Lekarskie

Founded in 1889

2021
Vol. 90, No. 3

QUARTERLY

Indexed in:
DOAJ, Crossref, Google Scholar,
Polish Medical Bibliography, Index Copernicus,
Ministry of Education and Science

eISSN 2353-9801
ISSN 2353-9798

www.jms.ump.edu.pl

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PUBLISHER
Poznan University of Medical Sciences
10 Fredry Street, 61-701 Poznań, Poland
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www.ump.edu.pl

eISSN 2353-9801
ISSN 2353-9798
DOI: 10.20883/ISSN.2353-9798

Publishing Manager: Grażyna Dromirecka
Technical Editor: Bartłomiej Wąsiel

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Ark. wyd. 10,3. Ark. druk. 9,3.
Zam. nr 127/21.

The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

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The possible role of molecular mimicry in SARS-CoV-2-mediated autoimmunity: an immunobiochemical basis

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Keywords: autoimmunity and COVID19, molecular mimicry and COVID19, SARS-CoV-2 autoimmunity, molecular mimicry

Published: 2021-10-10

How to Cite: Kovačić D, Jotanović J, Laković J. The The possible role of molecular mimicry in SARS-CoV-2-mediated autoimmunity: an immunobiochemical basis. Journal of Medical Science. 2021 Oct. 10;90(3):e560. doi:10.20883/medical.e560

DOI: https://doi.org/10.20883/medical.e560

ABSTRACT

Coronavirus Disease 2019 (COVID-19), caused by the novel Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), persists as a threat to global health and continues to be a rapidly evolving condition. Although COVID19 is negatively correlated with the existing comorbidities in terms of the clinical outcome, the ability of SARS-CoV-2 to mediate the novel, or to exacerbate the existing autoimmune conditions, has generated considerable interest, due to its potential implications both with regard to patients suffering from autoimmune conditions, as well as to the long-term consequences of the disease. However, although molecular mimicry has been postulated as a potential causative factor in post-COVID19 autoimmunity and multi-organ damage, a substantial body of research needs to emerge in order to achieve a more definitive conclusion. We investigated the possibility of SARS-CoV-2 peptide sequences behaving as molecular mimics with a potential to trigger an autoimmune response. Thus, on the basis of analysis in silico, we were able to develop a plausible case for the molecular mimicry as a potential aetiological mechanism of SARS-CoV-2-mediated autoimmunity, both in a multi-organ damage context or outside of the viral phase of infection. Interestingly, this is the first time that the peptide sequence of MACROD1 has been implicated in the COVID-19 autoimmunity. Additionally, we also confirm that PARP9 and PARP14 may be involved in the process.

Introduction

Coronavirus Disease (COVID-19) Pandemic, resulting from by the novel Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), persists as a threat to global health and continues to be a rapidly developing situation [1]. Due to the potential implications both with regard to

patients suffering from autoimmune conditions, as well as to the long-term consequences of COVID-19, COVID-19 has generated considerable attention, although it has been negatively correlated with the existing comorbidities in terms of the clinical outcome, the ability of SARS-CoV-2 to mediate the novel, or to exacerbate the existing autoimmune conditions [2–4]. In spite of the respiratory involvement, as the most prominent symptom, the prospect of the virus triggering the novel autoimmune conditions, or exacerbating the existing ones, is becoming increasingly substantiated by a growing body of research [5–8]. It has been established that, depending on the disease severity, COVID-19 is characterised by cytokine dysregulation, which results in a tissue-damaging cytokine storm in the critically ill patients [5, 9–12]. Single cell RNA sequencing revealed that proinflammatory cytokines are predominantly secreted by monocyte-derived inflammatory macrophages [13]. This causes the onset of acute respiratory distress syndrome (ARDS), with interleukin (IL)-6, IL-1β and tumour necrosis factor (TNF)-α closely correlated with severe ARDS cases, accompanied by multi-organ damage [1, 6, 14–16]. Considering that IL-6 and IL-1β strongly mediate the recruitment of neutrophils and T cells, it is not at all surprising that these cytokines have been particularly strongly correlated with a severe immunopathology [17–19]. In fact, most COVID-19 patients are either asymptomatic or exhibit mild-to-modest symptomatology. However, the subpopulations predisposed to autoimmunity, or those with pre-existing autoimmune conditions, may experience detrimental effects; either in an acute clinical context of COVID-19, or through a potential triggering of novel autoimmune conditions via a number of virus-mediated mechanisms [4, 20, 21].

Research concerning the underlying mechanisms is currently developed, although clinical reports suggest a link between SARS-CoV-2 and autoimmunity, both as a potential causative factor and an exacerbator. For instance, a recent study reported the presence of antiphospholipid antibodies (aPLs) in 47% (31/66) of critically ill COVID-19 patients, as compared to the non-critically ill ones [22]. Moreover, SARS-CoV-2-induced autoimmunity is also indicated by reports of anti-nuclear antibodies (ANAs), lupus anticoagulant, anti-interferon (IFN) and anti- melanoma differentiation-associated protein 5 (MDA5) antibodies in severely ill patients. A study conducted by Vojdani et al. 2020 demonstrated a potential cross-reactivity of anti-SARS-CoV-2 spike antibodies and the following human tissue proteins, i.e. transglutaminase 3, transglutaminase 2, myelin basic protein, mitochondria, nuclear antigen, α-myosin, thyroid peroxidase, collagen, claudin 5+6, and S100B. This, in turn, further contributes to the hypothesis that SARS-CoV-2 may be an aetiological factor of autoimmunity [23]. Furthermore, it has been estimated that approximately 4% of uninfected patients over the age of 70 possess anti-IFN autoantibodies which contribute to ~20% of COVID-19 fatalities. This fact has been attributed to mutations in genes involved in the regulation of type I and type III IFN immunity [24, 25]. However, whether SARS-CoV-2 is able to elicit the production of *de novo* anti-IFN autoantibodies, remains to be substantiated. Nevertheless, it would be tempting to suggest that mutations in genes associated with IFN immunity may contribute to the ability of SARS-CoV-2 to trigger the production of autoreactive antibodies which target the IFN pathways and IFN itself.

Autoimmune conditions, such as Guillain–Barré syndrome (GBS), Miller Fisher syndrome, paediatric immune thrombocytopenia (ITP), immune thrombocytopenic purpura (ITPP), systemic lupus erythematosus (SLE) and Kawasaki disease (KD), have been reported in COVID-19 patients regardless of the acute phase of infection [26–33]. Additionally, multisystem inflammatory disorders in children, phenotypically consistent with the clinical presentation of the Kawasaki disease, have been substantially correlated with acute COVID-19 in a recent report by Rubens et al. [34].

The possible correlation between ITP and COVID-19 is particularly remarkable. In view of two thirds of children diagnosed with ITP have suffered a viral infection, such as cytomegalovirus, hepatitis C, herpes, varicella zoster, rubella, Epstein-Barr virus, approximately one month prior to the diagnosis, it is impossible to exclude that SARS-CoV-2 could potentially be a novel aetiological factor of this autoimmune condition [29, 35–39]. The aforementioned paediatric case report of COVID-19-associated ITP could be indicative of the fact that SARS-CoV-2 may act as a viral trigger for autoimmunity in younger indi-

viduals, or it may exacerbate the existing ITP. The idea of SARS-CoV-2 which would exacerbate the stable ITP (and possibly other autoimmune conditions) may be supported by the case report of a previously stable ITP with infrequent flare-ups transitioning towards acute ITP after immunization with the Pfizer-BioNTech mRNA COVID-19 vaccine [40].

A possible mechanism by which SARS-CoV-2 may trigger or exacerbate autoimmunity is the phenomenon of molecular mimicry, which presumably occurs when T or B cells, induced by pathogen-derived peptides become cross-activated by self-peptides (of the host). In theory, therefore, it is possible that sequence similarities between foreign and self-peptides are sufficient to result in the aforementioned cross-activation [41, 42]. Structural homology is very important in the theory of molecular mimicry as confirmed by findings of single antibodies or TCR (T cell receptor) being activated by merely a few crucial residues [42, 43]. In fact, molecular mimicry may occur at the following levels:

1) complete identification of the viral and host protein,
2) homology between the host and viral protein/s,
3) common or sufficiently similar native or modified (glycosylated) amino acid sequences/epitopes between the virus and the host,
4) structural similarities between viral or environmental agents. Furthermore, viral infections may be followed by epitope spreading, which is a process of an immune response being elicited against viral epitopes which are not pathogenicity factors and display no cross-reactivity with such epitopes [44–47].

Nevertheless, although molecular mimicry has been postulated as a potential causative factor in post-COVID-19 autoimmunity and multi-organ damage, more research is necessary with regard to this hypothesis. By means of *in sillico* analysis, we investigated whether SARS-CoV-2 peptide sequences could behave similarly to molecular mimics, with a potential to trigger an autoimmune response. The immunogenic potential of the retrieved homologous sequences was validated in terms of their potential to elicit T and B cell responses in terms of their sequential and structural information. In particular, the study comprised the analysis of binding affinity between human leukocyte antigen (HLA)-encoded proteins and the molecular mimics, along with the immunogenicity of continuous and discontinuous predicted B cell epitopes which share ≥ 3 amino acid sequences (viral proteins).

Methods

Protein sequences

SARS-CoV-2 reference protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) database. All of the 28 SARS-CoV-2 proteins were queried when running the BLASTp tool (**Table 1**).

Table 1. List of NCBI database protein accession numbers used in the BLASTp query. Accession numbers presented in bold are SARS-CoV-2 proteins for which the BLASTp program returned significant E values.

Protein Accession Number	Protein name
YP_009724389.1	**ORF1ab polyprotein**
YP_009725295.1	**ORF1a polyprotein**
YP_009724390.1	surface glycoprotein
YP_009724391.1	ORF3a protein
YP_009724392.1	envelope protein
YP_009724393.1	membrane glycoprotein
YP_009724394.1	ORF6 protein
YP_009724395.1	ORF7a protein
YP_009725318.1	**ORF7b**
YP_009724396.1	ORF8 protein
YP_009724397.2	nucleocapsid phosphoprotein
YP_009725255.1	ORF10 protein
YP_009742617.1	nsp10
YP_009742616.1	nsp9
YP_009742615.1	nsp8
YP_009742614.1	nsp7
YP_009742613.1	nsp6
YP_009742612.1	3C-like proteinase
YP_009742611.1	nsp4
YP_009742610.1	**NSP3**
YP_009742609.1	nsp2
YP_009742608.1	leader protein
YP_009725312.1	nsp11
YP_009725311.1	2'-O-ribose methyltransferase
YP_009725310.1	endoRNAse
YP_009725309.1	3'-to-5' exonuclease
YP_009725308.1	helicase
YP_009725307.1	RNA-dependent RNA polymerase

Homology search

The BLASTp online tool was utilized to compare SARS-CoV-2 protein accessions (**Table 1**) to the human proteome, and the query was limited to *Homo sapiens* (taxid: 9606) in the UniProtKB/Swiss-Prot database. Both default and modified parameters (expected threshold modified to 1) were used in the analysis. BLASTp results were visualized using Kablamm. The retrieved human proteins with homologous sequences were queried using the Open Targets online repository for human proteins implicated in the disease in order to establish whether any of the retrieved human proteins have been correlated with either autoimmunity, or general inflammatory phenotypes.

Prediction of B cell epitopes

In order to explore the possibility whether the conserved regions may act as B cell epitopes, the prediction was conducted on the basis of the Immune epitope database (IEDB). From a variety of algorithms available on IEDB, Emini surface accessibility, Kolaskar and Tongaonkar and Bepipred were used. Homologous SARS-CoV-2 amino acid sequences retrieved from the BLASTp results were queried in their full length. Validation of whether the retrieved sequences could act as B cell epitopes on a structural level, was performed using the ElliPro server, which uses a protein data bank (PDB) format as input. PDB crystal structures for each of the 28 SARS-CoV-2 proteins were retrieved from PDB and prepared for docking using PyMOL [48]. ElliPro retrieves both linear and discontinuous antibody epitopes based on the provided crystal structure, using algorithms rooted in the protein shape approximation along with the clustering of neighbouring residues, and the residue protrusion index. Default search parameters were maintained during the utilization of each of the aforementioned tools.

Prediction of T cell epitopes

The selected viral protein sequences were subjected to HLA-II binding analysis on 3/16/2021 using the IEDB analysis resource SMM-Align (ver. 1.1) tool [49]. Outputs generated by the SMM-Align method are given in units of inhibitory concentration 50 nM, therefore, lower IC50nm values indicated high binding affinity. According to the guidelines provided on IEDB, inhibitory concentration (IC) 50 values < 50nM are considered to be binders of high affinity, < 500 nM of intermediate affinity, whereas < 5000 nM of low affinity.

Potential binders (IC50 < 100) generated by the MHC-II binding prediction tool were further analysed and visualized using LigPlot+, a tool which generates 2D ligand-protein interaction diagrams (50). Docking simulations between the potential binders and MHC-II alleles were conducted through the GalaxyPepDock online docking server, using crystal structures of HLA-DRB1 (PDB ID: 6BIN), HLA-DQ2.3 (PDB ID: 4D8P) and HLA-DR (PDB ID: 4H26) (51). Prior to docking simulations, the resident peptide, the accompanying solvent (water), and any other ligands provided within the PDB files, were removed.

Results

Homology between SARS-CoV-2 proteins and human proteins implicated in autoimmune and non-autoimmune conditions

Interestingly, out of the 28 SARS-CoV-2 proteins which were queried against the human proteome, homology was found between the viral open reading frame (ORF) 1a, ORF1ab, ORF7b and the multi-domain non-structural protein 3 (NSP3), as well as the human proteins mono-ADP-ribosyltransferase (PARP14), mono-ADP-ribosyltransferase (PARP9), ADP-ribose glycohydrolase (MACROD1) and the low-density lipoprotein receptor-related protein 2 (LRP2) (refer to **Table 1**).

In terms of the default BLASTp search parameters, ORF1a and ORF1ab and NSP3 were found to have sequences homologous with PARP14, PARP9 and MACROD1 (**Figures 1–9**), whereas homology between ORF7b and LRP2 was obseved only after running a second query where the expected threshold was modified to 1 (**Figure 10**). The homologous regions between ORF7b and LRP2 returned no significant results once analysed for their ability to act as T and B cell epitopes.

PARP9 and PARP14 play pivotal roles in the eukaryotic physiology, and have been strongly implicated in COVID-19. In spite of the fact that PARP protein family is generally relatively obscure with regard to their functionality under normal physiological conditions, both

Table 2. BLASTp results obtained following the query of 38 SARS-CoV-2 proteins against the human proteome

Viral protein	Human protein	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
ORF1a	PARP14	56.2	56.2	2%	5.00E-07	32.26	1801	Q460N5.3
	PARP9	50.1	50.1	2%	3.00E-05	30.99	854	Q8IXQ6.2
	MACROD1	42.7	42.7	3%	0.003	28	325	Q9BQ69.2
ORF1ab	PARP14	56.2	56.2	1%	8.00E-07	32.26	1801	Q460N5.3
	PARP9	50.4	50.4	1%	4.00E-05	30.99	854	Q8IXQ6.2
	MACROD1	42.7	42.7	1%	0.005	28	325	Q9BQ69.2
ORF7b	LRP2	26.2	26.2	51%	0.55	60.87	4655	P98164.3
NSP3	PARP14	57.4	57.4	5%	9.00E-08	32.26	1801	Q460N5.3
	PARP9	51.2	51.2	6%	7.00E-06	30.99	854	Q8IXQ6.2
	MACROD1	41.6	41.6	7%	0.003	27.81	325	Q9BQ69.2

Figure 1. Diseases and phenotypes associated with PARP9

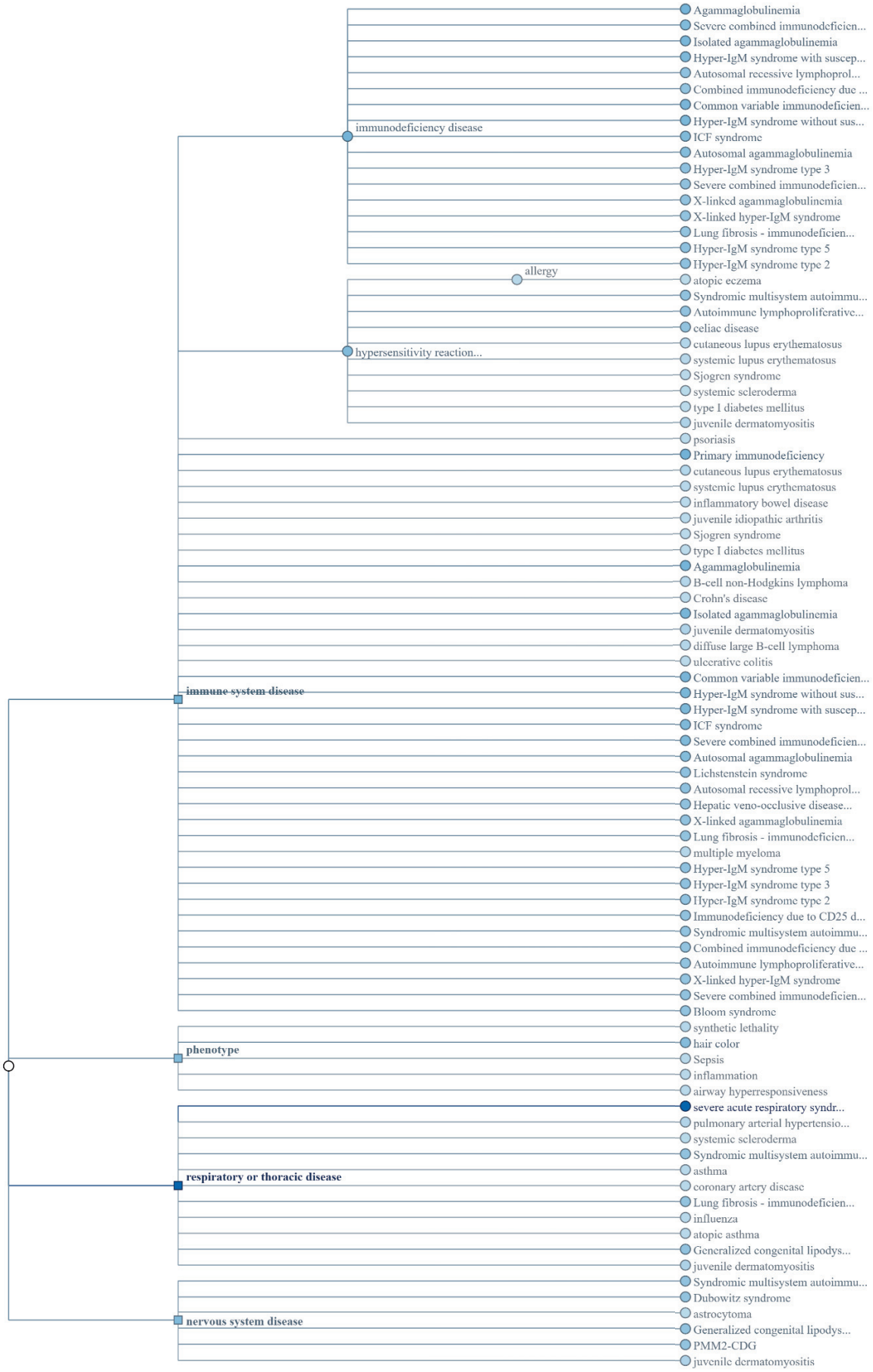

Figure 2. Diseases and phenotypes associated with PARP14

Figure 3. Diseases, phenotypes and biological processes associated with MACROD1

of these proteins hold significant roles in host interferon (IFN)-mediated antiviral defence and DNA repair. Specifically, PARP9 and PARP14 play two opposing roles in IFNγ-induced macrophage activation, where PARP9 promotes IFNγ responses, whereas PARP14 suppresses them by preventing the phosphorylation of STAT1. Additionally, MACROD1 is notably a promiscuous mitochondrial protein that regulates mitochondrial function and plays a role in DNA repair. Although knowledge on the function of MACROD1 remains incomplete, it is highly enriched in tissues which are energetically demanding, such as the heart or the musculoskeletal system. Furthermore, recent studies have correlated MACROD1 and MACROD2 knockouts with neurological dysfunction and cancer. Querying PARP14, PARP9 and MACROD1 in the OpenTargets platform revealed a strong association with a wide spectrum of human diseases spanning across multiple organ systems, a great number of which possess an autoimmune component in their aetiology (**Figures 1–3**).

Potential B cell epitopes

In order to further verify the hypothesis that molecular mimics play a role in COVID-19 associated autoimmunity, we analysed identified homologous sequences for the presence of structural and linear B cell epitopes, which, in turn, measure their ability to stimulate the auto-reactive antibodies production. Interestingly, out of all the proteins containing homologous regions, only NSP3 – a replication/transcription SARS-Cov-2 protein – contained a tripeptide (*LKH*) homologous with the human proteome on a conformational level, at two different regions. Moreover, a search for linear epitopes using the aforementioned three algorithms, did not provide significant results in terms of antibody production potential for the specific conserved sequences (data not shown) (**Figures 4–5**).

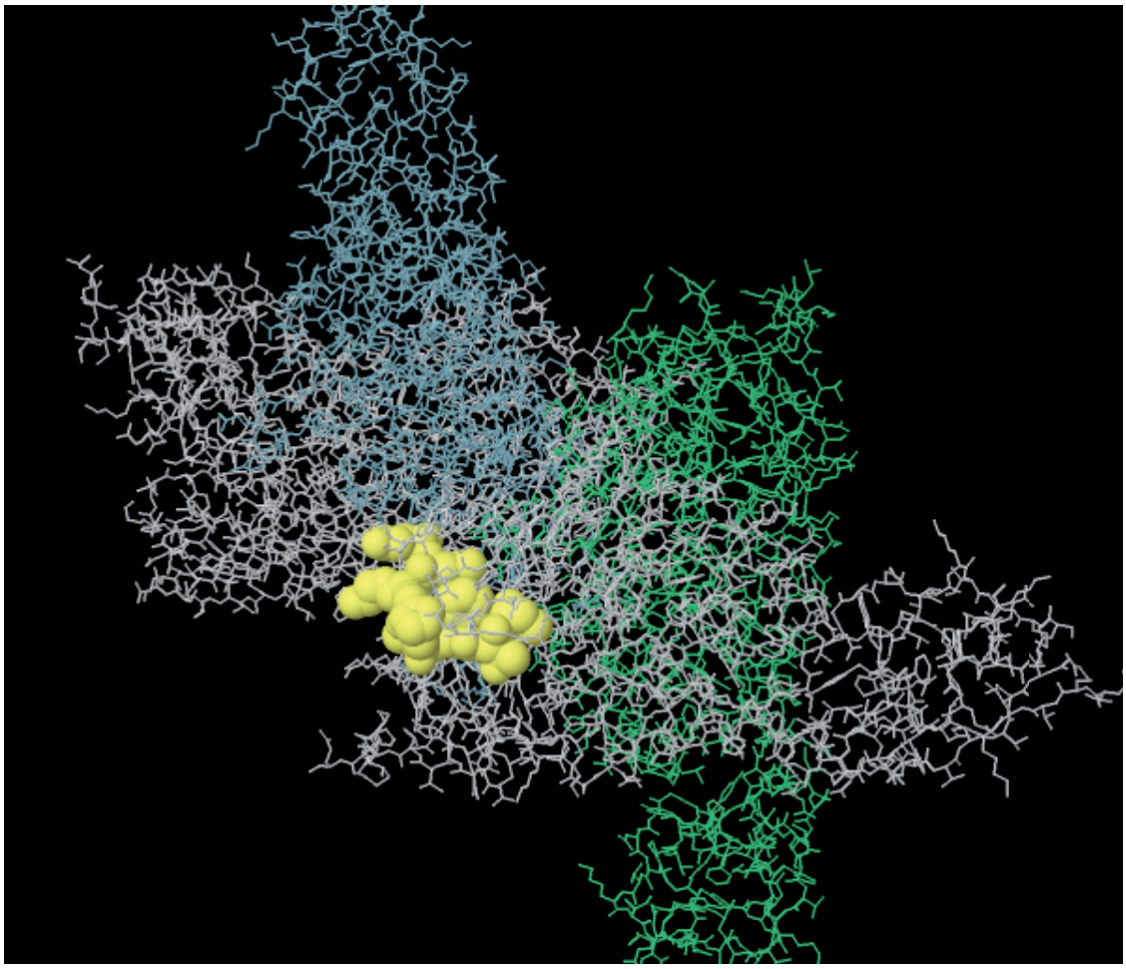

Figure 4. Three-dimensional render of the region within the NSP3 SARS-CoV-2 protein which contains the conformational LKH epitope (highlighted in yellow). The image was rendered using the ElliPro online server

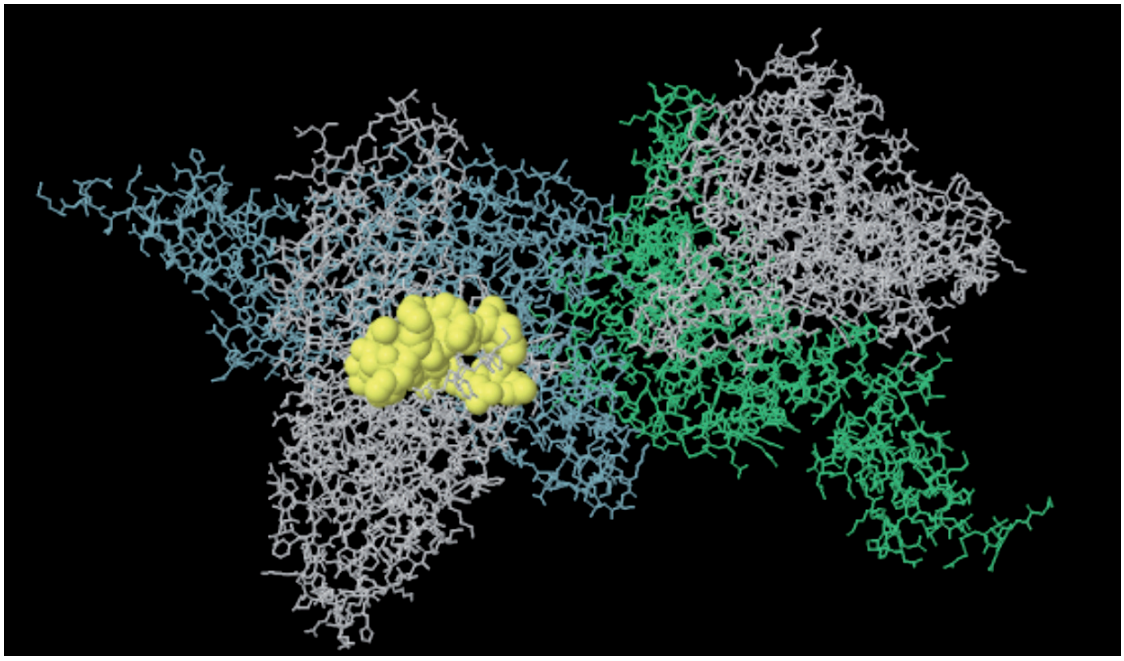

Figure 5. Three-dimensional render of the region within the NSP3 SARS-CoV-2 protein which contains the conformational LKH epitope (highlighted in yellow). The image was rendered using the ElliPro online server

Homologous peptides may act as HLA-II binding motifs

In terms of molecular mimicry, in order for breakdown of immunological tolerance towards self-peptides to occur, the intensity of the immune response elicited by human mimic peptides should presumably be sufficiently similar to that of viral peptides. Therefore, the HLA class II encoded HLA-DR-peptide and HLA-DQ-peptide complex structures were evaluated for their ability to bind both viral-derived and human-derived peptides within the binding groove of HLA. The HLA molecules selected in this study predominantly present exogenous antigens to CD4+ T helper cells (T_h), and their correlation with a broad spectrum of autoimmune disease has been well established [52–58]. In order to determine whether any of the homologous sequences retrieved by

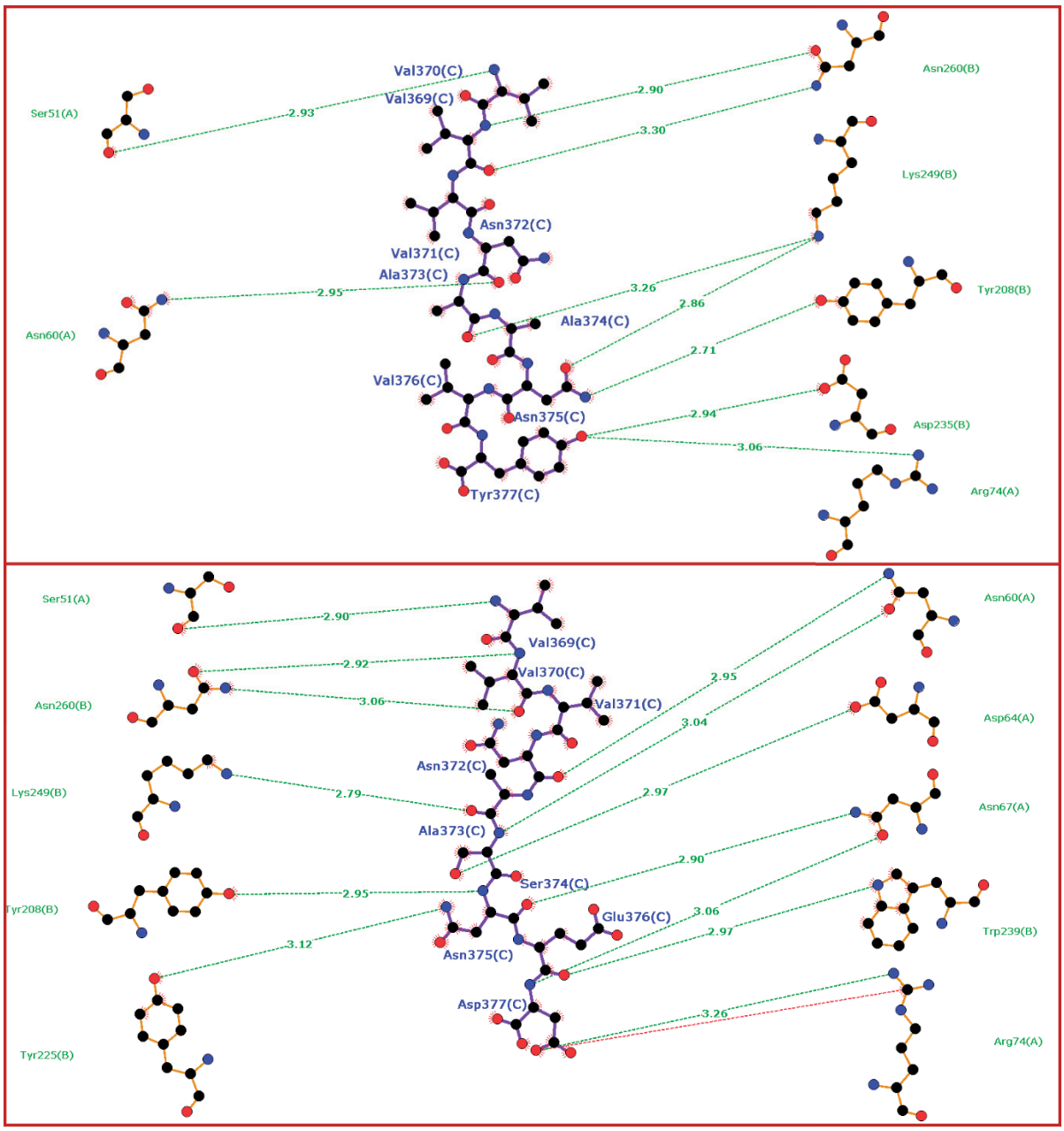

Figure 6. LigPlot-generated diagram of homologous viral (top) and human (bottom) peptides docked with the HLA-DRB1 binding groove. The viral peptide (**VVVNAANVY**) stems from ORF1a, while the human homologue (**VVVNASNED**) stems from MACROD1. Carbon atoms are coloured black, oxygen atoms are coloured red, nitrogen atoms are coloured blue. The bonds between C atoms coloured orange belong to HLA-DRB1 residues, whereas the bonds between C atoms belonging to the ligand peptide are coloured blue. Hydrogen bonds are represented with green lines. The red lines represent salt bridges

the BLASTp query could act as T cell epitopes, potential binders were generated after analysing the sequences using the IEDB analysis resource SMM-Align tool. The SMM-Align tool analysed the sequences for their ability to bind to 12 HLA-DR and 12 HLA-DQ alleles, the results of which are summarized in **Table 2**. Although the SMM-Align method automatically calculates binding energy and thus offers considerable insight into the immunogenicity of the peptide in the context of MHC-II, LigPlot diagrams were generated as to to visualize the key residues which would play a part in these MHC-II-peptide dockings. SMM-Align generates both lone core sequences and core sequences with flanking residues; however, the binding affinity of flanked core sequences is sig-

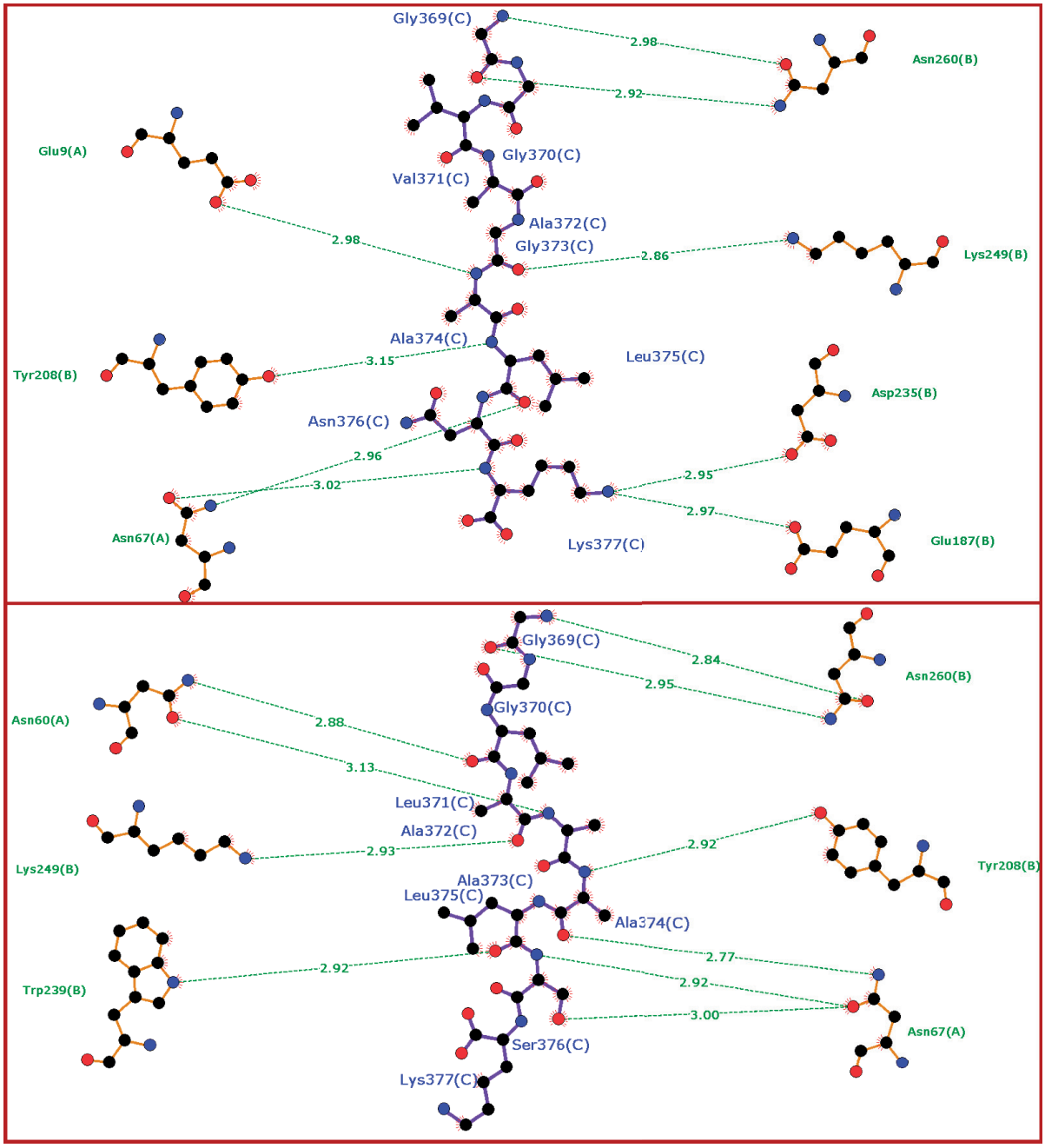

Figure 7. LigPlot-generated diagram of homologous viral (top) and human (bottom) peptides docked with the HLA-DRB1 binding groove. The viral peptide (**GGVAGALNK**) stems from ORF1a, while the human homologue (**GGLAAALSK**) stems from PARP9. Carbon atoms are coloured black, oxygen atoms are coloured red, nitrogen atoms are coloured blue. The bonds between C atoms coloured orange belong to HLA-DRB1 residues, whereas the bonds between C atoms belonging to the ligand peptide are coloured blue. Hydrogen bonds are represented with green lines

nificantly influenced by the core itself (59,60). Hence, both core and flanked core sequences with low IC50 values were docked with the HLA binding groove. Docking simulations with flanked cores can be found as PDB files in **Supplementary File 1**, with the SMM-Align results being available in **Supplementary File 2**. The length of hydrogen bonds between residues in the HLA binding pockets, as well as the peptides themselves were taken as indicators of peptide stability within the pocket binding groove. A distance of ≤ 3.5 Angstroms (Å) was considered indicative of good peptide stability.

The viral peptide *VVVNAANVY* and its human counterpart *VVVNASNED*, docked with HLA-DRB1, display high stability within the binding groove,

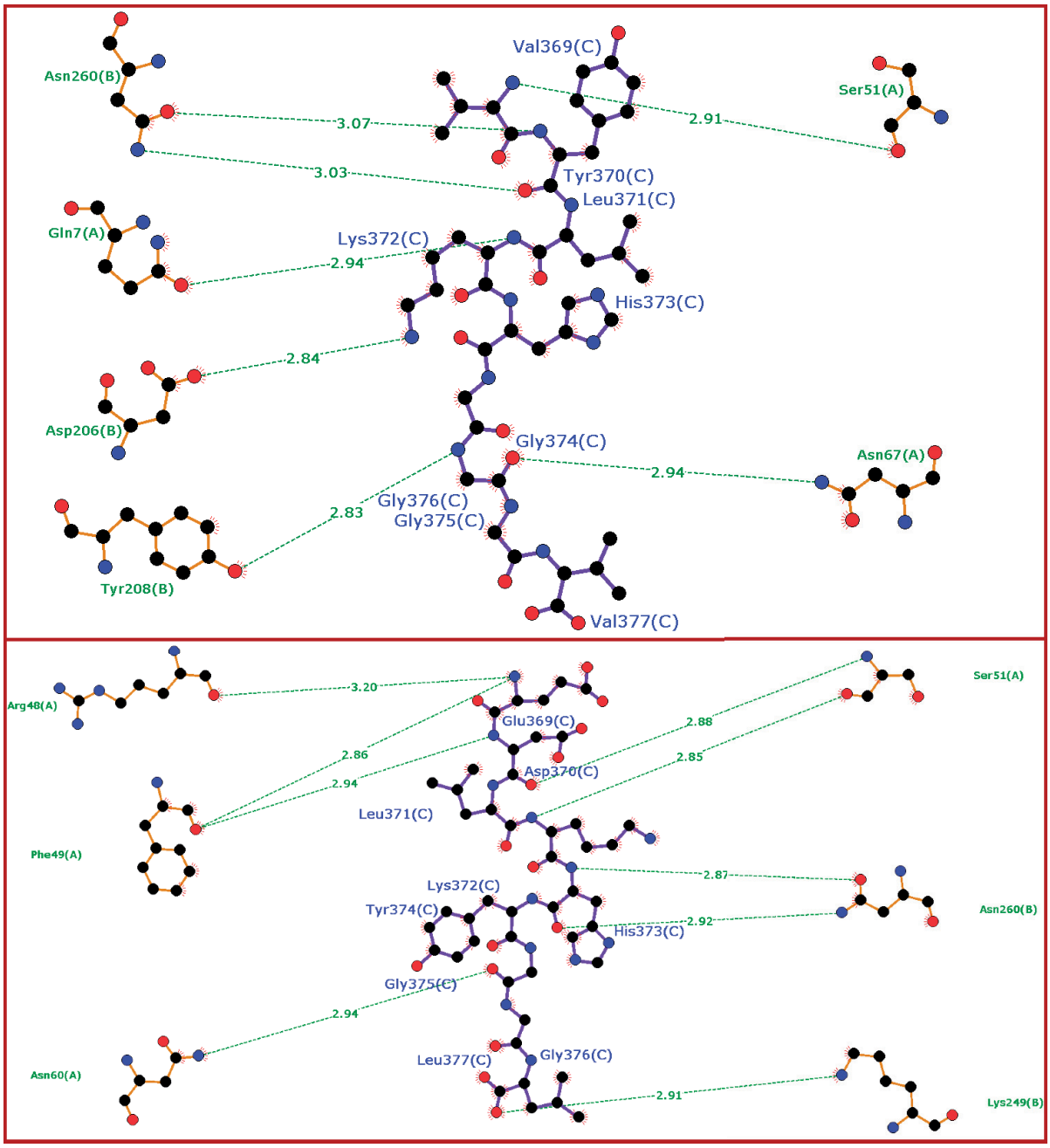

Figure 8. LigPlot-generated diagram of homologous viral (top) and human (bottom) peptides docked with the HLA-DRB1 binding groove. The viral peptide (**VYLKHGGGV**) stems from ORF1a, while the human homologue (**EDLKHYGGL**) stems from PARP14. Carbon atoms are coloured black, oxygen atoms are coloured red, nitrogen atoms are coloured blue. The bonds between C atoms coloured orange belong to HLA-DRB1 residues, whereas the bonds between C atoms belonging to the ligand peptide are coloured blue. Hydrogen bonds are represented with green lines

with a notable number of identical HLA residues (Ser51, Asn60, Asn260, Lys249, Tyr208, Arg74) interacting with both peptides at similar hydrogen bond lengths, within the 3.5 Å threshold (**Figure 6**). Furthermore, the peptides *GGVAGALNK* (viral derived) and *GGLAAALSK* (human derived) demonstrate high stability within the HLA-DRB1 binding groove, similarly interacting with several identical binding groove residues (Asn260, Asn67, Lys249, Tyr208) (**Figure 7**). **Figure 8** presents the docked viral (*VYLKHGGGV*) and human (*EDLKHYGGL*) peptides interact with fewer identi-

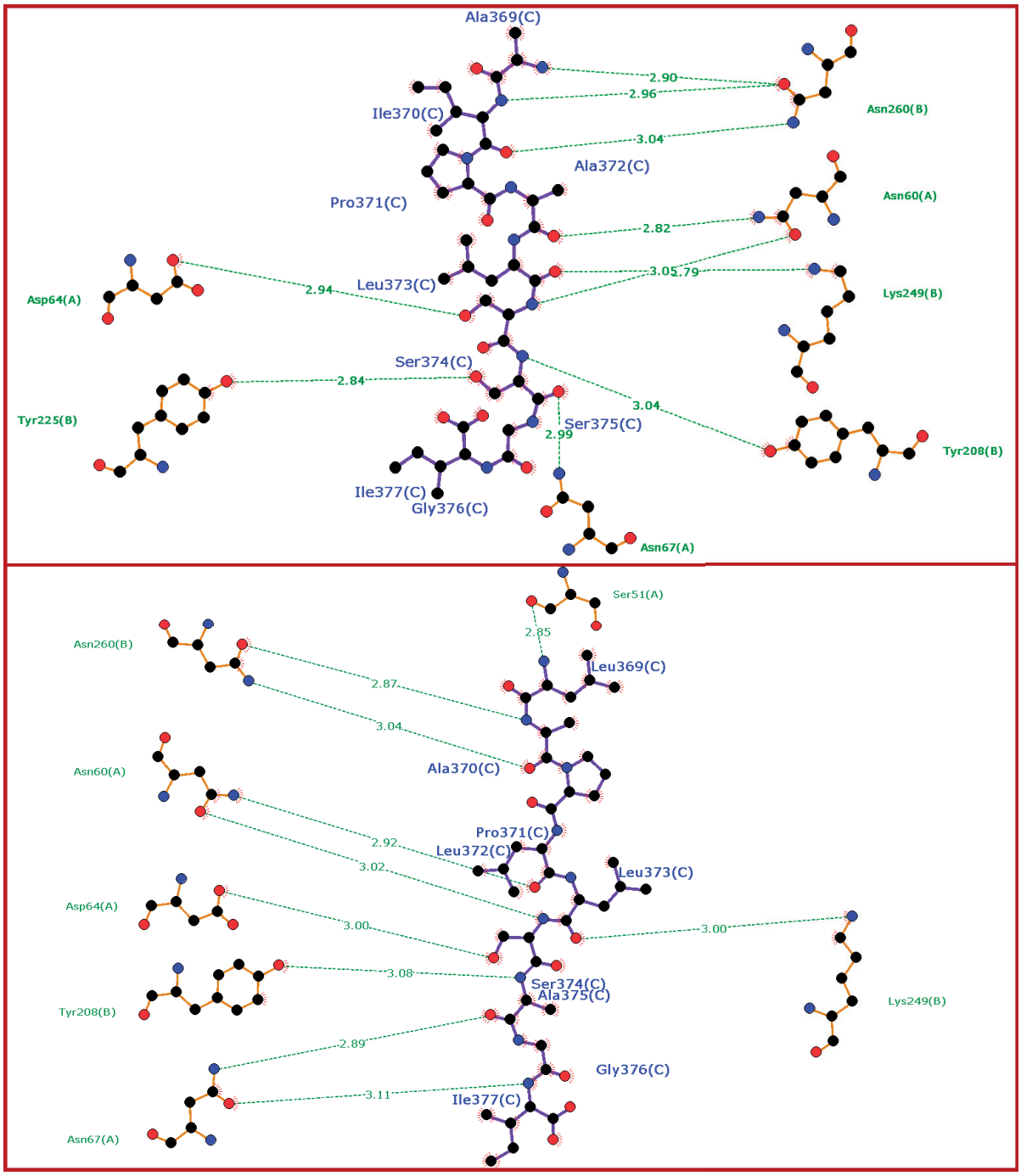

Figure 9. LigPlot-generated diagram of homologous viral (top) and human (bottom) peptides docked with the HLA-DRB1 binding groove. The viral peptide (**LAPLLSAGI**) stems from ORF1a, while the human homologue (**AIPALSSGI**) stems from PARP14. Carbon atoms are coloured black, oxygen atoms are coloured red, nitrogen atoms are coloured blue. The bonds between C atoms coloured orange belong to HLA-DRB1 residues, whereas the bonds between C atoms belonging to the ligand peptide are coloured blue. Hydrogen bonds are represented with green lines

cal residues, namely Asn260 and Ser51; however, the valine present in the viral-derived sequence and the leucine present in the human-derived sequence are functionally equivalent. In contrast, the viral peptide *LAPLLSAGI* and its human homologue AIPALSSGI demonstrate much higher binding homogeneity, with both peptides similarly interacting between identical residues (Asp64, Asn260, Asn60, Lys249, Tyr208, Asn67) at acceptable hydrogen bond lengths. In spite of the evident differences in amino acids which constitute these peptides, they share a high percentage of functionally equivalent amino acids (**Figure 9**).

Discussion

Viral infections have been intensely investigated for their potential to trigger a breakdown of immunological tolerance through the phenomenon of molecular mimicry, where viral protein sequences homologous with sequences of the human proteome may elicit T cell or B cell responses through cross-reactivity. Prior to correlating this process with SARS-CoV-2-mediated autoimmunity, several crucial factors of autoreactivity must be considered. Firstly, autoreactive antibodies are a natural part of the human immunoglobulin repertoire, although their binding affinity towards self-antigens is relatively weak, thus failing to contribute to phenotypes characteristic of autoimmunity. In fact, autoreactive antibodies appear to contribute to numerous homeostatic repair mechanisms, which is probably possible due to their weak binding affinity towards self-antigens. Moreover, autoreactive T cells are equally prominent in healthy individuals and their homeostatic maintenance is conferred through various tolerogenic mechanisms [61, 62]. In spite of the fact that majority of these mechanisms are not completely understood, the correlation between pathogen-induced inflammatory responses and the breakdown of immunological tolerance has been well documented for a number of virological agents. Nevertheless, the breakdown of immunological tolerance implies the dysregulation of the existing auto-reactive T cells (and B cells – for that matter), stimulating the production and subsequent inclusion of the novel auto-reactive T cells into the T cell repertoire. This process may be triggered during viral infections through intermolecular and intramolecular epitope spreading; with molecular mimicry as a prelude to this process. It is vital to note that the same mechanism may apply for B cells that produce autoantibodies, particularly when discussing the role of autoreactive $CD4^+$ cells and their role in autoimmunity overall. However, in either case, arguably the most elegant and definitive examples of epitope spreading stem from research on demyelinating autoimmune conditions, such as multiple sclerosis, and revolve around interactions between professional antigen-presenting cells (APCs), autoreactive $CD4^+$, $CD8^+$ T cells and autoreactive B cells, as key mediators of sustained demyelination [63]. In fact, these key mechanisms appear to be similar in other autoimmune conditions.

The mechanisms by which SARS-CoV-2 molecular mimics may initiate post-infection autoimmunity are multiple, and primarily rooted in the concept of epitope spreading. Based on findings from the previous research on this phenomenon, it may be suggested that local antigen APCs initiate this process when they come to contact with autoreactive T and B cells which had migrated to the inflammation site [63, 64]. This, however, does not exclude dendritic cell (DC)-mediated priming of autoreactive lymphocytes within lymphoid organs. Thus, we suggest that, upon coming to contact with professional APCs presenting SARS-CoV-2 molecular mimics, autoreactive T and B cell subpopulations may promote three distinct and non-mutually exclusive immunopathologic continua, i.e.:

1) the lymphocytes may promote extensive organ damage at the site of infection and immune dysregulation (**Figure 10**) [65];
2) creation of a "fertile field" as a prelude to post-COVID-19 autoimmunity [42, 66–68];
3) initiation of intermolecular and intramolecular epitope spreading (**Figure 11**) [43–45, 64, 69, 70].

The first scenario implies the absorption of SARS-CoV-2, or its fragments, particularly these belonging to the ORF1a polyprotein, from the apoptotic infected cells by resident APCs, such as macrophages and DCs, which possibly depends on TCR specificity [71, 72]. $CD4^+$ cells expressing TCRs which promiscuously interact with self-peptides and their presumed molecular mimics, may immunomodulate macrophages and natural killer (NK) cells to destroy tissues expressing self-pro-

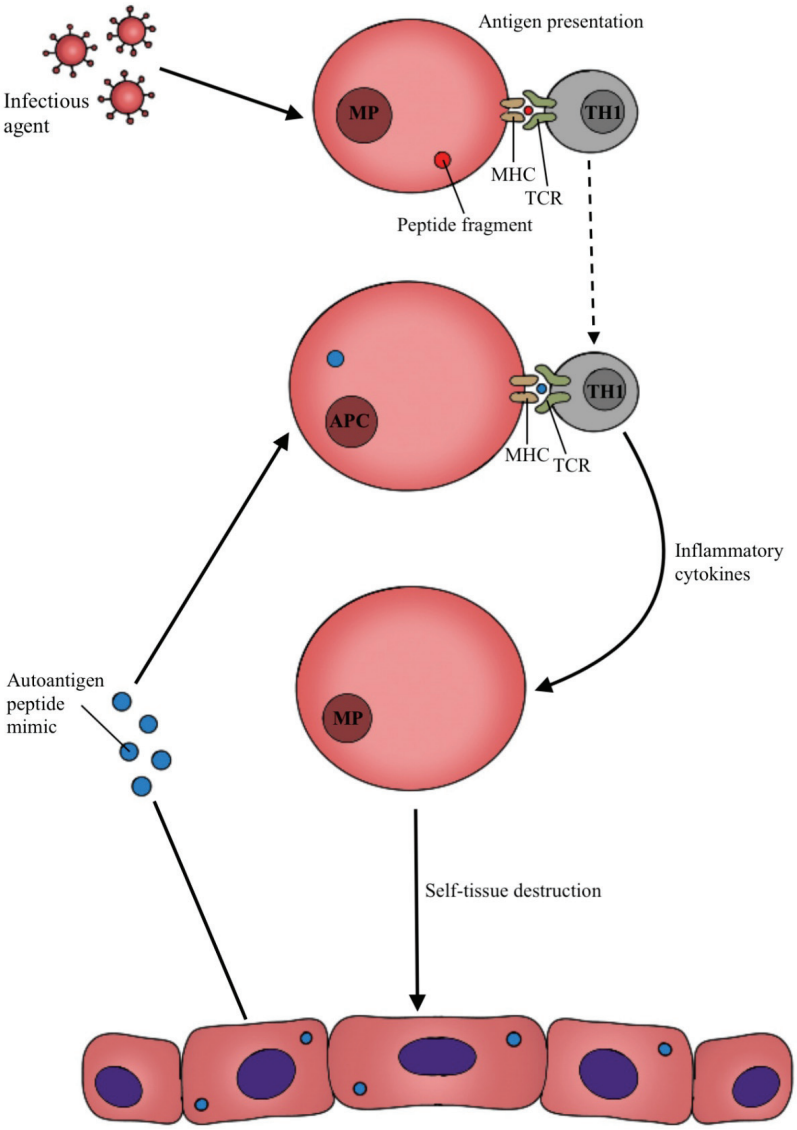

Figure 10. This illustration shows the case of acute self-tissue destruction mediated by the phenomenon of molecular mimicry, during the viral stage of COVID-19. Once a macrophage (MP) has processed the infectious agent, it presents the pathogen peptides containing the molecular mimic, to autoreactive $CD4^+$ T helper cells (TH1) via the major histocompatibility complex (MHC) II. This may be followed by a subsequent interaction between the same $CD4^+$ T helper cell and another antigen-presenting cell (APC) presenting the human-derived peptide mimic. Consequently, this cross-reactivity may prime the autoreactive lymphocyte to modulate self-destructive activities of macrophages via cytokine secretion

teins containing mimic regions; in this case - MACROD1, PARP14 and PARP9 [73–75]. Alternatively, one lymphocyte may express two TCRs which promiscuously interact with two or more molecular mimics, thus, increasing the chance of self-oriented immunopathology and further epitope spreading [61, 76, 77]. Alternatively, a single T cell may interact with a macrophage which presents a human peptide fragment containing the mimic, followed by a subsequent interaction with an APC which presents the viral-derived mimic peptide [78]. Haematological findings from COVID-19 patients support the first scenario, as macrophages and NK cells have been substantially correlated with the extensive immunopathology documented in severe cases, where macrophages comprise for the key mediators of severe cytokine storm and overall pathogenesis

enhancement [79, 80]. Although this process is well within the viral phase of infection rather than within the scope of the post-infection autoimmunity, it adequately complements the current clinical data regarding organ damage associated with COVID-19 patients, and maintains the mimicry hypothesis.

Secondly, cross-reactivity with molecular mimics does not necessarily have to induce an immediate autoimmune response directed towards the tissues which abundantly express MACROD1, PARP14 and PARP9. Alternatively, much as other autoimmunity-associated viruses, SARS-CoV-2 could create a "fertile field" – a term which encompasses molecular mimicry, epitope spreading and viral persistence [81]. This would be induced by the substantial immune dysregulation caused by SARS-CoV-2, where the optimal conditions for autoreactive $CD4^+$ and $CD8^+$ T cells and B cells to become primed against regions within PARP14, MACROD1, PARP9, and potentially other self-proteins, would be created. Nonetheless, whether these primed cells initiate an immune response during the viral stage of COVID-19, likely depends on the level of immunopathology in the course of infection. For instance, an increased pro-inflammatory cytokine expression has been reported as a prerequisite for the creation of the "fertile field" in certain virus-mediated autoimmunity models, and cytokine dysregulation is certainly considered a hallmark of COVID-19 [9, 67, 82, 83]. Once the "fertile field" has been established, subsequent infections with the unrelated pathogens – viral or bacterial – may trigger a profound autoimmune response mediated by the primed autoreactive lymphocytes.

The third scenario, which represents an integrative component of the aforementioned two, entails not only cross-reactivity with PARP9, MACROD1 and PARP14, but also the potential to extend the epitopes containing molecular mimics beyond the ones we have identified for these proteins (intermolecular spreading). In addition, a formation of an immune response against PARP14, MACROD1 and PARP9, may trigger epitope spreading directed towards other proteins, including those belonging to the PARP protein family, or MACROD2 (intramolecular spreading). Consequently, this results in T and B cell epitope diversification, which may account for the fact that a variety of autoimmune diseases have been reported in patients who have recovered from COVID-19 [2, 4, 21, 26, 27, 29, 30, 32, 33, 84].

Concluding, we have identified several homologous regions between the SARS-CoV-2 proteome and the human proteins PARP14, PARP9 and MACROD1, which may potentially behave as molecular mimics. The results obtained from molecular docking simulations between MHC-II and the identified regions, both viral and their

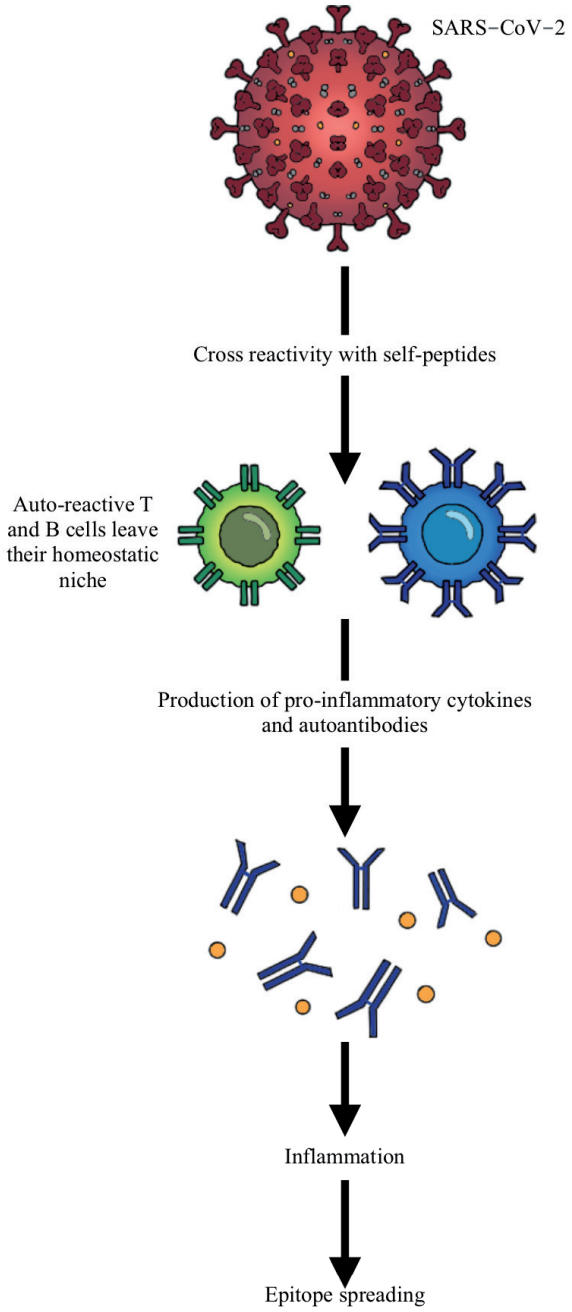

Figure 11. A schematic representation of the process of molecular mimicry-initiated process of epitope spreading, without an immediate initiation of self-tissue destruction

counterparts, prompted the conclusion that these potential mimics may be presented to autoreactive $CD8^+$ and $CD4^+$ T cells, in an identical or similar way to their human homologues, thus priming this lymphocyte subset towards an autoimmune response. Moreover, a prerequisite for these naturally-occurring T cells to leave their homeostatic niche, would be the creation of a "fertile field" by SARS-CoV-2-induced immune dysregulation; particularly in the context of cytokine dysregulation. This may result in either immediate and severe tissue destruction of cells containing the mimic peptides by autoreactive T cell-mediated macrophages, or in priming of the autoreactive repertoire of T cells in the absence of autoimmunity. In this case, these inert, yet primed, lymphocytes, may be prompted to leave their homeostatic niche and direct self-tissue destruction where PARP14, PARP9 and MACROD1 are abundant. In terms of the autoantibodies, although the number of linear and conformational B cell epitopes identified in our research is scarce, the results suggest the possibility that these homologous regions may prompt autoreactive B cells to produce antibodies for these epitopes, particularly the *LKH* tripeptide. Therefore, we were able to develop a plausible case – based on *in sillico* analysis - where molecular mimicry potentially constitutes an aetiological mechanism of SARS-CoV-2-mediated autoimmunity, whether in a multi-organ damage context, or outside the viral phase of infection. This and other studies further support the notion that COVID-19 survivors may be prone to the elicitation or exacerbation of autoimmune conditions, due to the extensive immune dysregulation caused by the virus. Epidemiologically, this may be evaluated by means of a broader epidemiological surveillance of COVID-19 survivors, supplemented by serological cohort studies aimed at detecting autoantibodies and the presence of potential IFN I and IFN III gene mutations which could contribute to post-COVID-19 autoimmunity. The aforementioned findings would be indispensable in understanding the role of SARS-CoV-2 as a potential trigger of autoimmunity. Interestingly, this is the first time that the peptide sequence of MACROD1 has been implicated in COVID-19 autoimmunity. We also confirm that PARP9 and PARP14 may be involved, which is consistent with the previously published findings concerning the effects of these two proteins in COVID-19-related autoimmunity [8]. Moreover, our findings corroborate those of the previous study of homologous sequences between PARP14 and SARS-CoV-2 ADP ribose 1'-phosphate. In fact, our approach focused on structural biology which further enhanced the aforementioned and other bioinformatic studies pertaining to this topic. An encouraging finding of this study is the lack of significant BLASTp results related to the homology between the human proteome and the SARS-CoV-2 S protein, particularly since the S protein sequence forms the antigen employed in the currently-approved COVID-19 vaccines. Furthermore, more extensive *in vivo* research should be conducted with regard to these peptide sequences, in order to determine their relevance for autoimmunity to address the limitations of *in sillico* analysis, which been thoroughly discussed in recent years, particularly with regard to the immunoinformatic approaches. Nevertheless, the limitations of this study need to be taken into account, and the study should be regarded as indicative rather than definitive. As more data are collected to complement the existing immunoinformatic algorithms, along with improvements in molecular docking technology, the precision of such studies shall undoubtedly increase. Presently, the tools used to conduct this study, as well as the results obtained by their use, are of sufficiently high quality to supplement and clarify the hypothesis presented in this paper. However, *in vivo* studies are indispensable to confirm any results obtained within *in sillico* research.

Acknowledgements

Special thanks go to prof. Andrej Gajić from National Geographic, for his continuous support and mentoring throughout this and other works of the author related to tuberculosis; prof. Monia Avdić, Ph.D., from International Burch University, for her mentoring in the subject of immunology and microbiology, and to all friends and colleagues who have supported this work with their unbiased critique.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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ORIGINAL PAPER

JMS Journal of Medical Science

Evaluation of neurological deficiency in rats with cerebral ischaemia following the administration of omega polyunsaturated fatty acids

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DOI: https://doi.org/10.20883/medical.e529

Keywords: cerebral ischaemia, rats, neurological deficiency, omega-3 polyunsaturated fatty acids

Published: 2021-09-21

How to Cite: Bon L, Maksimovich NY. Evaluation of neurological deficiency in rats with cerebral ischaemia following the administration of omega polyunsaturated fatty acids. Journal of Medical Science. 2021 Sep. 21;90(3):e529. doi:10.20883/medical.e529

ABSTRACT

Aim. The aim of the study was to assess the degree of neurological deficit in rats with experimental cerebral ischaemia following the administration of omega-3 polyunsaturated fatty acids.

Material and Methods. The experiments were conducted on 42 male outbred white rats weighing 260 ± 20 g. The modelling of cerebral ischaemia was performed with intravenous thiopental anaesthesia (40-50 mg / kg), and the research involved models of subtotal, partial and stepwise subtotal cerebral ischaemia. Subtotal cerebral ischaemia (SCI) was modelled by simultaneous ligation of both common carotid arteries (CCA). Partial cerebral ischaemia (PCI) was modelled by ligating one CCA on the right. Stepwise subtotal CI (SSCI) was performed by sequential ligation of both CCA within the intervals of 1 day (subgroup 1), 3 days (subgroup 2), or 7 days (subgroup 3). In order to investigate the effects of omega-3 polyunsaturated fatty acids (ω-3 PUFA), animals with CI were injected intragastrically with the drug "Omegamed" (SCI+ω-3 PUFA) at a dose of 5 g / kg of body weight for the duration of one week. The control group consisted of sham-operated rats of the same sex and weight. Neurological deficits were assessed with regard to the "muscle strength", "swimming test" and "open field" tests after 5-6 hours of the ischaemic period.

Results. Following a stepwise bilateral ligation of both common carotid arteries with a 1 day interval, neurological disorders were most prominent, which indicates an aggravation of neurological deficit with a reduction in the time between CCA dressings. In rats with SCI, the changes were more visible than with PCI, although they were less observable than in the group with SCI. The least noticeable changes were noted in the 3rd subgroup (with a 7 day interval between CCA dressings). Research has demonstrated a dependence of the severity of brain damage in SSCI on the interval between the blood supply cessation in both CCA. In the course of a 7 day interval between CCA dressings, the compensatory mechanisms were activated, which prevented the development of morphological changes and neurological deficits. When CCA was ligated within 1 day interval, the degree of neurological deficit was maximal, indicating an insufficient implementation of compensatory mechanisms. In comparison with the control group, the rats of the "SCI+ω3-PUFA" group retained neurological deficit, the muscle strength indicator was decreased by 86% ($p < 0.05$), the swimming

duration by 63% ($p < 0.05$), the number of crossed squares by 55% ($p < 0.05$), the number of washes by 62% ($p < 0.05$), the number of racks by 62.5% ($p < 0.05$) and the number of bowel movements by 60% ($p < 0.05$). However, the neurological deficit was less prominent as compared with the SCI group. In fact, an increase in muscle strength by 67% ($p < 0.05$) was observed, in swimming duration by 37.5% ($p < 0.05$) and in the number of squares crossed in the "open field" test by 31% ($p < 0.05$), which indicates the presence of a corrective action in the ω3-PUFA preparation.

Conclusion. The administration of the preparation of ω-3 polyunsaturated fatty acids has a corrective effect in subtotal cerebral ischaemia, contributing to a reduced severity of the neurological deficit symptoms (an increase in muscle strength, duration of swimming and the number of squares crossed in the "open field" test).

Introduction

Acute disorders of cerebral circulation are one of the most pressing problems in modern medicine. The incidence of strokes varies in different regions of the world from 1 to 4 cases per 1000 individuals per year, increasing significantly with age. Cerebrovascular diseases of ischaemic aetiology tend to increase, rejuvenate, are associated with a severe clinical course, high rates of disability and mortality [1–4]. The primary mechanism by which stroke causes injury is the focal deprivation of blood supply to the cerebral parenchyma. Although a variety of phenomena can result in such ischaemia, large-artery atherosclerosis is most prevalent. In atherosclerosis, accumulations of fatty deposits in the arterial subintima aggregate platelet clumps. These, in turn, attract thrombin, fibrin, and erythrocyte debris which ultimately coagulate to a size which poses a stenotic risk to the cerebral vasculature. Local blood supply stagnation due to a low wall shear stress is thought to predispose certain areas of the vasculature, such as the carotid bulb, to atherosclerotic plaque development. Subsequently, the resulting thrombus deprives cells of the cerebral parenchyma of the required oxygen, resulting in the pathology. Nevertheless, the development of plaque and the consequent stenosis are not necessarily *in situ*. In fact, plaques can also travel to the cerebral circulation from another location, and then they are referred to as emboli. As a result of atrial fibrillation, the heart constitutes their most common source, although they can originate also in other diseased parts of the arterial system.

There are many other pathogenic routes to cerebral ischaemia. In addition to the previously discussed large-vessel infarcts, involving the carotid, vertebral, and basilar arteries, as well as major branches of the circle of Willis, small-vessel (or lacunar) infarcts are also a major aetiology. Most frequently as a result of lipohyalinosis or micro-atheroma, but also occasionally due to the same mechanism by which larger arteries are blocked, the blockage of these small, penetrating arteries running at right angles to the major branches produces the focal deficits characteristic of stroke. Some less frequently observed causes include acute arterial dissection secondary to fibromuscular dysplasia, hematologic disorders such as sickle cell anaemia, and recreational use of cocaine or amphetamines [5, 6].

In order to study the degree of neurological and behavioural disorders in adult animals with cerebral ischaemia, a number of methods can be used: Bederson's test, the test for assessing the modified depth indicators of neurological deficit, the Garcia test, as well as the angular test, the leg extension test, and the "open field" test. They allow for the monitoring of the impaired motor function to register such elements as discoordination, trembling, paresis, paralysis. In terms of the Bederson's test, it is performed in the following manner: the rat is held by the tail at a distance of 1 meter above the floor and the mobility of the forelimbs is monitored. Normally, rats pull their extremities towards the floor. The test involves placing the rats on a slippery smooth surface and pressing gently from the side behind the shoulder until the forelimbs begin to slide, and the animals must equally resist sliding in both directions. In order to asses indicators of the depth of neurological deficit, a scale has been introduced which includes tests for detecting motor activity when hanging an animal by the tail, features of walking

on a horizontal plane, coordination of movements when walking on a beam, the severity of reflexes. On the other hand, the Garcia test includes an assessment of spontaneous activity in the cage for 5 min, the symmetry of the stretching of the forelimbs when the animals are suspended by the tail, the ability to climb the wall of the ethmoid cage, as well as the response to touching each side of the rat's body, and the response to touching vibrissae.

Angle test evaluates space perception disorders and gaze paresis. The rat is placed between two vertical planes. Intact rats easily turn both to the right and to the left, whereas in a number of pathologies, including cerebral ischaemia, neglect is observed, i.e. a state when the animal is not able to perceive a certain part of the space. Furthermore, the "paw extension" test allows to identify and evaluate disorders of the forelimb motor activity. The rat's extremities during the test should hang without support, then it is raised to the edge of the platform so that its vibrissae touch the surface of the plane. The animal is held by hands and pulled to the side on a smooth surface. The number of movements of the forelimbs performed on the side from which the rat is pushed is recorded. Simultaneously, intact rats perform many movements using their front paws.

The "open field" test comprises the assessment of the number of crossed squares, activity in the horizontal and vertical planes, grooming (washing), the number of bowel movements, and the search for depressions and holes for animals [7–13].

One of the most promising concepts of modern science is the search for new methods of stroke prevention and treatment. Omega-3 polyunsaturated fatty acids (ω-3 PUFA), such as eicosapentaenoic acid and docosahexaenoic acid, are widely regarded as vasoprotective. Several large-scale, randomized clinical trials have demonstrated that a dietary intake of omega-3 PUFAs improves the prognosis of patients with symptomatic heart failure, or after a recent myocardial infarction. Omega-3 PUFAs can be incorporated into the phospholipid bilayer of cell membranes and can affect membrane fluidity, lipid microdomain formation, and signalling across membranes. Furthermore, omega-3 PUFAs also modulate the function of membrane ion channels, such as Na and L-type Ca channels, to prevent lethal arrhythmias. Moreover, omega-3 PUFAs also inhibit arachidonic acid conversion into pro-inflammatory eicosanoids by serving as an alternative substrate for cyclooxygenase or lipoxygenase, which results in the production of less potent products. In addition, a number of enzymatically oxygenated metabolites derived from omega-3 PUFAs were recently identified as anti-inflammatory mediators. These omega-3 metabolites may contribute to the beneficial effects against vascular that are attributed to omega-3 PUFAs [14, 16–19].

Aim

The aim of this paper was to assess whether PUFA administration affects the degree of neurological deficit in rats with experimental cerebral ischaemia.

Methods

The experiments were performed on 42 male 3-month-old outbred white rats weighing 260 ± 20 g in compliance with the Directive of the European Parliament and of the Council No. 2010/63 / EU of 22.09.2010 on the protection of animals used for scientific purposes. Modelling of cerebral ischaemia (CI) was conducted with intravenous thiopental anaesthesia (40–50 mg/kg). The research involved models of subtotal (SCI), partial (PCI) and stepwise subtotal (SSCI) cerebral ischaemia. **Table 1** shows the experimental groups and the number of animals included.

Subtotal cerebral ischaemia (SCI) was modelled by simultaneous ligation of both common carotid arteries (CCA). Partial cerebral ischaemia (PCI) was modelled by ligating one CCA on the right. Stepwise subtotal CI (SSCI) was performed

Table 1. Experimental groups

Experimental groups		Number of animals
SCI		6
PCI		6
SSCI	subgroup 1 (1 day)	6
	subgroup 2 (3 days)	6
	subgroup 3 (7 days)	6
SCI + Omega-3 PUFAs		6
Control		6

by sequential ligation of both CCA with an interval of 1 day (subgroup 1), 3 days (subgroup 2), or 7 days (subgroup 3). In order to investigate the effects of ω-3 PUFA, animals with CI were injected intragastrically with the drug "Omegamed" (SCI + ω-3 PUFA) at a dose of 5 g / kg body weight for the duration of one week. The control group consisted of sham-operated rats of the same sex and weight. Neurological deficits were assessed in terms of the "muscle strength", "swimming test" and "open field" tests after 5–6 hours of the ischaemic period. The abovementioned methods were chosen as fairly simple to perform and providing a complete picture of the impairments to the animals' motor activity. "Muscle strength" and "swimming test" components were employed to study physical activity. The "muscle strength" is assessed by placing the rat on a horizontal 60 cm long metal mesh with a centimetre division scale which determines the retention time when the mesh is turned to a vertical position.

To conduct a "swimming test", the animal is placed in a glass reservoir with water (21° C) and the retention time the animal stays on the water surface is determined. The "open field" test is performed for 5 minutes on a flat surface, lined with 36 squares, enclosed around the perimeter.

On the basis of the "open field" test, disorders of motor activity can be observed, registering discoordination, the disappearance of voluntary movements or their limitation. The motor activity of animals in the horizontal plane includes movement in different direction, as well as walking in a circle. In this case, the participation in the movement of all extremities of the rat is evaluated. One crossed square is taken as a unit of movement for the visual registration of activity. Motor activity of rats in the vertical plane is represented by two types of racks: slimbing (climbing) – the hind extremities remain on the surface, and the front extremities rest against the wall of the "open field", and rearing ("rear" – "stand on their hind legs") – the front extremities remain on weight. Grooming can be short – in the form of quick circular movements of the front extremities around the nose and vibrissae, and long – washing the eyes, the area behind the ears, the entire head, paws, sides, back, anogenital region, and tail. Exploring holes in the floor is demonstrated by sniffing the edges or putting a muzzle into the holes.

The study was performed 6 hours after the simulation of the CI. Quantitative continuous data were obtained, which were processed using the licensed computer program Statistica 10.0 for Windows (StatSoft, Inc., USA). Since the experiment used small samples which presented an abnormal distribution, the analysis was conducted on the basis of nonparametric statistics. Data are presented as Me (LQ; UQ), where Me is the median, LQ is the lower quartile value; UQ is the upper quartile value. Differences between groups were considered significant at $p < 0.05$ (Kruskal-Wallis test with Bonferroni's correction).

Results

When assessing the neurological deficit in animals with SCI, a decrease in "muscle strength" by 95% ($p < 0.05$) was observed, and the retention time on the water surface in the "swimming test" decreased by 76% ($p < 0.05$). These data are presented in **Table 2**.

Furthermore, motor activity assessment in the "open field" test also confirmed the development of neurological deficits. When conducting this study, the number of crossed squares decreased by 64% ($p < 0.05$) in comparison with the indicators in animals of the control group, and the number of short washes was reduced by 67% ($p < 0.05$), the number of racks by 62.5% ($p < 0.05$), the number of defecations by 60% ($p < 0.05$). Compared to the control group rats, the animals with PCI demonstrated a decrease in the "muscle strength" indicator and the duration of swimming by 75% ($p < 0.05$) and 41% ($p < 0.05$), respectively. In terms of the "open field" test, the number of crossed squares decreased by 26% ($p < 0.05$), the number of short washes by 33% ($p < 0.05$), the number of "climbing" racks by 25% ($p < 0.05$), the number of defecations by 40% ($p < 0.05$). Prolonged washing and rearing was observed only in intact animals ($p > 0.05$).

The results of behavioural tests indicate the development of a minor neurological deficit in rats with PCI. In comparison with the "control" group, in the 3rd subgroup of SSCI (interval of 7 days), the "muscle strength" indicator decreased by 81% ($p < 0.05$), the duration of swimming by 45% ($p < 0.05$), the number of crossed squares in the "open field" test by 40% ($p < 0.05$), the number

Table 2. Indicators of changes in motor function in rats with cerebral ischaemia, Me (LQ; UQ)

Experimental groups		Muscle strength, min	Swimming test, min
Control		21(20; 23)	21,5(18;25)
SCI		1(1;1) *	5(4;5) *
SSCI	1 sg	1 (1;1) *	5 (4;5) *
	2 sg	3 (3;3) *+	8 (7;9) *+
	3 sg	4 (4;5) *+	12 (12;14) *+
PCI		5(4;5) *+	13(12;15) *+
SCI+ ω-3PUFA		3(2;3) *+	8(7;8) *+

Test "open field"

Experimental groups		Number of squares crossed	Number of short washes	Climbing	Number of defecations
Control		72(64;75)	6,5(5;8)	6,5(5;8)	5(4;6)
SCI		23 (21;23) *	2(1;2) *	3(3;3) *	2(2;2) *
SSCI	1 sg	23 (21;24) *	2 (1;2) *	3 (3;3) *	2 (1;2) *
	2 sg	33 (29;33) *+	3 (2;3) *	4 (3;4) *	3 (2;3) *
	3 sg	43 (41;45) *+	3 (3;4) *+	4 (4;4) *+	3 (2;3) *
PCI		53(52;55) *+	4(3;4) *+	6(5;6) *+	3(3;3) *
SCI+ ω-3PUFA		33 (30;33) *+	3 (2;3) *	4(3;4) *	2(1;3) *

* – p <0.05 compared with the control group; + – p <0.05 compared with SIGM; SCI – subtotal cerebral ischaemia; SSCI – subtotal stepwise cerebral ischaemia; PCI – partial cerebral ischaemia; ω-3PUFA – ω-3 polyunsaturated fatty acids; sg – subgroup

of washes by 54% (p < 0.05), the number of climbing racks by 50% (p < 0.05), the number defecations by 40% (p < 0.05). In the 2nd and 1st subgroups, the changes were more evident. So, the indicator of "muscle strength" decreased by 86 (p < 0.05) and 95% (p < 0.05), swimming duration by 63% (p < 0.05) and 77% (p < 0, 05), the number of squares crossed by 55% (p < 0.05) and 68% (p < 0.05), the number of washes by 54% (p < 0.05) and 69% (p < 0.05), the number of climbing racks by 57% (p < 0.05) and by 62.5% (p < 0.05), the number defecation by 50% (p < 0.05) and by 60% (p < 0.05), respectively. Compared with the 3rd subgroup of SSCI, in the 2nd subgroup the indicator of "muscle strength" decreased by 24% (p < 0.05), the duration of swimming by 33% (p < 0.05), the number of squares crossed in the test "open field" by 24% (p < 0.05), and in the 1st subgroup these indicators were reduced to the greatest extent by 75% (p < 0.05), by 58% (p < 0.05), by 47% (p < 0.05), respectively. In addition, in the 1st subgroup, there was a decrease in the number of washes by 33% (p < 0.05), the number of climbing racks by 25% (p < 0.05) and the number defecations by 33% (p < 0.05), and compared with the 2nd subgroup, there was a reduction in muscle strength by 67% (p < 0.05), swimming duration by 37.5% (p < 0.05) and the number of crossed squares in the "open field" test by 29% (p < 0.05). In the 3rd subgroup of SCI, the indicator of muscle strength and duration of swimming did not change (p > 0.05) in comparison with the "PCI" group, although during the "open field" test, a decrease in the number of crossed squares by 19% (p < 0.05) was observed and the number of racks by 33% (p < 0.05). Compared with SCI, in the 3rd subgroup of SCI with an interval between dressings of both CCA for 7 days, the muscle strength indicator was 75% higher (p < 0.05), the duration of swimming by 58% (p < 0.05), the number of crossed squares by 48% (p < 0.05), the number of washes and climbing racks by 33% (p < 0.05). In the 1st and 2nd subgroups of SCI, the manifestations of neurological deficit were more observable than in rats with PCI: muscle strength index by 40% (p < 0.05) and 80% (p < 0.05), swimming duration by 39% (p < 0.05) and 62% (p < 0.05), the number of squares crossed by 39% (p < 0.05) and 57% (p < 0.05), the number of racks climbing by 42% (p < 0.05) and 50% (p < 0.05), respectively. The number of washings and defecations in the 2nd subgroup did not differ from the values of indicators in the PCI group (p > 0.05), but in the 1st subgroup their number was 50% less (p < 0.05). In the 2nd subgroup of SSCI, in comparison with the group SCI, the indicator of muscle strength was 67% more (p < 0.05), the duration of swimming by 37.5% (p < 0.05), the number of squares crossed by 31% (p < 0.05) and washing by 33% (p < 0.05).

In comparison with the control group, the rats of the "SCI+ω3-PUFA" group retained neurological deficit, the muscle strength indicator was 86% less ($p < 0.05$), the swimming duration by 63% ($p < 0.05$), the number of crossed squares by 55% ($p < 0.05$), the number of washes by 62% ($p < 0.05$), the number of racks by 62.5% ($p < 0.05$) and the number of bowel movements by 60% ($p < 0.05$). However, in comparison with the SCI group, the neurological deficit was less evident. There was an increase in muscle strength by 67% ($p < 0.05$), swimming duration by 37.5% ($p < 0.05$) and the number of squares crossed in the "open field" test by 31% ($p < 0.05$), which indicates the presence of a corrective action in the ω3-PUFA preparation.

Discussion

In the study no differences in the degree of neurological deficit were observed between single-stage SCI and the 1st subgroup of SSCI with a 1 day interval between dressings ($p > 0.05$). With a stepwise bilateral ligation of both common carotid arteries with a 1 day interval, neurological disorders were most noticeable, which indicates an aggravation of neurological deficit with a reduction in the time between CCA dressings. In rats with SCI, the changes were more distinct than with PCI, but less than with SCI. The least prominent changes were noted in the 3rd subgroup (the interval between CCA dressings was 7 days). Studies demonstrated the dependence of the severity of brain damage in SSCI on the interval between the cessation of blood supply in both CCA. At a 7 day interval between CCA dressings, compensatory mechanisms were activated which prevented the development of morphological changes and neurological deficits. When CCA was ligated with an interval of 1 day, the degree of neurological deficit was maximal, which in turn indicates insufficient implementation of the compensatory mechanisms. Therefore, the rats with the experimental CI presented poorer muscle strength, less physical activity, although they showed behavioural disorders. The morphological basis of the revealed changes in CI is damage to the brain neurons as a result of the destabilization of nervous processes (the ratio of excitatory and inhibitory reactions), which affects the implementation of brain functions. In animals with SCI, as well as in the 1st "SSCI" subgroup, more evident disorders were observed in comparison with the 3rd "SSCI" subgroup and the "PCI" group. Therefore, it is possible to claim that with these methods of CI modelling, the adaptation processes occur which prevent the development of distinct morphological changes and allow neurons to adapt to conditions of moderate hypoxia. According to the literature, due to the development of compensatory mechanisms, 7 days after hypoxia resulting from CCA ligation, a tendency is observed to improve microcirculation: capillary patency is restored, their number and diameter increase, leading to an improvement in cerebral blood supply, which is one of the crucial compensation effects, and is based on an increase in the blood vessels density [3]. The corrective effect of polyunsaturated fatty acids on the state of neurons in subtotal cerebral ischaemia may stem from the improvement in the rheological properties of blood due to a decrease in the production of thromboxane A by platelets and to an increase in the tissue plasminogen activator, as well as to an improvement in the neuronal membrane fluidity, and a decrease in blood viscosity. Interestingly, not all Omega-3 PUFAs trials have shown reductions in vascular disorders. However, several adequately powered observation and intervention trials have strongly supported the efficacy of Omega-3 PUFAs for the prevention of vascular disorders. Furthermore, experimental studies have revealed multiple underlying molecular mechanisms, including membrane modification, attenuation of ion channels, regulation of pro-inflammatory gene expression, and the production of lipid mediators. It remains unclear which mechanism contributes most to the cardioprotective effects of Omega-3 PUFAs observed in vivo; nevertheless, the pleiotropic anti-inflammatory effects of Omega-3 PUFAs could be valuable, particularly in the setting of atherosclerosis and cardiac remodelling. Although further research is necessary to address the molecular relationship between Omega-3 PUFAs and vascular pathology, it might be useful to consider bioactive Omega-3 PUFA-derived metabolites, such as 18-hydroxyeicosapentaenoic acid, as endogenous anti-inflammatory molecules and potential new therapeutic targets for vascular disorders [17–19].

In addition, omega 3-PUFAs also show an anti-inflammatory effect due to their incorporation into the phospholipid layer in cell membranes of monocytes, leukocytes, endothelial cells, which is accompanied by a decrease in the production of inflammatory mediators and a decrease in the adhesion of leukocytes to the endothelial wall. Moreover, polyunsaturated fatty acids, affecting the synthesis of prostaglandins, regulate vascular tone and prevent catecholamine-induced vasoconstriction, which results in a moderate hypotensive effect [15, 16].

Conclusion

Concluding, the severity of neurological deficit depends on the severity of the ischaemic injury. The most severe consequences occurred with subtotal one-stage ischaemia and stepwise ischaemia with a minimum 1 day interval between arterial ligation. In contrast, stepwise ischaemia with an interval between dressings of 7 days and partial ischaemia did not lead to such pronounced disorders of the neurological status. The introduction of the ω-3 polyunsaturated fatty acid preparation shows a corrective effect in subtotal cerebral ischaemia, contributing to a decreased severity of neurological deficit symptoms (an increase in muscle strength, duration of swimming and the number of squares crossed in the "open field" test).

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
State scientific program «To study the processes of damage and adaptation of the brain during its ischaemia and the use of correction»..

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ORIGINAL PAPER

JMS Journal of Medical Science

Maternal and foetal outcome of pregnancy in women with connective tissue diseases

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DOI: https://doi.org/10.20883/medical.e525

Keywords: systemic connective tissue diseases, perinatal outcomes, pregnancy complications

Published: 2021-09-28

How to Cite: Klimaszyk K, Wender-Ożegowska E, Kędzia M. Maternal and foetal outcome of pregnancy in women with connective tissue diseases. Journal of Medical Science. 2021 Sep. 28;90(3):e525. doi:10.20883/medical.e525

ABSTRACT

Introduction. The majority of patients diagnosed with systemic connective tissue diseases are women of childbearing age.
Aim. The analysis of obstetric results and exacerbation rates in the pregnancies involving systemic connective tissue diseases.
Material and Methods. We retrospectively reviewed perinatal outcomes of fourteen women with systemic connective tissue diseases hospitalised in the Department of Reproduction in the period between September 2019 and July 2021.
Results. Median duration of a pregnancy was 37 weeks. One pregnancy ended in a stillbirth in week 28. Of the 13 live births, preterm delivery occurred in 5 cases. Of the 13 live-birth neonates, preterm delivery occurred in five cases. The Caesarean section rate was 57.1% and vaginal delivery rate was 42.9%. The mean birth weight of the live neonates was 2787g (SD 892), and the median Apgar score in the 1st and the 3rd minute was 10.
In total, all 4 patients with the active disease at the time of conception and 1 who did not decide to undergo the recommended mitral valve surgery prior to pregnancy experienced symptoms indicating a disease flare-up in the course of pregnancy. None of the patients who planned their pregnancy experienced an exacerbation of the disease.
Conclusions. All patients diagnosed with systemic connective tissue diseases should receive multidisciplinary care prior to conception, during pregnancy and in the postpartum period. Furthermore, they should be monitored by a team of specialists, due to the risk of a disease exacerbation and high rates of maternal and foetal complications resulting from the underlying condition.

Introduction

Systemic connective tissue diseases are a group of autoimmune diseases which occur significantly more frequently in women than in men [1]. The majority of the diagnosed patients are women of childbearing age.

Their prevalence ranges from 2–4 per 10,000 people in terms of the most common rheumatoid arthritis, to 5–10/100,000 in scleroderma and systemic lupus. In fact, other systemic diseases are even less common [1].

Various large retrospective studies have proven an increased rate of adverse pregnancy outcomes, such as hypertensive disorders of pregnancy (gestational hypertension and pre-eclampsia), foetal growth restriction (FGR), premature delivery in patients with rheumatoid arthritis, as well as systemic lupus erythematosus [2–4]. However, the effects of other systemic connective tissue diseases have not been as extensively characterised.

The disease activity, extra-articular symptoms, and patient antibody status play a vital role in the risk stratification of these pregnancies. In fact, foetal atrioventricular block and the neonatal lupus syndrome constitute one of the most characteristic neonatal complications of pregnancy in women suffering from Sjögren's-syndrome-related antigen A (anti--SSA) and/or antigen B (anti-SSB) autoantibody-positive [5].

In the past, women with rheumatoid arthritis, systemic lupus erythematosus, systemic scleroderma, or the mixed connective tissue disease have been advised not to become pregnant due to the risk of a disease flare-up, adverse pregnancy outcomes, and the lack of evidence for safe treatment options during pregnancy. Nevertheless, with the increased knowledge regarding the management of patients with systemic connective tissue diseases, obstetric outcomes in these populations have also improved [6]. These advances highlight the importance of careful planning of the conception, multidisciplinary monitoring and treatment provided for women in this patient group.

Aim

The aim of the study was to analyse the obstetric results and exacerbation rates in the pregnancies of patients suffering from systemic connective tissue diseases.

Material and Methods

We retrospectively reviewed the pregnancy outcomes of fourteen women with systemic connective tissue diseases at the Department of Reproduction for the period between September 2019 and July 2021.

The inclusion criteria of our retrospective observational study were the following: pregnant women diagnosed with diseases classified by the American Rheumatism Association as one of the systemic connective tissue diseases, who were under the care of the Department of Reproduction, Department of Obstetrics, Gynecology, and Gynecological Oncology, Poznan University of Medical Sciences, Poland. All study participants were followed up at regular intervals, with clinical and laboratory assessments during the entire term of the pregnancy. In patients with anti--SSA or anti-SSB antibody, an ultrasound assessment of foetal atrioventricular time intervals was performed.

The data analysed in this study were collected from the paper and electronic medical records of the study participants. Among these 14 women, 3 suffered from systemic lupus erythematosus, 3 from rheumatoid arthritis, 3 from the mixed connective tissue disease, while 2 had been diagnosed with overlap syndrome of rheumatoid arthritis and systemic lupus erythematosus, 1 suffered from the primary Sjögren's syndrome, 1 from

Table 1. Diagnoses of patients

Diagnosis	Number of patients
Systemic lupus erythematosus	3 (21.4%)
Rheumatoid arthritis	3 (21.4%)
Mixed connective tissue disease	3 (21.4%)
Overlap syndrome of rheumatoid arthritis and systemic lupus erythematosus	2 (14.3%)
Primary Sjögren's syndrome	1 (7.1%)
Systemic scleroderma	1 (7.1%)
Dermatomyositis	1 (7.1%)

systemic scleroderma, and 1 presented with dermatomyositis (**Table 1**).

The mean age of the patients was 30 years (SD 5.9), whereas the mean age at the time of systemic connective tissue disease diagnosis was 23.6 years (SD 6.6). The average disease duration prior to the current pregnancy was 6.4 years (SD 4.0).

In the group of fourteen analysed pregnancies, one was a dichorionic diamniotic twin pregnancy, and thirteen were singletons. Eight pregnancies (57.1%) in the study group were the first pregnancies for the patients, four (28.5%) were the second, one was the third (7.1%), and one was the fourth (7.1%). Three patients (21.4%) had a history of miscarriages prior to the current pregnancy (**Table 2**).

The following pregnancy outcomes were analysed: premature birth, stillbirth, number of women with preeclampsia, gestational week of delivery, Caesarean section rate, birth weight, Apgar score in the 1st and the 3rd minute, number of infants with FGR, and number of infants hospitalised in the Neonatal Intensive Care Unit (NICU).

In the study, the following definitions with regard to a pregnancy outcome were adopted:

- Spontaneous abortion - spontaneous loss of a foetus before the 22nd week of gestation; premature birth (preterm) – live birth before the 37th week of gestation. Stillbirth was defined as the death of a viable foetus (after the 22nd week of gestation).
- Preeclampsia - the evidence of proteinuria (> 300 mg/24 hours) and hypertension, with or without oedema, in patients with normal blood pressure and no evidence of proteinuria prior to 20 weeks of gestation, or a significant increase in blood pressure and proteinuria, or new abnormalities in platelet count, or liver enzyme levels in the presence of pre-existing hypertension and proteinuria.
- FGR – according to the Delphi consensus: for early FGR (< 32 weeks), three solitary parameters (abdominal circumference (AC) < 3rd centile, estimated foetal weight (EFW) < 3rd centile and absent end-diastolic flow in the umbilical artery (UA)), and four contributory parameters (AC or EFW < 10th centile combined with a pulsatility index (PI) > 95th centile in either the UA or uterine artery). For late FGR (≥ 32 weeks), two solitary parameters (AC or EFW < 3rd centile) and four contributory parameters (EFW or AC < 10th centile, AC or EFW crossing centiles by > two quartiles on the growth charts and cerebroplacental ratio < 5th centile or UA-PI > 95th centile).

Table 2. Characteristics of patients. The numbering of cases in Table 2 corresponds to the numbering of cases in Table 3

Case no.	Diagnosis	Obstetric history (term births-preterm births- abortions)	Age at the time of diagnosis (years)	Disease duration (years)	Pregnancy planning
1.	Rheumatoid arthritis	(0–0–1)	19	6	No
2.	Systemic lupus erythematosus	(0–0–0)	15	4	No
3.	Overlap syndrome of rheumatoid arthritis and systemic lupus erythematosus	(0–0–0)	31.5	0.5	No
4.	Mixed connective tissue disease	(0–1–0)	39	5	No
5.	Systemic lupus erythematosus	(0–0–3)	20	12	No
6.	Overlap syndrome of rheumatoid arthritis and systemic lupus erythematosus	(0–0–0)	28	6	Yes
7.	Rheumatoid arthritis	(0–0–2)	20	16	Yes
8.	Systemic lupus erythematosus	(0–0–0)	24	7	Yes
9.	Rheumatoid arthritis	(0–0–0)	18	6	Yes
10.	Primary Sjögren's syndrome	(0–0–0)	31	3	Yes
11.	Mixed connective tissue disease	(0–0–0)	23	6	Yes
12.	Systemic scleroderma	(0–0–0)	26.5	0.5	Yes
13.	Dermatomyositis	(1–0–0)	15	10	Yes
14.	Mixed connective tissue disease	(1–0–0)	21	7	Yes

Results

We identified fourteen women with systemic connective tissue diseases treated in the Department of Reproduction for the period between September 2019 and July 2021.

Ten pregnancies (71.4%) occurred during the period of disease remission, and four of these pregnancies began while the patient was administered corticosteroid medications (prednisone or methylprednisolone).

One patient (7.1%), with the remission of systemic lupus erythematosus 9 months prior to conception, dediced not to undergo the recommended mitral valve surgery before pregnancy.

Five of the pregnancies (37.7%) were unplanned, and in three of these cases, drugs which are contraindicated in pregnancy were administered in the first trimester, including methotrexate in two cases and mycophenolate mofetil in one case.

Effects of systemic connective tissue diseases on pregnancy

Median duration of pregnancy was 37 weeks. One pregnancy ended in the 28th week of gestation with stillbirth, whereas a preterm delivery occurred in five cases (38.5%) out of the 13 live birth pregnancies. In the 36th week two of these pregnancies were associated with a premature rupture of membranes, whereas one twin pregnancy in the 32nd week was linked to the inevitable labour. Moreover, in one pregnancy in the 32nd week we observed symptoms of foetal distress in CTG and high bile acid levels as a result of intrahepatic cholestasis. Additionally, in yet another one in the 30th week we found increasing respiratory insufficiency of the patient (**Table 3**). Nevertheless, two patients could not receive the recommended prophylactic dose of acetylsalicylic acid, one due to the acetylsalicylic acid induced asthma, and one due to thrombocytopenia. In the study, Caesarean section rate was 57.1%, and the vaginal delivery rate was 42.9%. Furthermore, three pregnancies (21.4%) occurred in patients with lupus nephropathy, with two ending before the 37th week. Antiphospholipid syndrome was diagnosed in one woman (7.1%) with an obstetric history of three miscarriages, whereas preeclampsia occurred in one patient (7.1%) (**Table 4**).

Effects of systemic connective tissue diseases on the neonatal outcome

The mean live neonatal birth weight was 2787 g (SD 892) and the median Apgar score in the1st and the 3rd minute was 10.

There was one stillbirth in the 28th week of gestation in a patient with the mixed connective tissue disease, pregestational diabetes classes RF/ H, multidrug resistant hypertension with renal, cardiovascular and proliferative retinopathy complications. This pregnancy was unplanned, began when the patient was taking methotrexate, and the disease exacerbation occurred in the 15th week of gestation. The neonate's birth weight was 760 g, below the 1st percentile for the gestational age. In terms of other disorders, there were four cases (28.6%) of FGR and no cases of neonatal lupus or congenital complete atrioventricular block. Four infants (26.7%) were hospitalised in the neonatal intensive care unit (**Table 3**).

Effects of pregnancy on systemic connective tissue diseases

In total, all four patients with an active disease at the time of conception and one who would not undergo the recommended mitral valve surgery before pregnancy experienced symptoms indicating a disease flare-up during pregnancy. The patients presented with a lupus nephritis proteinuria level which increased from 0.5 g/24h before pregnancy to 2 g/24h in the first trimester. Five women experienced the exacerbation of articular symptoms, including two patients diagnosed with systemic lupus erythematosus, one with rheumatoid arthritis, one with the mixed connective tissue disease, and one with the overlap syndrome of rheumatoid arthritis and systemic lupus erythematosus. The patient suffering from rheumatoid arthritis needed an intra-articular injection of corticosteroids. The patient with systemic lupus erythematosus who did not decide to undergo the recommended mitral valve surgery before pregnancy developed respiratory insufficiency with pulmonary oedema, due to the exacerbation of mitral regurgitation in the complex defect of the mitral valve, whereas the pregnancy in the case of the patient with the mixed connective tissue disease pregnancy ended with stillbirth.

Table 3. Detailed characteristics of pregnancy and foetal outcomes of women with connective tissue diseases. The numbering of cases in Table 2 corresponds to the numbering of cases in Table 3

Case no.	Gestational age (weeks)	Route of delivery	Indication for Caesarean section	Pharmacotherapy during pregnancy	Sex of neonate birth weight (g); Apgar score	NICU admission	FGR
1.	37	cs	exacerbation of articular symptoms	– methotrexate continued until the 3rd week of pregnancy – methylprednisolone – enoxaparin – hydroxychloroquine – acetylsalicylic acid	M;2900 g; 10,10	No	No
2.	38	vag	-	– mycophenolate mofetil continued until the 6th week of pregnancy – hydroxychloroquine – methylprednisolone azathioprine – levothyroxine – acetylsalicylic acid	F;2200 g; 10,10	No	Yes
3.	38	cs	exacerbation of articular symptoms	– methylprednisolone – nadroparin – methyldopa – salbutamol – fluticasone	M;2980 g; 10,10	No	No
4.	28	still-birth-cs	high blood pressure and risk of uterus rupture during labour induction due to previous Caesarean section	– methotrexate continued till the 5th week of pregnancy – methylprednisolone insulin glargine – insulin lispro – methyldopa – labetalol – nitrendipine – nadroparin – acetylsalicylic acid levothyroxine	M;760 g; 0,0	No	Yes
5.	30	cs	increasing respiratory insufficiency	– hydroxychloroquine prednisone – nadroparin – acetylsalicylic acid verapamil – levetiracetam – oxcarbazepine	M;1380 g; 8,8	Yes	No
6.	37	cs	lack of labour progress	– hydroxychloroquine – acetylsalicylic acid – levothyroxine	F;3220 g; 10,10	No	No
7.	32	cs	symptoms of foetal distress in CTG and high bile acid levels as a result of intrahepatic cholestasis	– hydroxychloroquine – methyldopa – verapamil – acetylsalicylic acid – enoxaparin – ursodeoxycholic acid – sulfasalazine	F; 1490 g; 8,8,9	No	Yes
8.	39	cs	to lack of progress in labour	– methylprednisolone – nadroparin – acetylsalicylic acid	M;3760 g; 10,10	No	No
9.	32	cs	inevitable labour and breech presentation of a first foetus in a twin pregnancy	-	F;1746 g;7,7,8	Yes	No
					M;1960 g; 7,6,8	Yes	No
10.	36	vag	-	– hydroxychloroquine – acetylsalicylic acid	F;2840 g; 10,10	No	No
11.	36	vag	-	– hydroxychloroquine – methylprednisolone – levothyroxine	M;2065 g; 10,10	Yes	Yes
12.	39	vag	-	– hydroxychloroquine – acetylsalicylic acid insulin aspart	M;3360 g; 9,10	No	No
13.	38	vag	-	– methylprednisolone acetylsalicylic acid – human insulin	F;4220 g; 10,10	No	No
14.	39	vag	-	– hydroxychloroquine – enoxaparin – acetylsalicylic acid	M;3960; 10,10	No	No

cs – Caesarean section; vag – vaginal delivery; M – male; F – female; NICU – Neonatal Intensive Care Unit; FGR – foetal growth restriction

Table 4 Perinatal outcomes of patients with systemic connective tissue diseases

No. of pregnancies	14
Stillbirths	1 (7.1%)
Premature deliveries (< 37 week)	5 (38.5%)
Term deliveries (≥37 week)	8 (61.5%)
Gestation duration (median)	37 weeks (min 28; max 39)
Birth weight mean, (SD)	2787 g (892)
Apgar score at the 1st minute (median)	10
Apgar score at the 3rd minute (median)	10
NICU admission	4 (26.7%)
Foetal Growth Restriction	4 (28.6%)
Preeclampsia	1 (7.1%)

Discussion

In our study, we described the courses of pregnancy and obstetric results in fourteen women with systemic connective tissue disease.

We found that all patients with active disease at the beginning of their pregnancy experienced the disease exacerbation during the pregnancy. The exacerbation involved articular symptoms, an increase in the proteinuria level, and pulmonary oedema, due to deterioration of the mitral valve function. As a result, four out of five patients gave birth by Caesarean section resulting from the exacerbation of the disease. One pregnancy ended in a vaginal delivery of the child with FGR. Moreover, two preterm deliveries occurred, one due to increasing respiratory insufficiency of the patient in the 30th week of gestation, and one due to the intrauterine death of a 780 g foetus in the 28th week of gestation.

Of the nine patients with systemic connective tissue disease who planned their pregnancy during a remission period, none experienced the exacerbation of their underlying disease during pregnancy. In this group, four women gave birth prematurely, stemming from the reasons not related to the underlying disease (two experienced pPROM in the 36th week of gestation, one underwent Caesarean section due to inevitable preterm labour in the 32nd week of gestation in a twin pregnancy, and another ended in the 32nd week due to increasing cholestasis and pre-eclampsia.

Multiple previous studies have confirmed an increased risk for the mother, as well as foetal adverse pregnancy outcomes in women suffering from systemic connective tissue diseases. In fact, the authors of the study published in 2006 analysed the hospitalisations of 3,264 pregnant women with systemic lupus erythematosus, and 1,425 pregnant women with rheumatoid arthritis. The results of this analysis demonstrated a more than threefold increase in the risk of hypertension and IUGR compared to the general population [7]. Furthermore, Zucchi et al. retrospectively assessed the course of 100 pregnancies in 81 pregnant women with undifferentiated connective tissue disease between 2000–2018. Obstetric complications occurred in 26 out of 89 pregnancies which ended in a live birth (29%), including 1 case (1%) of pre-eclampsia. In contrast, in other cases, a single pregnancy was affected by more than one complication. The stillbirth rate was 11%. In 13 cases, there was the exacerbation of the underlying disease, with 3 cases which included the development of systemic lupus erythematosus with renal involvement [8]. In our study, 5 patients (35.7%) out of 14 experienced the exacerbation of the underling disease, two of those cases included patents with lupus nephritis diagnoses.

The increased risk of an abnormal course of pregnancy in patients with systemic connective tissue diseases was not only due to the underlying disease, but also to the comorbidities or complications which occurred in the course of pregnancy. In 2019, Radin et al. assessed the obstetric outcomes of women with the mixed connective tissue disease in a multicentre study. Data for the 2000–2017 year period included 203 pregnancies in 94 women and were analysed retrospectively. The live birth rate was 71.9%, the stillbirth rate was 8.9%, and FGR rate 5.4% [9]. In the analysis of our group of patients, the rate of

live births and FGR was significantly higher than in the cited study, and were, respectively, 92% and 28.6%.

Nevertheless, in the last 50 years, the incidence of pregnancy morbidity in women with systemic lupus erythematosus has significantly decreased from 40% to < 15% [10]. The main cause for this improvement is associated with the pre-conception counselling, including the advice to choose a period of the disease quiescence for conception. Furthermore, the identification of the high risk factors related to pregnancy complications (a history of lupus nephritis, the presence of SSA and/or SSB antibodies and the coexistence of antiphospholipid syndrome), followed by appropriate monitoring of those patients is also essential.

The European League Against Rheumatism in 2017 published the first recommendations related to the management of pregnant women with systemic lupus erythematosus and/or antiphospholipid syndrome [10]. In fact, the coexistence of antiphospholipid syndrome is a very important risk factor of adverse obstetric outcomes in patients with systemic lupus erythematosus.

The risk of obstetric failures includes intrauterine foetal death, pre-eclampsia, placental insufficiency, and foetal growth restriction [11–14]. The current recommendations regarding pregnancy planning in this population suggest conception in a period when the disease symptoms have been reduced for at least six months. Additionally, the recommended pharmacotherapy during pregnancy involves a prophylactic dose of low-molecular-weight heparin and acetylsalicylic acid [14]. The abovementioned therapy of antiphospholipid syndrome increases the live birth rate from 20% to 70%, compared to the group with the untreated disease [15]. However, a completely successful, full-term pregnancy and delivery of a healthy child cannot be guaranteed.

In the group of patients analysed in our study, one woman was diagnosed with systemic lupus erythematosus and antiphospholipid syndrome. Her three previous pregnancies ended with miscarriage. During her fourth pregnancy, the one analysed in our study, nadroparin and acetylic acid were administered, and her pregnancy ended with a preterm Caesarean section delivery of a live, preterm neonate. Nevertheless, most patients with systemic lupus develop secondary or steroid-induced cardiovascular complications. They are not the most common manifestations of systemic lupus, but the most dangerous ones, constituting the main cause of death in this patient group [16].

Another risk factor for an increased rate of pregnancy complications in patients diagnosed with systemic connective tissue disease is the coexistence of hypertension. Interestingly, arterial hypertension develops in approximately 70% of patients with systemic lupus erythematosus [17]. This is usually a consequence of chronic renal disease, due to lupus nephritis, or a result of chronic steroid therapy. In fact, every increase in the daily dose of steroids by 10 mg elevates arterial blood pressure by 1.1 mm Hg [17]. The recommended treatment in patients with lupus and arterial hypertension includes the lowest effective doses of steroids and intensive hypotensive therapy [17]. In fact, the coexistence of hypertension and systemic connective tissue disease was observed in four of our patients (28.6%). Three of them were diagnosed with nephritis, while one patient developed preeclampsia.

Following atherosclerosis and pericarditis, mitral regurgitation is one of the most common cardiac complications in the lupus patients [16]. The prevalence of this disorder is approximately 28%, and in patients with systemic lupus and concurrent antiphospholipid syndrome, mitral regurgitation is found significantly more often (38%) than in the population of patients without antiphospholipid syndrome (12%) [16]. Additionally, patients with a major mitral regurgitation are significantly more often at risk of stroke, peripheral embolism, the need of heart valve replacement and death, compared to patients with systemic lupus, but without a major defect (86% vs. 25%, respectively; p = 0.003) [18]. In fact, major mitral insufficiency is more often observed in patients with high levels of IgG anticardiolipin antibodies (50% vs. 3%) [14]. In our study, one of patients, with a complex defect of the mitral valve, was qualified for mitral valve surgery before pregnancy. However, she decided not to undergo this operation, which resulted in the development of respiratory insufficiency with pulmonary oedema due to the exacerbation of mitral regurgitation. This precipitated an urgent preterm Caesarean section delivery in the 30th week of gestation.

In conclusion, as our study demonstrated, the significance of pregnancy planning and appropriate pre-contraceptive treatment in patients with systemic connective tissue diseases should be emphasised. In our material, the live birth rate was 92%. Nevertheless, it is worth bearing in mind that the most severe perinatal complications, including intrauterine death and urgent preterm Caesarean section delivery due to respiratory failure, were found in the group of patients insufficiently prepared for pregnancy. Therefore, these pregnant patients should be referred for treatment in the reference centres, so that the delivery time can be chosen in the case of sudden deterioration of their condition, providing a chance of survival for the neonate and the mother. In fact, none of the patients who planned the pregnancy experienced the exacerbation of the disease, which contributed to better obstetric outcomes.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Cardiovascular imaging in the acute phase of coronavirus disease 2019 (COVID-19)

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DOI: https://doi.org/10.20883/medical.e532

Keywords: SARS COV-2, echocardiography, cardiac magnetic resonance, computed tomography, multimodality imaging, COVID-19

Published: 2021-09-28

How to Cite: Sacharczuk W, Dankowski R, Marciniak A, Szałek- Goralewska A, Szyszka A. Cardiovascular Imaging in the Acute Phase of Coronavirus Disease 2019 (COVID-19). Journal of Medical Science. 2021 Sep. 28;90(3):e532. doi:10.20883/medical.e532

ABSTRACT

The coronavirus disease 2019 (COVID-19) has become the most critical healthcare issue worldwide since the pandemic was announced in March 2020. Although respiratory symptoms remain the critical characteristic feature of COVID-19 (with acute respiratory syndrome as the leading cause of mortality), the disease also affects other organs. In fact, the involvement of the cardiovascular system during COVID-19 may include acute coronary symptoms, acute heart failure and myocarditis, arrhythmias, cardiac tamponade, pulmonary embolism, and right ventricular failure due to a high-pressure mechanical ventilation. It is vital to note that all of the abovementioned disorders require specific, pandemic-adapted imaging algorithms.
This brief review aims to discuss different cardiac imaging modalities to demonstrate their effectiveness in managing patients in the acute phase of COVID-19.

Introduction

Cardiovascular involvement among patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is common and significantly deteriorates the prognosis [1].

Hence, it requires quick diagnostic diagnosis and immediate treatment. In addition, several cardiac complications have been reported in the acute phase of COVID-19, such as myocarditis [2], pericarditis [3], acute myocardial infarction [4], stress-induced cardiomyopathy [5], cardiac tamponade [6], and / or right ventricular failure due to a high-pressure mechanical ventilation [7]. All these clinical situations require imaging techniques as a primary diagnostic tool.

Due to the sanitary regime during the COVID-19 pandemic, the availability of diagnostics may be

limited. Consequently, new or modified diagnostic pathways have recently been developed [8].

In this short review, we address the relevant issues concerning cardiovascular and pulmonary imaging, and share our experience from the COVID-19 ward.

Computed tomography

Computed tomography (CT) is the primary imaging tool in COVID-19 patients. In fact, high-resolution CT (HRCT) allows for the assessment of pulmonary involvement: pneumonia and pulmonary fibrosis. Typical images include bilateral, multifocal, multi-lane frosted glass with or without sub-segment consolidation, or a "crazy paving" pattern in the circumferential distribution. Results are usually presented as a percentage of infiltrating pulmonary parenchyma (**Figure 1**).

Furthermore, CT also plays a crucial role in diagnosing pulmonary embolism (PE). The prevalence of PE at the time of hospital admission for COVID-19 reached 14.2%, and further increased in the course of hospitalization [8]. By means of CT pulmonary angiography (CTPA) it is possible to confirm blood clots in the pulmonary arteries, or in the right heart chambers (**Figure 2**). The calculation of the right ventricle enlargement is a simple method to assess ventricular overload [9].

CT may constitute a helpful tool in the diagnosis of cardiovascular diseases and their complications, as on the basis of CT it is possible to exclude coronary artery disease (CAD) before further diagnosis [10], or before diagnosing an aortic pathology in patients with chest pain. Another issue is atrial fibrillation, the most common type of arrhythmia in COVID-19, observed in 17% of hospitalized patients. CT successfully replaces transoesophageal echocardiography in thrombus detection prior to cardioversion [11].

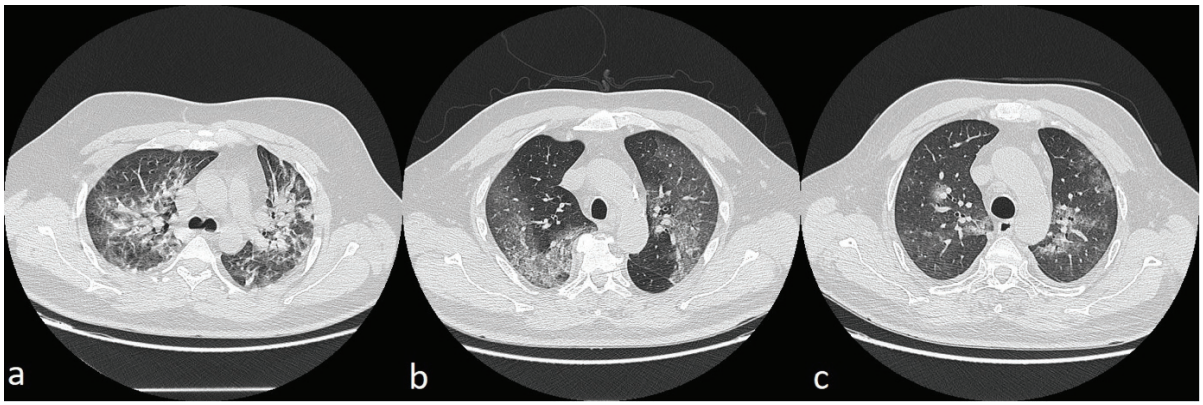

Figure 1. High-resolution CT (HRCT) presenting a percentage of pulmonary infiltration during SARS-COV 2 infection: (a) large involvement – more than 80% of infiltration, (b) 60% infiltration, (c) about 30% infiltration

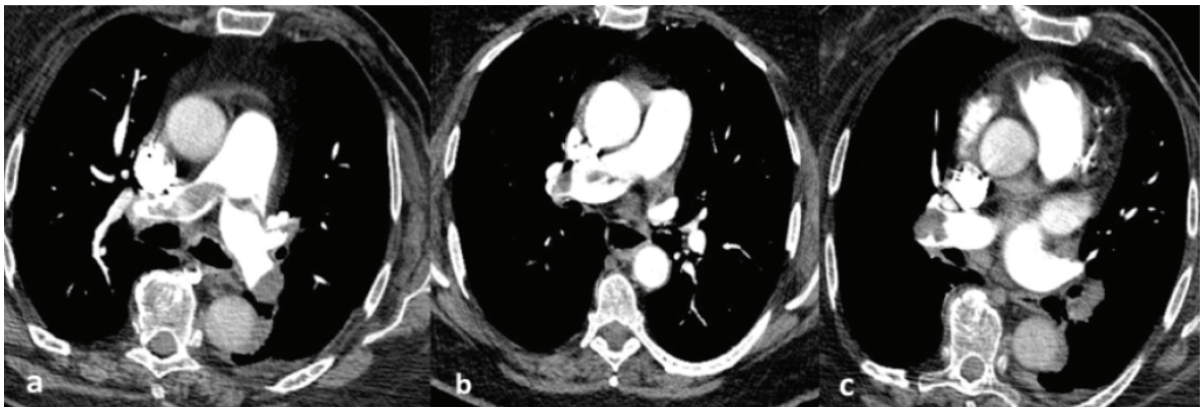

Figure 2. Pulmonary embolism in the acute phase of COVID-19: (a) large embolus in the pulmonary trunk and pulmonary arteries, (b) small embolus in the right pulmonary artery, (c) embolus in the proximal part of the right low lobe artery

Concluding, CT in COVID-19 patients enables the application of "the triple rule-out principle" concerning all the aforementioned conditions (**Table 1**).

Serious limitations in the use of CT technique include transporting the patient to the radiology department under the appropriate sanitary regime and sanitation processes.

Echocardiography

Although echocardiography is recommended as the first-line imaging tool for most cardiac conditions, it should not be routinely ordered in patients with COVID-19, particularly when no clinical benefits are expected. Transoesophageal echocardiography, which is an aerosol-generating procedure, should ideally be performed after the acute phase of COVID-19, due to an increased risk of virus transmission [12]. The primary echocardiographic modality in the management of COVID-19 patients is a point-of-care cardiac ultrasound (POCUS). Pocket size devices, such as a hand-held or a laptop-based scanner, are recommended, due to the speed of image availability, as well as bedside interpretation which allows reducing the scanning time and affects immediate patients' management. Easy transportability and a more efficient disinfection play a vital role in comparison to large machines [13]. An echocardiogram targeted at a specific clinical problem is referred to as a focused cardiac ultrasound (FoCUS), and its main goals are presented in **Table 2**, whereas typical echocardiographic images are demonstrated in **Figure 3**.

Careful evaluation of the right ventricle (RV) may be crucial in mechanically ventilated patients. Echocardiographers should pay particular attention to high positive end-expiratory pressures (PEEP) – induced cardiopulmonary overload, resulting in the symptoms of acute cor pulmonale. In fact, a dilated right ventricle with a flattened interventricular septum with a basal RV to LV ratio >1.0 are the most specific echocardiographic markers identifying this process [14]. Moreover, McConnell's sign, short RV outflow Doppler acceleration time, and high tricuspid regurgitation pressure gradient may indicate the presence of embolic material in pulmonary arteries [13, 15]. In addition, POCUS may also play an essential role in the PE diagnosis in COVID-19 patients with contraindications for CT angiography (e.g. pregnant women, allergy to contrast media).

Table 1. "Triple rule-out" CT Angiography Objectives in COVID-19 patients presenting the acute chest pain

The anatomical structure	Conditions which can be excluded by CT
Thoracic aorta	Aortic dissection
Coronary arteries	CAD[a]
Pulmonary arteries	PE[b]

Abbreviations: (a) coronary artery disease; (b) pulmonary embolism

Table 2. The focused cardiac ultrasound (FoCUS) parameters important in COVID-19 patients

Characteristics	Left Ventricle	Right Ventricle
Global Function	LVEF[a]	RVFAC[b] TAPSE[c]
Regional Contraction Abnormalities	Hypo-/akinetic regions ABS[d]	Hypo-/akinetic regions
Diastolic Function	LVEDd[e]	RVEDd[f]
Valves assessment		TRP[g]
Others	Pericardium thickening, pericardium effusion	

Abbreviations: (a) LVEEF – left ventricular ejection fraction; (b) RVFAC – right ventricular fractional area change, (c) TAPSE – tricuspid annular plane systolic excursion; (d) ABS – apical ballooning syndrome (typical in the Tako-Tsubo syndrome); (e) LVEDd – left ventricular end-diastolic dimension; (f) RVEDd – right ventricular end-diastolic dimension; (g) TRP – tricuspid regurgitation pressure gradient

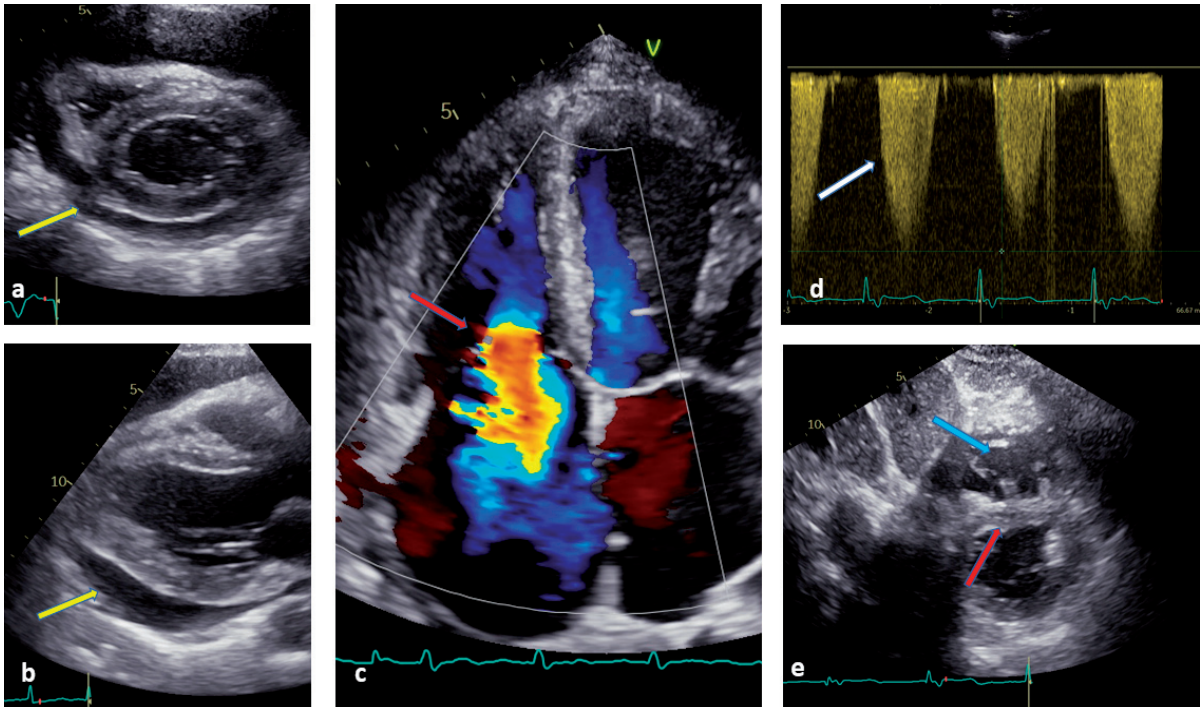

Figure 3. FoCUS images: (a) Parasternal Short Axis View (PSAX) with pericardial effusion – yellow arrow, (b) Parasternal Long Axis View (PLAX) with pericardial effusion – yellow arrow, (c) Apical 4-chamber View (4CH) with severe tricuspid regurgitation – red arrow, (d) Continuous-wave Doppler performing tricuspid regurgitation – white arrow, (e) Dilated right ventricle – blue arrow with a "flattening" of the interventricular septum as a result of high pressure in the right ventricle – red arrow

Lung point-of-care cardiac ultrasound (lung POCUS)

"Light beam" artifacts caused by subpleural consolidation can be early detected in COVID-19. They correspond to the "ground-glass" haze observed in CT imaging of COVID-19 pneumonia. The identification of the B lines in the POCUS examination may constitute an additional diagnostic value in tomography imaging [16, 17].

Cardiac magnetic resonance (CMR)

CMR is currently considered the gold standard for tissue imaging in the assessment of myocardial oedema and fibrosis by late gadolinium enhancement [18]. It is vital to bear in mind that in patients with COVID-19 it allows to distinguish the non-ischemic myocarditis type (**Figure 4**) from the ischemic mechanism of a myocardial injury [19]. Furthermore, when coupled with T1 and T2

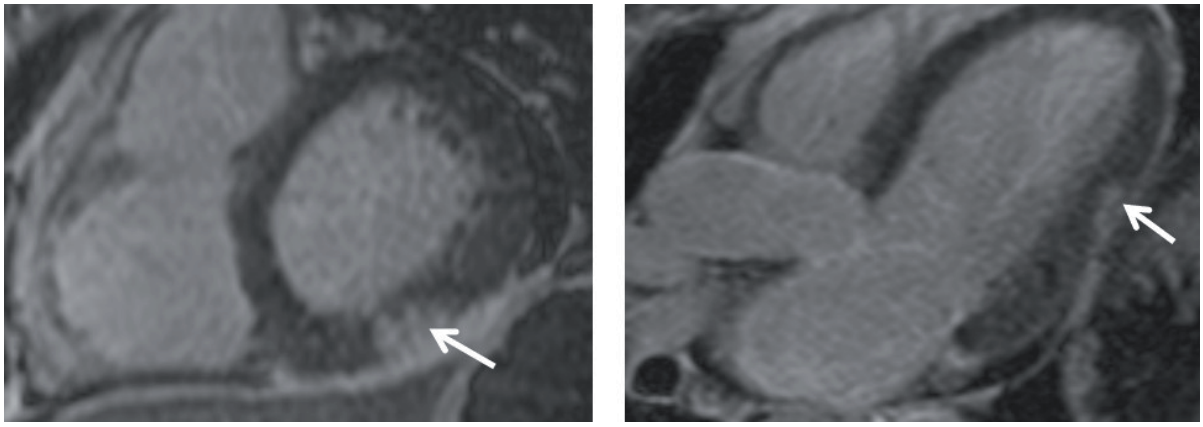

Figure 4. An example of a patient suffering from COVID-19, and an associated troponin increase with a subepicardial, myocarditis-pattern, late gadolinium enhancement in the mid-inferior wall extending to the inferolateral segment (white arrows)

mapping, CMR improves the diagnostic accuracy and identifies both acute and chronic changes of myocardial inflammation [20]. Interestingly, the most recent multicentre study has demonstrated that patients surviving severe COVID-19 who show elevated troponin levels and ongoing localized inflammation represent an emerging issue of clinical relevance. In patients with a severe COVID-19 course and a positive troponin assay, the evidence of residual inflammation on early CMR may play a role in the pathophysiology of dilated cardiomyopathy [19]. Nevertheless, a cost and availability issue for this technique may be a limiting factor for this modality.

Conclusions

The worldwide spread of SARS-CoV-2 disease revealed numerous weaknesses of healthcare systems, and changed our diagnostic pathways in patients suffering from COVID-19. Additionally, it also motivated the experts to create unique recommendations, algorithms, and practical guidelines to diagnose and treat infected patients, one of which is the employment of short protocols of cardiac imaging, which should be routinely applied due to the epidemic risk.

In our experience, CT may be selected as a "one-stop-shop" imaging method in COVID-19 patients, as it allows for obtaining various parameters in a single screening. Moreover, mobile devices, as well as POCUS and FoCUS protocols, should be the preferred choice when performing echocardiography, whereas CMR should be performed in troponin-positive patients, if available.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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REVIEW PAPER

JMS Journal of Medical Science

The possible negative effects of prolonged technology-based online learning during the COVID-19 pandemic on body functions and wellbeing: a review article

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DOI: https://doi.org/10.20883/medical.e522

Keywords: COVID-19, pandemics, technology-based learning, smartphones, tablets, body functions, cognitive function, sleep, headache, life style, food habits, earphones, headphones, eye fatigue, low back pain

Published: 2021-09-03

How to Cite: Yousof SM, Eid Alsawat R, Ali Almajed J, Abdulaziz Alkhamesi A, Mane Alsuhaimi R, Abdulrhman Alssed S, Salem IMW. Impacts of prolonged online learning practice during COVID-19 epidemic on body functions and wellbeing: a review article. Journal of Medical Science. 2021 Sep. 3;90(3):e522. doi:10.20883/medical.e522

ABSTRACT

The COVID-19 pandemic has had impact on life on a global scale, however, one of the most affected aspects are the teaching and learning practices. Advances in technology have made distance learning a good alternative option for on-site learning, as students can both interact with one another and with the tutor, use audio, video, text to learn, as well as use the internet for research purposes. However, this mode of education will extend throughout 2020 and early 2021, which could have negative implications on the health and body functions of university students. This review aims to shed light on the negative consequences of the prolonged technology-based, remote online learning on the students' wellbeing. Therefore, in this review we will discuss some of the physiological functions and body systems which could be affected during the COVID-19 pandemic in an attempt to suggest preventive measures in advance for safe technology-based learning.

Introduction

COVID-19 is a highly infectious disease transmitted through close contact, occurring due to infection with acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since the outbreak of the disease, it has affected the entire world and resulted in millions of deaths [1]. Internet-based learning is an academic method of distance education that has become common in the wake of the COVID-19 pandemic [2, 3]. Moreover, advances in technology have greatly facilitated student-teacher communication in the teaching process. This stems from the fact that students can interact with each other and with the teacher, use audio-visual and text messages in addition to using the internet for research, especially during of the COVID-19 pandemic [4].

The pandemic-related lockdown was double-bladed in regard to university students. This may be attributed to the positive attitudes which have developed in students, particularly students of medicine, towards their communities. In fact, a number of medical students in various countries have volunteered to assist in the ventilation therapy, and to help the nurses in the health care practice during the pandemic. Their participation met with encouragement and gratitude from their colleagues and families, which was subsequently enthusiastically welcomed by the students [5, 6]. Another positive aspect which emerged in the course of the pandemic is the students' involvement in the support of vulnerable groups, as well as in the COVID-19-related research practice [7]. Additionally, the learning practice has granted college students more time for studying instead of attending classes nearly every day, which in turn, has been positively reflected on their academic performance and grades [2].

In spite of the positive aspects that have been documented in many countries in response to COVID-19 pandemic emergency, there are certain drawbacks and negative consequences of the quarantine which may affect the learner's health. For instance, students have received their education on-line, seated in front of their laptop screens, mobile phones, or computers at their homes [8]. Notwithstanding the benefits of this technology-based remote education, this method presents several disadvantages, in particular as its effects may only appear following a long period of using technology [9]. Even though electronic devices may be equipped with cutting-edge display technology, looking at the screens of smart mobile devices can lead to severe vision strain and pain [10]. Furthermore, it is worth bearing in mind that excessive smartphone use has been linked to mental, cognitive, emotional, physiological, and neurological alterations, which should be taken into account [11]. In South Korean adolescents, excessive smartphone use was associated with impaired family and friendship relationships, impulsiveness, and low self-esteem. In fact, the studies demonstrate that individuals who use smartphones extensively present with poor concentration, diminished numeric processing skills, increased impulsivity, hyperactivity, and negative social involvement [11].

Therefore, in this review, we aim to highlight the negative consequences of technology-based learning on body functions during the pandemic. The underlying aim is not to incriminate new technology, but rather to identify the possible complications, and thus to prevent the side effects early, in order to maximize the benefits of modern technology in teaching and learning. A summary comparison regarding the advantages and disadvantages of online technology-based learning is shown in **Figure 1.**

Methods

In this narrative review, we used PubMed/Medline and google scholar as search engines for the studies related to our topic in the last 10-year period (2010–2021), as well as the related literature which appeared in the last 20 years. Moreover, a search via the related studies on PubMed has been performed. The keywords which have been used were as follows: "headphones or earphones or tablets or smartphones or technology-based learning, or online learning" and "tinnitus, otitis media, hearing loss, noise-induced hearing loss, telecom workers, infection, hyperacusis, asthenopia, eye strain, myopia, headache, migraine, sleep, memory, cognitive impairment, dietary habits and lifestyle". A summary of the possible negative consequences of prolonged technology-based learning is presented in **Table 1.**

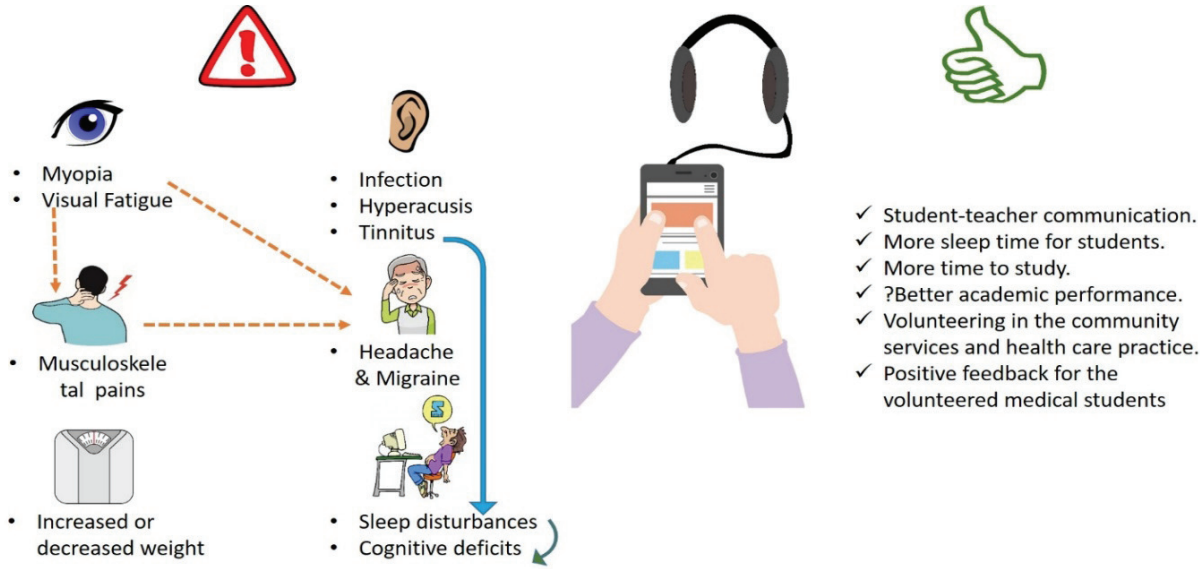

Figure 1. This graphical abstract shows the advantages and disadvantages of technology-based learning. Although there are numerous benefits of online learning, as demonstrated on the right-hand side of the figure, it is vital to note it may also negatively affect body functions as presented on the left-hand side of the figure. Additionally, certain disadvantages may result in other negative aspects, e.g. in tinnitus which, when severe, can lead to disturbed sleep. In fact, sleep disturbances are a known cause of cognitive and memory deficits. Moreover, visual fatigue increases the strain on the neck and shoulder, leading to pain, and may eventually cause headaches

Table 1. The possible negative effects of the technology-based learning on body organs and functions

Body Organ	Effects	References
Eyes	Blue light: toxic to photoreceptors.	[15,17]
	Asthenopia +/- corneal epithelial damage, conjunctival hyperaemia and a decrease in visual acuity.	[12]
	Affected posture control system.	[16,17]
	Computer Vision Syndrome.	[18]
	Tired, sore/aching, irritated, watery, and hot/burning eyes.	[10]
	Reduced blink rate and amplitude.	[21]
	Myopic shift.	[18]
Ears	Hearing loss.	[28]
	Tinnitus.	[31]
	Ear Infections.	[32]
	Hyperacusis.	[33]
Musculoskeletal system	Neck and shoulder pain.	[36]
	Tendonitis and carpal tunnel syndrome.	[37–39]
Sleep	Sleep disorders and anxiety.	[44]
	Memory and cognitive disturbances.	[46,47]
	Increased sleep time.	[48]
	Poor sleep quality.	[49]
Food habits and body weight changes	More frequent cooking instead of fast food consumption.	[53]
	Weight gain or weight loss.	[48,52]
Headache and migraine	Increased incidence of headache and migraine.	[58,59]
	Increased prevalence of aura.	[59]

Effects on the Eye

Smartphones have been widely used nowadays not only for telecommunication, but also as a means of learning, playing games, and social communication on the internet [12]. In fact, millions of students use computers and mobile phones also for learning, in addition to other daily uses, such as entertainment and social communication, particularly during the COVID-19

pandemic and the lockdown [13]. Interestingly, smartphones emit blue light which negatively affects the eyes [14], therefore, their prolonged use could result in vision damage, since blue light is highly toxic, due to the fact that it has a shorter light wavelength than other colors, and hence can damage the receptor cells [12, 15].

Asthenopia, Visual Acuity, and Blink Rate

Asthenopia (eye fatigue or strain) constitutes one of the conditions which affects the eyes as a result of a long-term daily use of computers and tablets. It may be accompanied by such symptoms as corneal epithelial cell damage, conjunctival hyperemia, and a decrease in visual acuity [12]. Moreover, prolonged visual fatigue can negatively affect visual feedback processing and the posture control system, which is associated with the integration disorders between the visual feedback, the vestibular organs, and the somatosensory control [16, 17]. In fact, studies demonstrate the emergence of "Computer Vision Syndrome; CVS" which affects more than 60 million people worldwide. This syndrome comprises symptoms including asthenopia, sensitivity to light, blurred vision, itchy eyes, and the sensation of a foreign body in the eye [18]. Moreover, the results have shown that visual fatigue is significantly increased if persons look at computer screen, or a tablet for 1 hour, even though these screens are equipped with state-of-the-art technology [10]. Another study has reported that the symptoms of CVS are common among health sciences students at King Saud Bin Abdulaziz University for Health Sciences (KSAUHS) in Jeddah, who use a variety of technological devices. Interestingly, female students, both observing the display glare and those wearing glasses, were considerably more likely to experience CVS symptoms. On the other hand, long-term device use has not been associated with an increase in the severity of CVS symptoms [19]. Nevertheless, another study involving adults (20–26 years old) has demonstrated that using computers has more adverse effects on the ocular surface and the tear film when compared with smartphones [20].

Blink rate data associated with smartphone and tablet use are contradictory, and may possibly be related to the complexity of the measurement, whereas research on blink amplitude is scarce. Blink rate and amplitude are frequently observed to be reduced when using a computer. Furthermore, digital devices, such as PCs, may also have a negative impact on tear stability. Although tear volume is reduced with computer use, there is little evidence to substantiate the impact of mobile devices on tear volume. In fact, the current literature does not present a definitive link between variations in binocular vision, blinking, or the ocular surface with ocular and visual discomfort complaints reported in relation to the use of hand-held digital devices [21]. However, as pointed out in the studies, 1 hour of using smart mobile phones increased the mean total asthenopia score from 19.59 8.58 to 22.68 9.39 ($p = 0.001$). Besides, looking at a smartphone screen substantially increased the ratings for five factors (tired eyes, sore/aching eyes, irritated eyes, watery eyes, and hot/burning eye). Considering the expanding openness to PC shown in everyday life, more rules and investigations are expected in order to maintain visual wellbeing [10].

Myopic Shift

Myopic shift is one of the most prominent effects following prolonged computer and mobile phone use, and is the consequence of a long-term effort to adapt to the constant use of laptops [18]. Myopia is a condition in which the patient can see the near objects clearly, but not the distant ones. In turn, myopia can later lead to further complications, such as retinal detachment and cataracts [22]. It is worth bearing in mind that mobile devices differ from desktop computers in terms of the location and viewing distance, screen size and brightness, as well as with respect to user behaviour. When using mobile devices, eye accommodations change with greater latency and less amplitude, whereas when using smartphones and tablets, fusion convergence is lower and the near convergence point may recede [21]. Decreased physical activity and an increased use of screen devices contributes significantly to the reported 25% prevalence of myopia in the group of healthy young individuals (16–17 years of age). Decreased physical activity for less than 3 hours per week, or using screen devices for more than 6 hours per day are more likely to contribute to myopia. Therefore, taking the abovementioned findings into account, it seems that physical activity may be a protective factor for myopia in teens [23].

Effects on the Ears

Distance education can also affect hearing and ears, particularly in view of a long-term use of headphones. Nevertheless, there are a limited numbers of studies which have described the effects of long-term headphone use on hearing in adolescents and young adults. However, certain studies have reported on the side effects of long-term headphone use by the telecom services employees, as well as on music-induced hearing impairments in young adults [24, 25].

Hearing loss

Hearing loss constitutes one of the most common communication issues in the 21st century, and it is a public health concern, since it impairs students' interaction, student achievement, and quality of life. According to the studies, the prevalence ranged from 0.88% to 46.70% [26]. Notably, Mazlan et al. have reported that 21.2% of their study population suffered from hearing impairment. Nevertheless, they have found an insignificant association between hearing loss and exposure to sound during headphone use, which stemmed from the fact that the high frequencies were uninvolved [27]. On the other hand, a Japanese study has reported that employees who use earphones in noisy workplaces may be exposed to noise-induced hearing loss (NIHL) risk factors, such as failure to use appropriate headphones or earphones, and the exposure to sound pressure from the earphones above the occupational exposure limit (Nakao et al. 2014). Similarly, in a study on adolescents, the researchers have found that a large percentage of the study population (79%) used portable music devices, and nearly half used them for prolonged periods of time. This population study indicated the incidence of hearing complaints, such as the need to repeat utterances and to increase TV volume, as well as tinnitus [28]. Another study has been conducted in Poland and found noise-related symptoms in telecom employees [24]. The authors of this study recommended further research related to the use of headphones by the employees due to a limited number of studies addressing this issue [24, 27]. Although the aforementioned studies did not focus on the use of headphones or earphones by students, they indicate the possible negative aspects of their long-term use.

Tinnitus

Tinnitus is the sound perceived in the ear as ringing, buzzing, whistling, occurring constantly or intermittently. Severe tinnitus can lead to anxiety, depression, irritability, sleep disturbances, stress, and sometimes, psychological counselling is necessary [29]. An interesting study has described the effects of different types of music (discos, concerts, and listening to music on headphones) on adolescents (14–18 years old) of both sexes. The researchers reported that 69% of students suffered from tinnitus after listening to different types of music. The females were more affected, and complained of tinnitus more frequently (41% females vs 27% males) [30]. Furthermore, in a Nigerian study on college students of medicine similar results were obtained. The study reported that the prevalence of earphone use and perceptual tinnitus among undergraduates were 95.6% and 20.6%, respectively. Over 90% of earphone users had used them for three to six years [31]. These results indicate the need to develop guidelines for headphones and earphones use by the students with regard to the type of instruments, the amplitude and frequency of the sounds generated, and the headphones are used.

Ear infections

Otitis and ear infections are other side effects which could result from wearing headphones [27]. These conditions are caused by the closure of the ear canal by the headphones, which prevents airflow to the ear,thus increasing the chance of ear infection. Additionally, the long-term use of earphones may lead to the growth of bacteria inside, which in turn leads to infection [32]. In a study assessing the effect of prolonged use of headphones in Customer Service Representative in Malaysia, it has been found that some subjects have suffered from chronic middle ear infections, whereas others suffered from a cerumen build-up [27].

Hyperacusis

Hyperacusis is one of the side effects of long-term headphones use. It is one of the auditory disorders characterized by extreme sensitivity to certain sound frequencies, which include everyday sounds, although the degree and the type of injury vary. Some patients find loud sounds highly uncomfortable in general, others complain when hearing a certain sound, whereas some may suf-

fer when hearing normal sounds. Therefore, this condition may not cause great inconvenience for some patients, although it may negatively affect the lives in another group [33].

Effects on the Musculoskeletal system

Incorrect body posture and position may result in actual medical issues [34]. In the 1990s and early 2000s, a significant increase in the prevalence of neck and shoulder pain (NSP), as well as in the low back pain (LBP) among young people was reported, where the use of the internet and new communication technologies constituted the vital risk factors [35]. Neck and shoulder pain has been considered as a mild musculoskeletal disorder to some extent, and performing excessive homework and incorrect sitting positions have been frequently described as predisposing factors for neck and shoulder pain among students [36]. Due to the fact that many students use their computers for long periods of time to maintain their grades, often without providing a convenient place at home, distance learning may contribute to pain and injury, and could subsequently lead to more severe injuries. Incorrect posture while sitting may cause discomfort, accompanied by pain which could develop within a short period of time. More severe repetitive stress injuries, such as tendonitis and carpal tunnel syndrome may develop in the course of months. It is also worth bearing in mind that remote learning during the COVID-19 pandemic involves students spending more time at computers, frequently at home which may not be as well prepared as a classroom [37–39].

Computer-related activities are an independent risk factor for NSP and LBP. The use of computers for more than 2 to 3 hours daily may be considered as a potential risk for developing NSP, whereas spending more than 5 hours in front of a computer is a risk factor of LBP [35, 40]. Considering the physical problems potentially caused by prolonged computer use for online tasks or for remote learning, neck pain has been linked to a low or high screen position and to a maladjusted keyboard. In turn, symptoms associated with shoulder joints have been associated with high screen positioning and shoulder elevation in individuals using a computer mouse. Daily use of a keyboard for four hours or more has been connected with shoulder and wrist pains, although not with neck pain. Moreover, work in front of monitors for more than 15 hours per week is considered a significant risk factor for NSP. Significant reductions in musculoskeletal discomfort in the shoulder, neck and upper back areas have been observed following correct positioning of computer users [35, 41, 42]. Based on the above findings, it is clear that proper positioning of computers and related equipment is essential to prevent LBP and NSP. Furthermore, time spent using the technology should be minimised, or at least include periods of rest and physical activity while studying.

Sleep Disturbances

Sleep cycle regulation is obtained by means of the circadian rhythm, also referred to as the sleep/wake cycle. The circadian rhythm is the brain's internal clock within the 24-hours which maintains the regularity of awakening, and sleepiness cycles via reacting to environmental light changes. Furthermore, both the body's physiology and behaviour are affected by the rotation of the earth around its axis. The significance of the circadian rhythm is rooted in its role in adapting to environmental changes and anticipating the changes in temperature, radiation and food availability [43].

Sleep Lack and Cognitive Impairments

Long-term mobile use has been associated with an increase in sleep disruptions and anxiety, which was alleviated when device use ceased [44]. Insufficient sleep affects memory, recall, and judgment, as well as fine motor skills. During sleep, the body rests while the brain is involved in memory processing. Therefore, a lack of sleep increases the liability of medical conditions involving obesity, hypertension, and diabetes [45, 46]. Moreover, imaging and behavioural studies refer to the crucial role sleep plays in the process of learning and memorizing, with researchers claiming that a lack of sleep impairs the ability to focus and to learn effectively. Additionally, they have reported that sleep is essential for memory consolidation to occur, so that information can be recalled in the future. A study where par-

ticipants who were subject to sleep deprivation demonstrated that they were more prone to think they were correct when they were, in fact, wrong [46, 47]. In contrast, a study on medical students in Croatia has found that the period of lockdown positively affected sleeping time, which was reflected by an increased time of sleep (+1.5 hrs.) and, therefore, improved the sense of refreshment following awakening [48].

Sleep Quality

According to a Chinese study, excessive smartphone use has been associated with poor sleep quality in a group of Chinese university students from a health vocational institution. Nevertheless, due to the study's limited sample representativeness and cross-sectional nature, the researchers recommended conducting large-scale prospective representative research to confirm these relationships [49]. Another study demonstrated that looking at a mobile phone screen for 8 hours or longer in a 24-hour period, or using the mobile phone for at least half an hour before sleep after turning off the lights and keeping the mobile near the bed lead to poorer sleep quality. Additionally, researchers have also found that mobile-related sleep risk factors are common among mobile phone users [50]. In fact, limiting cell phone use before sleep has been shown to decrease sleep latency and pre-sleep arousal and thus improve sleep duration and working memory. Therefore, moderate cell phone use is recommended for subjects with sleep disturbances [51].

Food Habits and Body Weight Changes

The lockdown obliged people to stay at home for long periods of time, and consequently resulted in changes in their dietary habits [52]. The availability of fresh food has been limited since the onset of the COVID-19 pandemic, with customers spending more time indoors and limiting their physical activity. On the other hand, spending more time at home may have generated some positive outcomes, such as more frequent cooking instead of eating fast food [53]. Nevertheless, online learning for long periods of time and reduced physical activity in the COVID-19 pandemic may have led to weight gain and its subsequent consequences. Additionally, it is important to note that even when daily caloric intake is maintained, reduced physical activity may lead to an increase in body weight [54].

In their study, Sidor and Rzymski have described the observed changes in dietary habits and food choices during the COVID-19 pandemic lockdown. According to their research, more than 43.0% of persons reported eating more, and about 52% and were snacking more. Moreover, these changes were observed to be higher in overweight and obese individuals. Nearly 30% of the study population have suffered from an increased body weight (mean ± SD 3.0 ± 1.6 kg). However, more than eighteen percent have suffered from weight loss (−2.9 ± 1.5 kg). Interestingly, the researchers have found that weight gain was more often present in overweight, obese, and older participants (36–45 and > 45), whereas weight loss was more frequent in underweight individuals [52]. An interesting study on the medical students in Croatia has assessed the changes in the lifestyle and food habits before and during the COIVD-19 related lockdown. The researchers have observed a change in eating habits which involved an increased consumption of fruits, legumes, sweets, and fish. In contrast, the consumption of dairy products and white cereals was decreased. Additionally, 19% of students have noticed weight gain, whereas about 30% have observed weight loss during lockdown [48].

Headache and Migraine

In migraine sufferers, using a smartphone has been shown to increase the time of headaches. In fact, its excessive use in migraine sufferers has been associated with poor sleep quality and daytime sleepiness [55]. Moreover, the visual problems have been shown to be a risk factor for developing headaches, as well as head and neck pains. An additional factor is the lack of vision correction, which increases the strain on the visual system and head and neck muscles [56,57]. Young adults with extensive exposure to screens are more likely to suffer from migraine, although no association was identified between screen exposure and non-migraine headache [58]. On the other hand, another study that included 400 patients with recent onset headache and/

or patients with primary severe headache who were and were not smartphone users found different results. According to the study, a higher prevalence of aura was found in the smartphone user group compared to non-smartphone users (7.7% vs 17.5%; p = 0.003), although headache characteristics were similar in both groups. In addition, the researchers determined that smartphone use was associated with a greater need for pain medication and a shorter period of relief following medication administration. However, they recommended a longitudinal study to confirm these findings [59].

Conclusion

The sudden change to remote online learning was necessary during the COVID-19 pandemic in order to fulfil the needs of the learning process and to simultaneously avoid infection. However, prolonged time spent in front of screens, laptops and computers can have an adverse effect on students' health and body functioning in the future. Therefore, urgent and prompt health education programmes for students are required aiming at increasing their awareness on how to maintain a healthy body during the online learning process.

Abbreviations

CVS: Computer Vision Syndrome; KSAUHS: King Saud Bin Abdulaziz University for Health Sciences; LBP: low back pain; NIHL: Noise-induced hearing loss; NSP: Neck–shoulder pain.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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JMS Journal of Medical Science

Contrast-enhanced spectral mammography in the radiological assessment of response to neoadjuvant chemotherapy in breast cancer

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Keywords: contrast-enhanced spectral mammography, breast cancer, Response Evaluation Criteria in Solid Tumors, neoadjuvant chemotherapy

Published: 2021-09-22

How to Cite: Grażyńska A, Antoniuk S, Steinhof-Radwańska K. Contrast-enhanced spectral mammography in the radiological assessment of response to neoadjuvant chemotherapy in breast cancer. Journal of Medical Science. 2021;90(3):e521. doi:10.20883/medical.e521

DOI: https://doi.org/10.20883/medical.e521

ABSTRACT

Accurate morphological assessment and measurement of the residual disease following neoadjuvant chemotherapy are vital for the effective surgical treatment in patients with breast cancer. Neoadjuvant chemotherapy response is measured by RECIST 1.1 criteria (Response Evaluation Criteria in Solid Tumors), and the classification of the specific therapeutic responses is based on the difference in the tumour size prior to and after chemotherapy. There are currently a few methods of imaging used in the assessment of the neoadjuvant chemotherapy response. Conventional mammography remains the most popular method, whereas magnetic resonance imaging is considered the most effective ones. Nonetheless, the available methods tend to be imperfect and limited, and therefore, new methods are constantly investigated. Contrast-enhanced spectral mammography is a relatively new method used in breast cancer diagnosis, which involves the phenomenon of neoangiogenesis of cancerous tumours, allowing contrast enhancement in the areas of vessel proliferation in the background of the surrounding breast tissue. Contrast-enhanced spectral mammography presents sensitivity similar to magnetic resonance imaging in breast cancer detection, and can be an efficient method used in monitoring neoadjuvant chemotherapy response.

Introduction

Breast cancer is the most commonly found cancer in women. It affects almost 1.7M patients each year and constitutes one of the most frequent causes of death in this patient group. In Poland alone, breast cancer accounts for 22.5% of all cancers diagnosed in women, as well as for 15% of deaths [1, 2]. Multidisciplinary treatment of patients with operable breast cancer combines

surgical therapy, radiotherapy, and systemic treatment which includes a wide range of medications. Drugs administered as systemic therapies comprise hormone therapy, chemotherapy, as well as targeted molecular therapy, which can be administered alone or used in multi-drug regimens. Depending on the timing of the therapy, it is possible to distinguish adjuvant therapy following the surgery, and neoadjuvant therapy preceding a surgical procedure. In terms of adjuvant therapy, it aims to remove latent micrometastases, whereas hormone therapy, chemotherapy, and anti-HER2 therapy based on different anti-cancer mechanisms can improve both disease-free and overall survival rates [3, 4].

Neoadjuvant chemotherapy (NAC) is intended to cases where the aim is to decrease the tumour and to remove the micrometastases prior to the radical breast surgery. An accurate morphological assessment and measurement of the residual disease following NAC are crucial for the effective surgical treatment [5, 6]. In addition to reducing a tumor and, thus offering better conditions for breast-conserving therapy, NAC provides professionals with unique opportunities to assess the sensitivity of tumour cells to chemotherapy in vivo, as well as to search for new biomarkers of therapeutic response. Furthermore, in the event of poor response and progression of the disease – it offers a chance to alter the treatment plan, or refer a patient for surgical treatment [7, 8]. In fact, achieving full response following neoadjuvant therapy and surgical resection is associated with a better prognosis and an increase in the 5-year survival rate. NAC response assessment is based on RECIST 1.1 criteria (Response Evaluation Criteria in Solid Tumors), which are effective in the assessment of the therapeutic response based on a radiological examination. The classification of the individual therapeutic responses is based on the difference between tumour size before and after NAC. The abovementioned criteria include: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) [9, 10].

RECIST methods in breast cancer

Currently, a few imaging techniques are available for the prediction of NAC response in breast cancer patients. Nevertheless, the most commonly used diagnostic modalities involve physical examination, ultrasonography (US), full-field digital mammography (FFDM), and magnetic resonance imaging (MRI). According to RECIST 1.1 guidelines, the US should not be used to measure tumour regression or progression of lesions due to its subjectivity, dependence on the operator, and no means of standardization [11, 12]. Similarly, physical examination which is not only subjective, but it is also characterized by a significant inadequacy compared to other methods. In fact, it only has a 57% effective rate in comparison with FFDM (74%) and US (79%). Moreover, the limited effectiveness of physical examination stems from the lack of differentiation between irregularly-shaped tumours, poorly separated lesions, lesions with fibrous components, or ones with central necrosis [13].

Conversely, according to RECIST, FFDM remains an incomplete method, in spite of its frequent use. In fact, accuracy evaluation of FFDM depends on breast structure and infiltration morphology (tumour or architectural distortion). Furthermore, similarly to physical examination, the effectiveness of FFDM is reduced in cases where the tumour possesses spiculated or blurred margins, with dense breast tissue, and residual infiltration masked by glandular tissue. Additionally, fibrous lesions which are complications of previous diagnostic biopsies, as well as the presence of microcalcifications also constitute a challenge. Interestingly, studies show that up to 44% of microcalcifications following the treatment do not correlate with the presence of malignant processes [13–16].

MRI is the method considered to be the most effective according to the RECIST evaluation. Its main advantage is the ability to form high-quality images and assessing additional functional parameters, such as vascularization and permeability of tumour vessels. Breast MRI has evolved from a primarily contrast-enhanced technique to a multiparametric method in which T2-weighted, and diffusion-weighted imaging (DWI) are routinely performed. This, in turn, allows for obtaining information regarding the tumour diffusion restriction and its biochemical status. [17]. Moreover, MRI is also particularly useful in the high-quality assessment of multifocal and multicentric lesions, with specificity amount-

ing to 90%. However, some studies suggest that MRI may underestimate or overestimate the size of residual lesions in as much as 18% of cases [18]. It is worth bearing in mind that an individual response to NAC can vary significantly with the molecular subtype of breast cancer. Previous studies have shown that regression occurs significantly more often as concentric shrinkage (as opposed to tumour fragmentation, or 'crumbling' into scattered foci) in the case of triple-negative breast cancer (TNBC) than in the case of HER2-positive tumours and ER-positive/HER2 negative. This fact affects the assessment of the response to NAC using imaging examinations. Nevertheless, MRI accuracy remains highest in TNBC and HER2-positive breast cancer and lowest in hormone receptor-positive cancer [16, 19].

Taking into account the disadvantages of the currently used diagnostic methods, new, effective modalities are constantly explored.

Contrast-enhanced spectral mammography

Contrast-enhanced spectral mammography (CESM) is a relatively new tool in the field of breast cancer imaging. CESM is a mammography technique involving double exposure of energy during a single compression of a single breast, following the administration of an iodinated contrast agent. Two minutes after injecting 1.5 mL/kg of contrast, classic mammography images are taken in the mediolateral oblique (MLO) and craniocaudal (CC) projections. Low-energy exposure uses the same X-ray energy spectrum as standard mammography, and the images obtained correspond to those of mammography. On the other hand, high-energy exposure is not suitable for diagnostic purposes, although it is used in post-processing in order to generate a recombined or iodine image showing areas of contrast enhancement. The images are created using the dual-energy weighted log subtraction technique, producing two sets of images. The combination of low-energy and high-energy images allows for the creation of a single image showing the impression of the contrast agent distribution within the breast, emphasizing the vascularity of the lesion [20, 21].

CESM is a useful tool in the examination of high-risk patients, which is also employed in the assessment of a very dense glandular tissue, in the diagnostic assessment of suspicious lesions, as well as in determining the pathological stage of breast cancer and in designing the treatment [22]. In a study comprising 547 patients with 593 breast cancer lesions, Steinhof-Radwańska [23] has shown that the sensitivity of CESM in malignant tumour detection amounts to 97.86%. This result is similar to that obtained for MRI, which indicates that these are the most sensitive methods used in breast cancer. However, as pointed out by Łuczyńska [24], the specificity of CESM is significantly reduced, with 59.4% and 60%. Despite its low specificity, CESM presents a high negative predictive value (NPV, 95.76%) which, possibly, allows to exclude cancer in the absence of pathological contrast enhancement [23].

Contrast-enhanced spectral mammography in RECIST

In recent years, only a few authors have engaged in investigating the effectiveness of CESM in RECIST criteria. Two authors compared the poten-

Table 1. A comparison of sensitivity, specificity, PPV and NPV of individual diagnostic methods in the detection of Complete Response (CR).

Author	Diagnostic method	Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]
Patel [18]	CESM	95	66.7%	55.8	96.7
	MRI	95	68.9	57.6	96.9
Iotti [19]	CESM	100	84	57	100
	MRI	100	60	32	92
Barra [20]	CESM	76	62.5	86.	45.4
	MRI	92	87.5	95	53.8
	FFDM	76	75	92	75

Abbreviations: PPV – positive predictive value, NPV – negative predictive value, CESM – contrast-enhanced spectral mammography, MRI – magnetic resonance, FFDM – mammography

tial of CESM and MRI in detecting the residual disease and CR with regard to the golden standard, i.e. a histopathological evaluation [25–27]. In the study of 65 patients, Patel [25] has shown that CESM is as effective as MRI in the assessment of residual tumour following NAC. Individual data concerning sensitivity, specificity, positive and negative predictive values are presented in **Table 1.**

However, as studies have shown, when correlating the sizes following NAC with the histopathological evaluation, MRI showed a higher compatibility with histopathology than CESM (Lin's concordance coefficient 0.75 (95% CI 0.62–0.83) for CESM, and 0.76 (95% CI 0.65–0.84) for MRI; Pearson correlation was 0.77 for CESM and 0.80 for MRI). Moreover, compared with the results of the histopathological examination, CESM decreased tumour size by 5 mm, whereas MRI reduced it by 5.4 mm. In the study by Iotti et al. [26] involving 46 patients, in the comparison of the tumour size following NAC with the histopathological examination, CESM showed greater consistency with histopathology than MRI (Lin's coefficient 0.81 and 0.59, respectively; CESM-MRI concordance difference 0.22, CI 0.07–0.58; PCC 0.85 and 0.67, respectively). Similarly, according to Patel et al. [26], both methods tend to underestimate the actual extent of a residual tumour (mean underestimation of 4.1 mm in CESM and 7.5 mm in MRI). The study of Barra et al. [27], comprising 33 patients, evaluated the CESM accuracy in the assessment of the residual disease following NAC as compared to MRI and FFDM. The concordance coefficient between the measurements of all the imaging methods and the size of the tumour was the highest for CESM (0.7 for CESM, 0.3 do FFDM, 0.4 for MRI). Furthermore, the Pearson correlation coefficient was also the lowest for CESM (0.8 for CESM, 0.3 for FFDM, and 0.5 for MRI). In comparison with the measurements performed using MRI, CESM, in 31.8% of the cases overstated the results by more than 1 cm with respect to the histopathological assessment.

Additionally, Tang et al. [28] in their meta-analysis demonstrated that the total sensitivity, specificity, positive likelihood ratio (PLR), negative odds ratio (NLR) and diagnostic odds ratio (DOR) of the pathological breast cancer response to NAC assessed by CESM were: 0.83 (95% CI, 0.66–0.93, 0.82 (95% CI, 0.68–0.91), 4.66 (95% CI, 2.59–8.41), 0.20 (95% CI, 0.10–0.43), 22.91 (95% CI, 8.66–60.62), respectively.

Underestimation of a residual lesion may result in an incomplete removal of the tumour and, thus, in the risk of re-operation. In contrast, overestimation may lead to an overly extensive surgery, and may result in poorer cosmetic results of a surgical procedure, as well as in the surrounding tissue damage. Therefore, in order to address this issue in the evaluation of NAC response, Xing et al. [29] suggested not to rely only on RECIST 1.1 criteria, but to create a mathematical model. This method is based on the combination of the largest tumour diameter measurements in the region of interest (ROI) and the subjective identification of the difference in the intensity of contrast uptake before and after neoadjuvant chemotherapy. Subsequently, a combination of the total number of pixels and their intensity within the area of interest before and after NAC is included. It should be noted that the implementation of this approach increases the sensitivity and specificity of CESM in the prediction and assessment of response to NAC, and reduces the frequency of inaccurate measurement of residual lesions.

Discussion

The aforementioned studies have demonstrated that CESM is equally effective as MRI in the assessment of residual lesions following NAC, which is currently considered the most effective examination method. CESM has been suggested as a primary tool for potential use instead of MRI, as it is less expensive, more accessible, and better tolerated by patients than breast MRI [30–32]. In fact, MRI lasts about 20–30 minutes in the prone position, and it is generally regarded as an unpleasant examination related to a forced body position, which additionally excludes patients suffering from such disorders as claustrophobia, or possessing older types of pacemakers. On the other hand, CESM lasts only about 7–10 minutes and the abovementioned inconveniences do not occur [33]. Moreover, CESM seems to be a better alternative for patients who are psychologically distressed by chemotherapy and face several repeated MRI examinations over the period of several months. Additionally, the possibility

of significant cost reductions compared to MRI renders CESM an appealing option in the economy of the health system [31]. Furthermore, CESM allows for the assessment of microcalcifications which is not possible with MRI [34, 35]. Nevertheless, CESM has certain limitations, such as exposure to iodine contrast media which limits its use in patients allergic to iodine contrast media and with severe renal failure. Additionally, CESM exposes patients to a higher dose of radiation which is not desirable in patients receiving radiotherapy. It is also essential to take into consideration that both MRI and CESM tend to underestimate [25, 26] or overestimate [27] the size of a residual tumour.

At present, apart from the previously mentioned methods (US, FFDM, CESM, MRI), nuclear imaging techniques are more frequently used, such as 2-deoxy-2[F-18]fluoro-D-glucose positron emission tomography (FDG-PET, assessment of glucose metabolism), fluorine 18 fluorothymidine positron emission tomography (FLT-PET, assessment of tumour proliferation), anti-1-amino-3–18F-fluorocyclobutane-1-carboxcylic acid positron emission tomography (FACBC-PET, assessment of amino acid metabolism) and C-choline positron emission tomography (assessment of choline metabolism). Although each of the available modalities has its limitations with regard to sensitivity and specificity, multiparameter (e.g. FTV / BPE / ADC) and multimodal (e.g. MRI / PET) methods should be implemented in order to improve the characteristics of the residual disease and to predict responses to NAC [16]. In addition, recent studies on radiomics-based analysis in predicting responses to NAC have produced very promising results. In terms of CESM, radiomics model achieved a significantly better discriminative ability compared to the standard clinical model (AUC, 0.81 vs. 0.55, $p < 0.01$) [36, 37]. Moreover, the development of deep-learning and machine-learning methods is also vital. The above-mentioned new techniques are expected to be employed in other breast imaging modalities and may play a crucial role in the detection, diagnosis and prediction of breast cancer outcomes. Therefore, additional studies are necessary, as well as exploring new methods for the most accurate assessment and a potential increase in the survival rate of patients.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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JMS Journal of Medical Science

Nutcracker syndrome – a mini review on current knowledge

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DOI: https://doi.org/10.20883/medical.e527

Keywords: nutcracker syndrome, venous compression syndrome, pelvic congestion syndrome, venous reflux, idiopathic haematuria

Published: 2021-09-22

How to Cite: Kramek JT, Krasiński Z, Stępak H. Nutcracker syndrome - a mini review on current knowledge. Journal of Medical Science. 2021 Sep. 22;90(3):e527. doi:10.20883/medical.e527

ABSTRACT

The Nutcracker Syndrome (NCS) constitutes an underdiagnosed condition defined as an external compression of the left renal vein, with a consequent blood outflow impairment. The majority of cases involve left renal vein entrapment between the abdominal aorta and the superior mesenteric artery. The exact epidemiology is unknown, due to the lack of consensus with regard to the diagnostic criteria, and the frequently asymptomatic course of the disease. NCS may occur at any age, with a peak presentation in the second and third decade of life, as well as with a slight prevalence in female patients. The most frequent presentations include micro and macroscopic haematuria, orthostatic proteinuria, orthostatic hypotension, flank pain. In addition, patients may develop pelvic congestion syndrome comprising dyspareunia, dysmenorrhea, abdominal pain, pelvic, gluteal, vulvar varicose veins and varicocele in men. The clinical suspicion of NCS, based on signs and symptoms, requires imaging confirmation involving such modalities as Doppler ultrasonography, computed tomography and magnetic resonance angiography, intravascular ultrasound and phlebography. Treatment options of NCS range from the conservative follow-up to nephrectomy, therefore, an appropriate approach should be based on clinical manifestations and the severity of symptoms. Patients presenting with mild to moderate haematuria and with other acceptable symptoms should be treated conservatively. In cases of severe symptoms, or when conservative management fails, invasive treatment should be considered. The recommended open surgical procedures include left renal vein distal transposition and renal autotransplantation. Additionally, the endovascular stenting approach seems encouraging, although due to uncertain mid and long term consequences this treatment option should be reserved for patients unsuitable for open surgery, or after a failed open surgical approach. Further long term follow-up is required to develop objective treatment guidelines.

Introduction

The Nutcracker Phenomenon (NCP), defined as the external compression of the left renal vein (LRV) and a consequent blood outflow impairment, was first described by Grant in 1937. The term "Nutcracker Syndrome" (NCS) is reserved for the symptomatic patients suffering from NCP [2].

The majority of cases involve left renal vein (LRV) entrapment between the abdominal aorta and the superior mesenteric artery (SMA) - anterior NCP. Posterior NCP describes the compression of LRV between the aorta and the vertebral column [3]. This rare anomaly may occur when LRV passes behind the aorta. Moreover, NCP also includes other rare aetiologies of LRV compression, such as tumours, lordosis, or LRV stretching over the aorta [4]. The exact epidemiology is unknown due to lack of consensus with regard to the diagnostic criteria and the frequently asymptomatic course of the disease. NCS may occur at any age, with a peak presentation in the second and third decade of life, as well as a slight prevalence in women [5]. There are reports suggesting that an asthenic body type with a decreased volume of retroperitoneal fat tissue, particularly in tall patients, may contribute to the development of NCS [6–8], which stems from the narrowing of the angle created by the aorta and SMA.

Clinical presentation

Numerous NCS cases remain asymptomatic. The most frequent presentations include microscopic haematuria (8.6–21.7%), as well as macroscopic haematuria (39.1–69.5%), proteinuria (4.3–26.1%), orthostatic hypotension, and flank pain (43.4–65.2%). Furthermore, patients may develop pelvic congestion syndrome (PCS) comprising dyspareunia, dysmenorrhea, abdominal pain, pelvic, gluteal, vulvar varicose veins and varicocele in men (8.7–21.7%) [4–6, 9]. The abovementioned conditions result from venous reflux, and elevated venous pressure [10], which may contribute to the creation and recurrence of lower extremity varices. The underlying mechanism of the haematuria is the distension of small veins of the renal fornix which rupture into the collecting calyces [11]. Consequently, impaired renal haemodynamics may lead to an increase in norepinephrine and angiotensin II levels, which presumably are the cause of proteinuria [12].

Diagnosis

In order to confirm the suspected NCS on the basis of clinical signs and symptoms, the presence of haematuria should be determined [13]. Positive laboratory result requires imaging confirmation with Doppler ultrasonography (DUS) (sensitivity: 69–90%, specificity: 89–100%) [14], computed tomography (CT), as well as magnetic resonance (MR) angiography, intravascular ultrasound (IVUS) (specificity of 90%) [14] and phlebography [6, 7, 15]. DUS constitutes the first line examination, the diagnostic value of which may be further enhanced by the calculating of the peak velocity (PV) ratio between the compressed segment of LRV at the hilar portion, the measurement of the angle between SMA and the aorta, as well as LRV diameter at the hilar portion [16]. Results of PV ratio exceeding 5 may suggest NCS [15]. Additionally, it is essential to perform the DUS examination in an upright position, since the SMA angle closes, haemodynamic results indicative of NCS [16]. In fact, the upright position and functional haemodynamic imaging represent the advantages of the ultrasound examination as compared to other imaging modalities. Both MR and CT may reveal LRV compression and the distention and varicosity of pelvic veins. In terms of the CT diagnostic criteria of NCS, they include „beak sign" (sensitivity: 91.7%, specificity: 88.9%), beak angle <32 degrees (sensitivity: 83.3%, specificity: 88.9%), LRV diameter ratio > 4.9 (sensitivity: 66.7%, specificity: 100%) (**Figure 1**), and an angle between SMA and the aorta <39 degrees (sensitivity: 100%, specificity: 55.6%) (**Figure 2**) [14, 15, 17]. It is worth noting that a definitive advantage of MR is the lack of radiation exposure, which is beneficial in younger patients. Furthermore, phlebography and venous pressure gradient measurement between the distal LRV and the inferior vena cava remain the gold standard. Pressure gradient over 3 mm Hg is significant for NCS [4, 5, 15, 17]. Nevertheless, both techniques are rendered invasive, although phlebography might allow for a simultaneous endovascular intervention addressing LRV com-

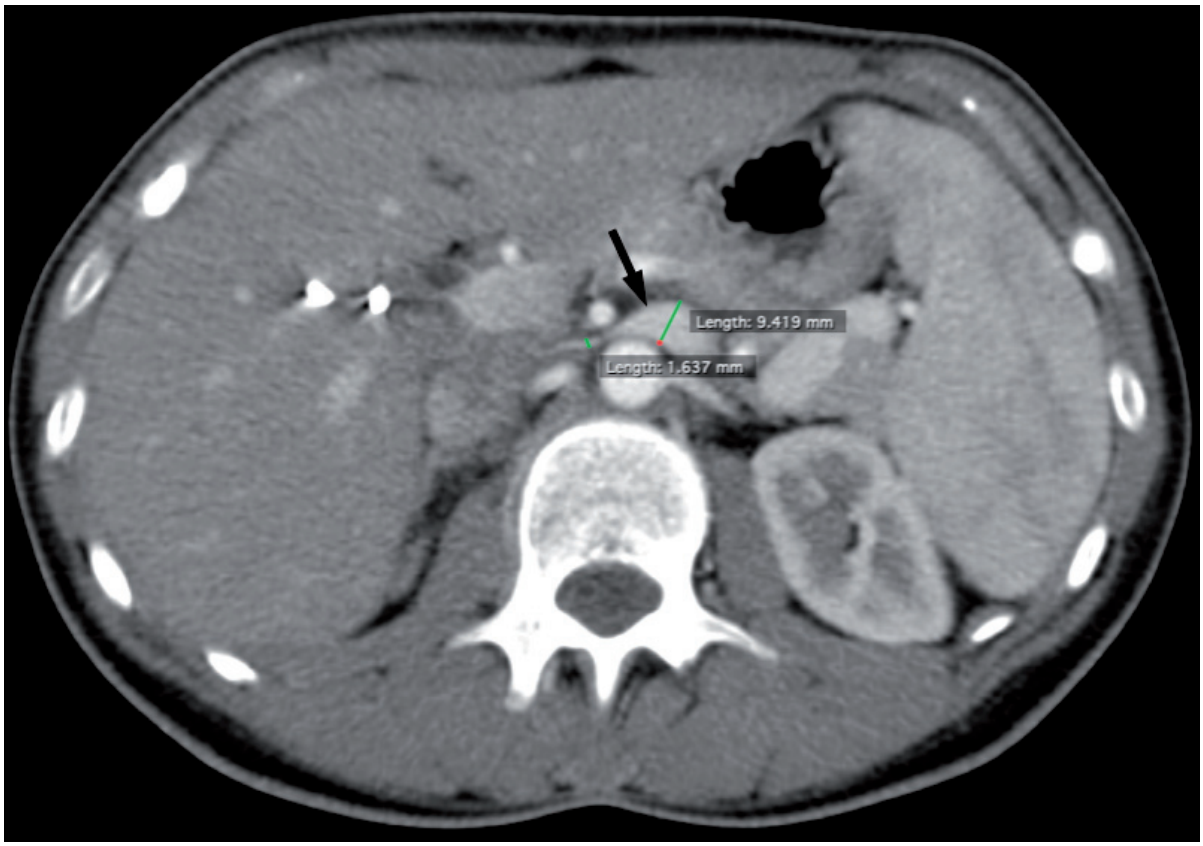

Figure 1. CT signs of NCS in a patient admitted to our department. The „beak sign" (black arrow). LRV diameter ratio exceeding > 4.9 (5,75 in this patient)

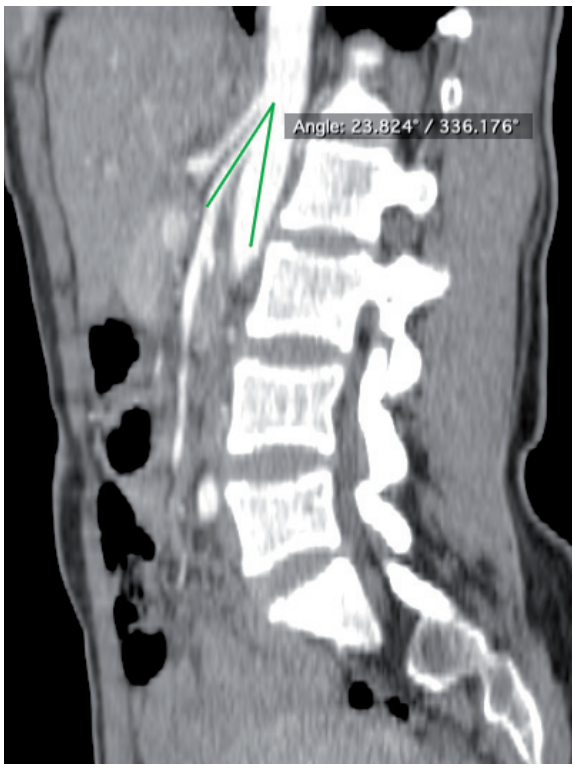

Figure 2. CT signs of NCS in a patient admitted to our department. The angle between SMA and the aorta < 39 degrees

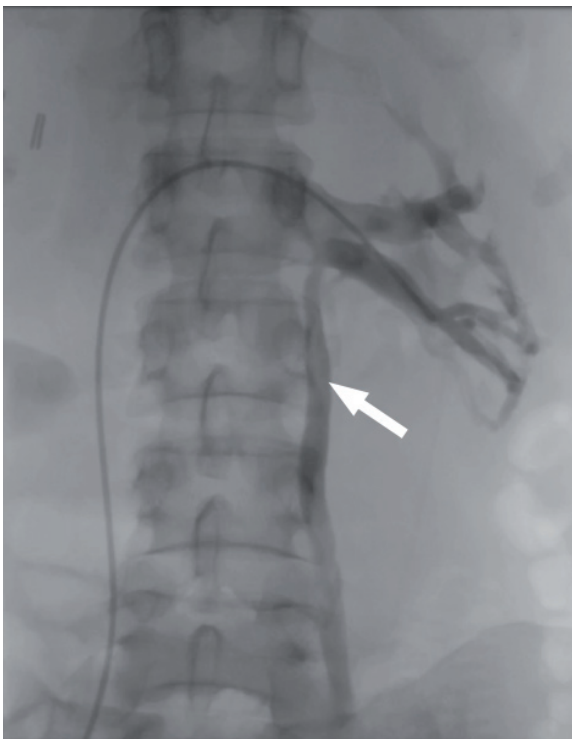

Figure 3. A patient admitted to our department (from Figures 1 and 2). Intraoperative phlebography presenting gonadal vein distention (white arrow)

pression (**Figure 3**). Therefore, IVUS is becoming a valuable supplementary tool of the diagnostic process [17].

Management

Treatment options of NCS range from conservative follow-up to nephrectomy, thus, the appropriate approach should be based on clinical manifestations, as well as the severity of the symptoms. Patients presenting with mild to moderate haematuria and with other acceptable symptoms should be treated conservatively. Such management includes: observation, ACE inhibitors in case of orthostatic proteinuria (especially alacepril), and acetylsalicylic acid in order to enhance renal perfusion [6, 14, 18]. Furthermore, a follow-up period of at least 2 years duration is also recommended in patients under 18 years of age. According to Kurklinsky et al., in 75% of this population, a resolution of symptoms will be observed [19], which can be attributed to a physical growth leading to an increase of the retroperitoneal fat and fibrous tissue, with a consequent correction of the anatomical relationships of SMA and the aorta [5, 15].

In cases of severe symptoms, or when the conservative management fails, invasive treatment should be considered. The recommended open surgical procedures comprise LRV distal transposition and renal autotransplantation [4, 6, 8, 10, 13, 20, 21]. The aim of the transposition is the reimplantation of the LRV into the inferior vena cava (IVC) distally to the origin of SMA. In order to allow a tension free anastomosis, an additional patch or a cuff from the venous autograft might be utilised. Moreover, LRV transposition is performed by laparotomy, and requires extensive retroperitoneal dissecting, thus, it might be associated with standard open surgical complications. Renal autotransplantation is even more challenging, and entails renal excision with a simultaneous reimplantation in the iliac fossa. Alternative surgical techniques include venous bypass between either the renal or the gonadal vein and IVC [8, 21].The treatment may also involve renal anterior nephropexy and pelvic varicosities excision or ablation in order to address LRV stretching over aorta and pelvic congestion, respectively, resulting from the venous reflux [22]. When other treatment options fail and severe haematuria reoccurs, nephrectomy might constitute the only solution [13, 23]. Interestingly, reported outcomes of laparoscopic interventions are comparable with open surgical procedures [24].

An endovascular stenting (EVS) approach seems encouraging, although due to uncertain mid and long term consequences, this treatment option should be reserved for patients unsuitable for open surgery, or after a failed open surgical approach [25]. Chen et al. reported improvement of clinical features in 95% of patients within a 5 year follow-up period in the group of 61 patients following EVS, with only 4 cases of stent migration [26]. The procedure allows for both diagnostic phlebography and pressure gradient measurement, as well as for the simultaneous treatment. EVS can be performed with either balloon-expandable or self-expandable stents (**Figure 4**) [26, 27]. Nevertheless, this technique has certain limitations. The possible complications include stent dislodgement and migration into the IVC, or even

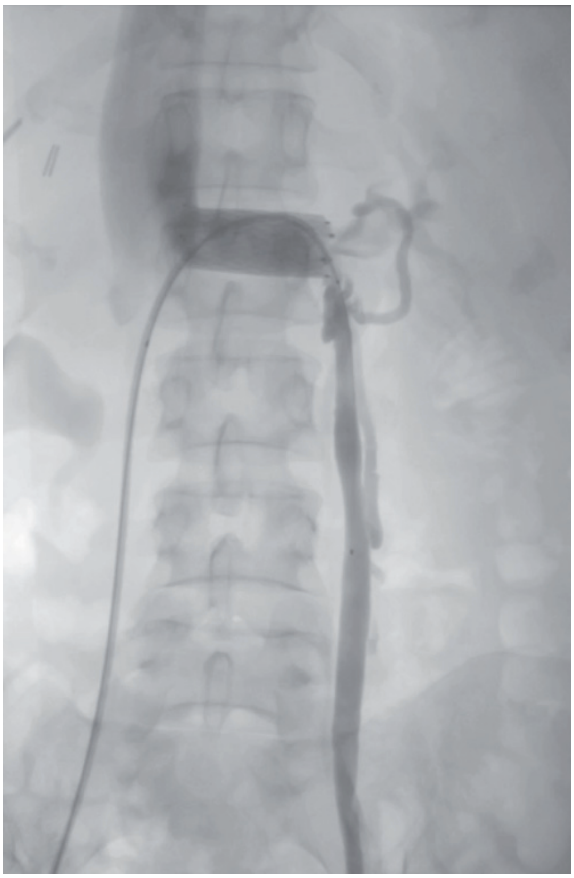

Figure 4. A patient admitted to our department (from Figures 1 and 2). Intraoperative phlebography presenting a successful stenting of LRV

into the right atrium with the necessity of a surgical removal [26, 28]. The stent might also displace distally into the renal hilum resulting in an occlusion. In terms of prevention of such complications, authors emphasise the importance of the adequate stent sizing [26]. Other possible complications involve stent thrombosis, fracture, or restenosis [26, 28, 29]. However, Jayaraj et al. described a hybrid approach to the treatment of NCS. According to their strategy, LRV distal transposition is followed by endovascular stent implantation and insertion of external stitches to prevent stent migration [30]. Although EVS remains less invasive, patients require anticoagulation and the administration of antiplatelet medication in the postoperative period [6].

Conclusion

NCS seems to be an underdiagnosed cause of haematuria, proteinuria and PCS. The diagnostic modalities include DUS, CT, MR and invasive techniques, such as phlebography and IVUS. The decision regarding the course of treatment should be based on the severity of the clinical presentation, with the aim of resolving the symptoms. The management is based on open vascular procedures – the transposition of LRV and renal autotransplantation, although minimally invasive strategies, e.g. laparoscopy and EVS, present promising results. Nonetheless, further long term follow-up is required to develop objective treatment guidelines.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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IMAGES IN CLINICAL MEDICINE

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Circulatory collapse after sheath removal in transfemoral transcatheter aortic valve implantation

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DOI: https://doi.org/10.20883/medical.e546

Keywords: TAVI, retroperitoneal hemorrhage, complications, abdominal compartment syndrome, diagnosis

Published: 2021-09-22

How to Cite: Iwata S, Ozaki M. Circulatory collapse after sheath removal in transfemoral transcatheter aortic valve implantation. Journal of Medical Science. 2021 Sep. 3;90(3):e530. doi:10.20883/medical.e546

ABSTRACT

An 87-year-old woman (146 cm, 42.2 kg) underwent transfemoral transcatheter aortic valve implantation (TF-TAVI) uneventfully. The patient successfully underwent emergency endovascular aortic repair using a covered stent to seal the vascular rupture. In order to treat the abdominal compartment syndrome, approximately 2,700 ml of haemorrhagic fluid was evacuated using ultrasound-guided abdominal paracentesis. RPH is a rare, although severe, complication of TF-TAVI, and has been reported in 0–2.2% of cases. Although the best management protocol for RPH remains controversial, conservative management should only be applied in stable patients. In cases of uncontrollable, ongoing bleeding, endovascular treatment or embolization should be the method of choice. Open surgical intervention is rarely require. Nevertheless, if treated inappropriately, the mortality rates remain high.

An 87-year-old woman (146 cm, 42.2 kg) underwent transfemoral transcatheter aortic valve implantation (TF-TAVI) uneventfully. Following the removal of the delivery sheath and achieving access-site haemostasis, hemodynamic instability became gradually obvious. Contrast-enhanced computed tomography (CT) revealed free fluid in the retroperitoneal and intraabdominal cavities, suggestive of retroperitoneal haemorrhage (RPH), which perforated the abdominal cavity with extravasation of the contrast material (arrow), thus indicating an ongoing haemorrhage (**Figure 1a**). The damaged site of the external iliac artery (arrow) was confirmed using digital subtraction angiography (**Figure 1b**). The patient successfully underwent emergency endovascular aortic repair using a covered stent to seal the vascular rupture. In order to treat the abdominal compartment syndrome, approximately 2,700 ml of haemorrhagic fluid was evacuated using ultra-

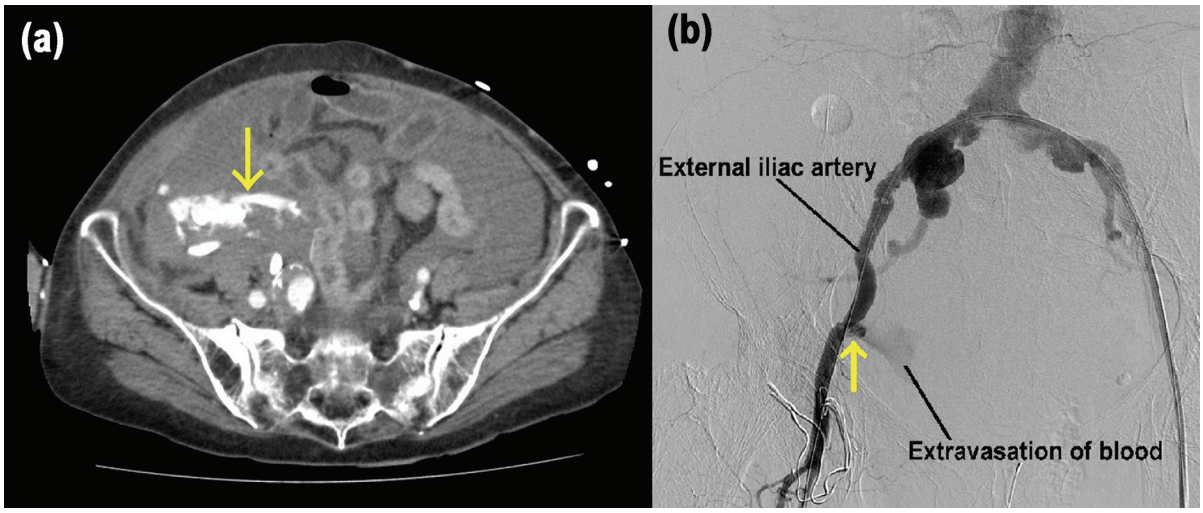

Figure 1. (a) Contrast-enhanced computed tomography image shows free fluid in the retroperitoneal and intraabdominal cavities, suggestive of retroperitoneal haemorrhage which perforated into the abdominal cavity with extravasation of the contrast material (arrow), indicating an ongoing haemorrhage. (b) Digital subtraction angiography shows bleeding at the damaged site of the external iliac artery (arrow)

sound-guided abdominal paracentesis. Eventually, she was discharged in a stable condition.

RPH is a rare, although severe, complication of TF-TAVI, and has been reported in 0–2.2% of cases [1]. It is associated with a damage to iliofemoral artery, and constitutes the most frequent complication associated with vascular access [2], with the main predictive factors being the dimensions of small vessels, moderate or severe calcification, and centre experience [1]. The diagnosis of RPH is often delayed due to the non-specific clinical presentations, such as flank, abdominal, back pain, and/or progressive hemodynamic instability [1, 2]. Although the best management protocol for RPH remains controversial, conservative management should only be applied in stable patients. In cases of uncontrollable, ongoing bleeding, endovascular treatment or embolization should be the method of choice. Open surgical intervention is rarely required [2]. Nevertheless, if treated inappropriately, the mortality rates remain high [2].

Acknowledgements

Author Contributions
SI wrote the initial draft of the manuscript and procured the clinical images. MO made a substantial contribution to the preparation of this manuscript. SI and MO approved the final version for submission.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acknowledgements

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1. Petrova NV, Kashirskaya NY, Vasilyeva TA, Kondratyeva EI, Marakhonov AV, Macek Jr M, Ginter EK, Kutsev SI, Zinchenko RA. Characteristics of the L138ins (p.Leu138dup) mutation in Russian cystic fibrosis patients. JMS [Internet]. 2020 Mar 31;89(1):e383. doi: 10.20883/medical.383.

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Personal author(s)

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JMS Journal of Medical Science

App-assured essential physical activity for the prevention of cognitive decline: changing paradigms in public health – a study protocol for a randomised controlled trial

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DOI: https://doi.org/10.20883/medical.e530

Keywords: physical activity, cognitive functions, mild cognitive impairment

Published: 2021-09-01

How to Cite: Jamka M, Makarewicz A, Wasiewicz-Gajdzis M, Brylak J, Wielińska-Wiśniewska H, Pawlak Z, Nowak JK, Herzig K-H, Mądry E, Walkowiak J. App-assured essential physical activity for the prevention of cognitive decline: changing paradigms in public health – a study protocol for a randomised controlled trial: A study protocol of the PA PROTECT study. Journal of Medical Science. 2021;90(3):e530. doi:10.20883/medical.e530

ABSTRACT

This study aims to assess the effect of an increase in daily physical activity to prevent cognitive decline, sustain brain volumes and maintain healthy biomarker levels in mild cognitive impairment (MCI) subjects

aged 50-70 years. In total, 198 subjects with MCI (assessed using the Montreal Cognitive Assessment test) will be recruited and randomised into two groups: active and passive. The active group will be instructed, encouraged and motivated to increase their physical activity to at least a moderate level (≥ 10,000 steps/day), whereas the passive group should maintain their normal activity levels. All subjects will undergo cognitive assessment, neuroimaging and biomarker tests prior to and after a one-year intervention. During the intervention, physical activity will be measured by the Fitbit Inspire HR wristband. The study was registered in the German Clinical Trials Register database (registration no. DRKS00020943, date of registration: 09.03.2020, protocol version: 1.0).

Research Project Objectives

The study aims to assess the effect of increasing daily physical activity on the prevention of cognitive decline, sustaining brain volumes, as well as on maintaining healthy biomarker levels in subjects aged 50–70 years affected by mild cognitive impairment (MCI). The study hypotheses are as follows:

1. Increased daily physical activity to at least a moderate level (> 10,000 steps/day) for one year will not affect cognitive function.
2. Higher levels of daily physical activity (> 10,000 steps/day) will not preserve brain volume and will not maintain proper values of healthy biochemical markers and anthropometric parameters.

Research Plan and Basic Concept

Basic Concept

MCI is a condition in which subjects demonstrate cognitive decline with minimal dysfunction of instrumental daily activities, which may also be a stage preceding dementia [1]. According to a recent systematic review, about 18% of MCI subjects develop dementia within two years, with the conversion rate increasing to 32% following five years [2]. In 2016, the global prevalence of dementia was 48.3 million [3], which is anticipated to increase to 80 million in 2030 [4]. Neurocognitive disorders significantly affect everyday living and place a substantial financial burden on healthcare systems. Advanced age and family history of neurocognitive disorders are important risk factors for developing dementia, as well as numerous modifiable risk factors, such as hypercholesterolaemia, hypertension, obesity, hyperglycaemia, poor education and physical inactivity [5]. Moreover, currently, there is no pharmacological treatment approved for MCI. Therefore, it is crucial to identify MCI subjects and attempt to mitigate the risk factors in this group [6].

To date, there have been several studies regarding the impact of physical activity on the prevention of cognitive decline [7–9]. A recent meta-analysis demonstrated that physical activity (aerobic, resistance training or tai chi) positively affects cognitive function in adults aged 50 years or older, regardless of their baseline cognitive status [7]. In addition, another meta-analysis showed that slow walking and jogging significantly improved attention, execution and memory processes [8]. Furthermore, improved daily physical activity, defined as walking a greater distance, helps to preserve grey matter volume in the frontal, occipital, entorhinal, and hippocampal regions, resulting in a reduced risk of cognitive decline [9, 10].

Although several studies reported a relationship between physical activity and cognitive functions preservation, there is no consensus regarding the exact frequency, duration, intensity and type of exercise necessary to prevent cognitive decline. Current physical activity guidelines recommend that adults should be involved in at least 150 minutes of moderate-intensity aerobic exercise a week [4, 11]. However, only less than 5% of adults were able to comply with the recommendations which indicates that the existing guidelines are too demanding for the elderly. Moreover, actual physical activity was lower compared to the declared level of activity in all forms of questionnaires [12].

Study design

The study was designed as a parallel-group prospective randomised controlled trial. The study protocol was registered in the German Clini-

cal Trials Register database (registration no. DRKS00020943, date of registration: 09.03.2020, protocol version: 1.0). The study protocol is reported in accordance with the Standard Protocol Items: Recommendations for Interventional Trials guidelines [13, 14]. The data in this study will be reported and presented according to the Consolidated Standards of Reporting Trials statement [15].

Ethical issues

The present study will be conducted according to the guidelines provided in the Declaration of Helsinki. The study protocol was approved by the Bioethics Committee of Poznan University of Medical Sciences (refs. 47/20, 169/20, 263/20, 481/20, 720/20, 296/21 and 555/21). The study personnel will obtain written informed consent from all study participants upon their enrolment. The study will not have a data monitoring committee, given that we do not anticipate severe adverse effects. Study-related personal damage of the participating subjects will be covered by the Poznan University of Medical Sciences insurance policy. Should the study protocol require amendments, a revised version will be submitted to the Bioethics Committee. The study team and the study participants will be also informed about all the changes.

Study population

In total, 198 subjects will be recruited to the study. Inclusion criteria are as follows: age 50–70 years, diagnosed MCI (the result of the Montreal Cognitive Assessment (MoCA) test: 19–26 points), community residence, and owning a smartphone. On the other hand, the exclusion criteria include: depression and/or the results of the Hamilton Depression Rating Scale (HAM-D) > 13 points, use of cognitive boosting medications or psychotropic medications, substance abuse disorders (e.g. alcohol > 15 drinks (units)/week), diagnosed psychiatric disorders, Parkinson's disease, Alzheimer's disease, dementia, anaemia, diabetes of at least 10 years, chronic renal and liver diseases, a history of cancer within the past five years, history of stroke, current evidence or a history of seizures in the past two years, head injury with loss of consciousness and/or immediate confusion following the injury, hypothyroidism with current misaligned thyrotrophic hormone levels, any chronic diseases which limit training and testing of cardiovascular and respiratory systems, current intensive physical activity (at least 10,000 steps/day), implanted pacemaker, neurostimulator and other metal components, including prosthetic implants, blindness, deafness, language difficulties or any other disability which may prevent subjects from participating, or cooperating in the protocol.

Recruitment

Participants will be recruited to the study from patients of medical clinics and medical centres in the Greater Poland region (Poland) in consultation with their physicians and directors of the clinics, by means of study promotion via workplace channels at the university and healthcare services, as well as via university newsletters and websites, posters, leaflets and email invitations sent to companies, offices, and institutions for distribution to their employees. The research team will contact the interested participants and send further information about the study. Prior to the commencement of the study, the potential subjects will be screened by a physician during an inclusion appointment to comply with the protocol requirements. In this phase, cognitive functions will be evaluated by the MoCA test and the HAM-D scale will be used to assess the occurrence of depression symptoms. Additionally, physical activity will be determined for at least one week before the enrolment using the Fitbit Inspire HR tracker. Subjects will receive information regarding the study, its purpose, putative benefits, and the possible risks. All subjects will be informed that participation in that study is voluntary, and that they may refuse to participate, or withdraw from the trial at any time without providing reasons.

Intervention

The study population will be randomised (allocation ratio: 1:1) into two groups: active (group A) or passive (group P). Group P (n = 99) will be asked to sustain their normal activity, whereas group A (n = 99) will be asked, instructed and motivated by the mobile application to increase their physical activity intensity to at least 10,000 steps/day. During the intervention period, all subjects will be instructed to maintain their current diet and

maintain their medications and, if they change, to record this in a diary. Prior to and after the one-year intervention period, cognitive functions, neuroimaging, and biochemical parameters will be assessed in all study subjects. Additionally, physical activity will be determined using the Fitbit Inspire HR tracker. Moreover, before, during and after the intervention period, anthropometric and densitometric parameters, body composition, as well as dietary habits will be assessed. A self-administered questionnaire regarding physical activity, health condition, medications, smoking, alcohol use, profession, and education will be distributed to subjects. Basic clinical examinations and measurements will also be performed. The scheme of this study is presented in **Figure 1**.

Adherence to the intervention

Adherence to the intervention will be assessed by data collected from the Fitbit, including the number of steps per day, distance travelled, estimated energy expenditure, sedentary behaviour, minutes of low, moderate and intensive activity, as well as sleep behaviour. The data will be wirelessly uploaded to the user's account and will

	STUDY PERIOD			
	Enrolment	**Allocation**	**Post-allocation**	**Close-out**
TIMEPOINT	$-t_1$	t_0	t_1 (6th month)	t_2 (12th month)
ENROLMENT:				
Eligibility screen	x			
Informed consent	x			
Medical examination	x			
Hamilton Depression Rating Scale	x			
Montreal Cognitive Assessment	x			
Physical activity (Fitbit)	x			
Allocation		x		
INTERVENTIONS:				
Active group (> 10 000 steps/day)		♦—	—	—♦
Passive group (< 10 000 steps/day)		♦—	—	—♦
ASSESSMENTS:				
Primary outcomes:				
Montreal Cognitive Assessment	x			x
Cambridge Neuropsychological Test Automated Battery		x		x
Secondary outcomes:				
Anthropometric parameters (body high, body weight, waist and hip circumferences, body mass index)		x		x
Biochemical markers (fasting glucose and insulin homeostasis markers, lipid profile, inflammatory markers, markers of neuronal growth and destruction)		x		x
Blood pressure		x		x
Body composition and densitometric parameters (DEXA)		x		x
Food Frequency Questionnaire		x	x	
Hamilton Depression Rating Scale	x			x
International Physical Activity Questionnaire		x	x	x
Magnetic resonance imaging		x		x
Physical activity (Fitbit)		x	x	x
Socioeconomic assessment		x		x
3-day dietary record		x		x

Figure 1. The study schedule of the enrolment, interventions, and assessments

be downloaded by our research team through the Fitbit website, or by means of the application programming interface. The Fitbit data will provide us with objective information regarding the level of adherence throughout the intervention period. Furthermore, in order to increase adherence to the intervention, phone calls will be scheduled to review the compliance with the physical activity guidelines, and all participants will also be given the option of additional calls if necessary. In addition, weekly emails, including the information about their average step count, will be sent to the study participants. Moreover, a check-up appointment will be conducted six months after starting the intervention in order to verify the subject's adherence to the intervention. The study participants who will not comply with the intervention will be excluded from the study, and the principal investigator will make a final decision regarding the exclusion. If a participant decides to withdraw from the study, no further data will be collected concerning this individual.

Minimum sample size calculation

The minimum sample size was calculated on the basis of a recent physical activity intervention study in subjects with an incident of cognitive impairment. It demonstrated that the executive function and memory scores were -0.33 ± 0.79 and -0.32 ± 1.29, in the low active and 0.31 ± 0.86, and 0.22 ± 1.05 in the highly active group [16]. However, we assume that due to the preselection, we will manage to obtain a more homogenous group, and thus achieve a more significant clinical effect and 1) the probability of a type-I error at an alpha cut-off level of 5% ($\alpha = 0.05$); 2) the probability of a type-II error at a beta cut-off level of 20% ($\beta = 0.2$); 3) the difference of the anticipated means equals to 0.62 standard deviation (SD); 4) the expected value of SD equals to 85% of the mean.

Randomisation and blinding

Randomisation will be performed via computer software (RRApp Robust Randomization App, the Icahn School of Medicine at Mount Sinai, New York, NY, USA [17]) and the data will be uploaded by an independent researcher. We will perform blocked randomisation (block size: six) with the stratification according to sex and prevalence of diabetes. The participants will be allocated in equal numbers to one of two groups, passive or active, as defined by the code. According to the character of the intervention, the study participants and researchers taking the physical activity measurements will not be blinded to the allocation. Only the outcome assessors and the study team members who will prepare the database and will perform the statistical analysis will be blinded.

Protection of data privacy

Quantitative data will be collected from the recruited subjects using anthropometric, clinical, biochemical and behavioural measurements to written and electronic files, and subsequently to permanent file formats for analysis. Subjects will be identified by non-personal codes and tied to metafiles. The data will be verified by investigators. Original written documents will be stored in a locked filing cabinet, whereas all the data will be collected in secure access computers. Documents and files will be retained as authorised by the Bioethics Committee. The final trial dataset will be accessed by the principal investigator, study coordinator and other team members.

Dissemination

The study results will be presented at local, national and international conferences, and will be published in open-access peer-reviewed journals. Authorship eligibility will be based on the International Committee of Medical Journal Editors. The data collected in this study will be available on request from the principal investigator. Study participants will be informed of the outcomes of the study.

Research Methodology

Primary and secondary outcomes

The primary outcomes of the study will be changes (Δ before – after) in cognitive function parameters assessed by the MoCA test and the Cambridge Neuropsychological Test Automated Battery (CANTAB), whereas the changes in biochemical parameters, neuroimaging, anthropometric parameters, body compositions and densitometric parameters will be regarded as the secondary outcomes. All the data, except neuroimaging, will

be collected in the Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences. Brain magnetic resonance imaging (MRI) will be performed at the Heliodor Swiecicki Clinical Hospital in Poznan. Blood samples will be collected by a commercial laboratory, while the biochemical parameters will be measured at the Laboratory of the Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, or by a commercial laboratory.

Anthropometric measurements

Basic anthropometric parameters (body height, body weight, hip and waist circumferences) will be measured before and after the intervention period. Body mass index will be calculated on the basis of body weight and body height measurements. During the anthropometric measurements, all participants will wear light clothes and will be barefoot, with an average of two measurements recorded.

Body composition and densitometric parameters

Before and after the intervention period, body composition (fat and free fat mass), bone mineral density and the content of the total body and lumbar spine (L1-L4) will be assessed by means of dual-energy X-ray absorptiometry methods using the Hologic Discovery analyser (Bedford, Massachusetts, USA).

Blood pressure

Blood pressure will be measured prior to blood sample collection according to the guidelines of the European Society of Hypertension. Blood pressure will be measured on the arm at the heart level, and will be expressed by three measurements of the systolic and diastolic pressure [18].

Assessment of dietary habits

Dietary habits will be assessed before and during the intervention period by means of 3-day dietary records covering two weekdays and one weekend day. Participants will be asked to give a detailed description of foods consumed and to estimate their quantity. To investigate food group intake, the Beliefs and Eating Habits Questionnaire created by the Behavioural Conditions of Nutrition Team, Committee of Human Nutrition Science of the Polish Academy of Sciences will be administered [19]. The energy intake and the basic nutritional compounds (carbohydrates, proteins, and fats), selected vitamins and minerals, dietary fibre, cholesterol, saturated, monounsaturated and polyunsaturated fatty acids intake will be assessed using the Aliant software (Anmarsoft, Gdańsk, Poland). The nutrition standards for the Polish population will be applied to determine whether individual dietary intakes meet the nutritional recommendations [20].

Physical activity

Physical activity will be determined using the Fitbit Inspire HR tracker (Fitbit Inc., San Francisco, USA). In addition, the International Physical Activity Questionnaire will be used to assess physical activity before, during and after the intervention period.

Fitbit Inspire HR is a wrist-worn wearable wireless sensor with an accelerometer recording physical activity throughout the day, which can synchronise with a smartphone application and a computer. Therefore, participants will be instructed to download the Fitbit app, and will be asked to wear the Fitbit all day, except when showering, bathing, and swimming. Participants will be instructed to wear the Fitbit on their non-dominant wrist for one year. In general, the Fitbit requires the creation of individual user accounts to download the stored data using a Web-based software application. Nevertheless, for the purpose of this study, user accounts will be created by the study team which can only be accessed by the researchers. Physical activity data will be stored on the individual accounts of study participants and will be downloaded of each participant's wearing period by the study team.

Hamilton depression rating scale

The HAM-D scale was used during the inclusion visit and after the intervention to assess the prevalence of depression symptoms [21]. The scale predominantly assesses cognitive and vegetative symptoms, with relatively few items related to social, motor, anxiety and mood factors. The 17-item HAM-D was employed in the present study, each item is scored from 0 to 2 or from 0 to 4, with total scores ranging from 0 to 52. The following cut-off points were used: ≥ 23 – very severe depression, 18–22 – severe depres-

sion, 14–18 – moderate depression 8–13 – mild depression and < 7 – not depressed [22].

Cognitive assessments

The CANTAB and MoCA tests will be employed in this study as the primary outcome parameters. The included tests comprise the following categories: executive functioning, processing speed, memory and abbreviated memory. The following test batteries will be involved in the present study: Motor Screening Task, Reaction Time, Paired Associates Learning, Spatial Working Memory, Pattern Recognition Memory, Delayed Matching to Sample, Rapid Visual Information Processing.

Neuroimaging protocol

Brain magnetic resonance imaging will be performed on all subjects using a Siemens Skyra 3T magnetic resonance imaging (MRI) System. The following MRI sequences will be used in every examination: 1) T2-weighted (fast-spin echo) and modified T2-weighted fluid-attenuated inversion recovery sequence for the detection and localisation of ischaemic lesions; 2) diffusion-weighted with an apparent diffusion coefficient map for the detection of acute ischaemic focuses; 3) susceptibility-weighted imaging for identification of intracerebral haemorrhagic and microhaemorrhagic lesions; 4) 3D angiographic time of flight sequence for visualisation of blood flow in intracerebral arterial vessels; 5) T1-weighted 3D spoiled gradient-recalled echo sequence (3D volumetric sequence) for estimation of total and segmented brain volume.

Blood collection and biochemical analysis

Blood samples will be collected from the antecubital vein via standard venepuncture performed by registered staff nurses. The samples will be taken from the participants after 12-h fasting. The following blood biomarkers will be measured: fasting glucose and insulin homeostasis markers, lipid profile, inflammatory markers (interleukin 6, interleukin 1 receptor type alpha, tumour necrosis factor-alpha, high-sensitivity C-reactive protein), as well as neuronal growth and destruction markers (brain-derived neurotrophic factor, amyloid β-40, amyloid β-42 ratio and phosphorylated Tau protein). Other biochemical and genetic analyses are planned as optional if further funding is available.

Sociodemographic and medical history questionnaires

Background, place of residence, education, family status, and economic status will be assessed before and after the intervention using a sociodemographic questionnaire. The participants will also answer questions regarding lifestyle factors, including tobacco smoking habits and alcohol consumption. A medical history questionnaire will be used to assess the health status of the study participants and to verify whether the subjects receive any medications or dietary supplements.

Statistical analyses

The STATISTICA (StatSoft, Tulsa, USA) software, or equivalent, will be used for the statistical analysis. A two-sided p-value < 0.05 will be considered statistically significant. The overall characteristics of subjects will be expressed as a mean and SD with 95% confidence interval, median and interquartile range, or as frequencies and percentages. The outcomes will also be expressed as changes between the post- and pre-intervention values (Δ value at 1 year). The normality of the variable distribution will be verified on the basis of the Shapiro-Wilk normality test. Comparisons between two unpaired groups will be determined using t-tests or Mann-Whitney U tests, respectively. The Wilcoxon test will be used to analyse the statistical significance of the pre- and post-intervention variables. The above non-parametric tests will be used, if the data either do not conform to normality or cannot be normalised by log-transformation. Otherwise, an analysis of covariance will be used to compare the differences between two groups with the baseline data as the covariate and the potential confounders added to the model. Contingency tables will be used to assess relationships between the categorical variables. Depending on the data distribution, parametric (Pearson's) or nonparametric tests (Spearman's) will be applied to assess correlations. Uni- and multivariate logistic and linear regression analyses will be used to identify independent determinants of cognitive functions. Potentially confounding factors from these univariate analyses will subsequently be entered in a multivariate linear regression analysis. In a stepwise multivariate analysis, factors

for inclusion will be set at $p < 0.1$. In terms of the categorical variables, dummy variables should be entered in the linear regression analysis. If any data are missing, we will assume that they all follow a multivariate normal distribution and adopt multiple imputation approaches. There are no planned interim statistical analyses, or formal stopping rules with regard to efficacy. For the main-outcome parameters, a correction for multiple testing will be applied, unless a multivariate model can be used which produces one single test.

Measurable Effects

This study will potentially provide additional information which allows a more efficient and precise planning of daily physical activity for MCI subjects. We expect that the study will produce exact values for the physical activity intensity required to protect against cognitive decline. The study findings might also be useful for developing first physical activity guidelines aiming to protect against cognitive impairment.

Expected Results

In the proposed randomised controlled trial, a 12-month physical activity intervention will be performed in a group of 198 subjects with MCI and aged 50–70 years. On the basis of both the current literature and new findings, we aim to establish thresholds of the intensity and frequency of physical activity which will serve to develop novel physical activity guidelines to protect against cognitive decline in high-risk adults. We will also investigate associations between physical activity, cognitive function, brain volume, and blood biomarkers. We assume that there are thresholds of physical activity frequency and intensity, which improve global cognitive functions in at-risk individuals, preserving brain volumes and maintaining biomarker levels within the normal limits. The expected findings will allow us to develop the first specific, cognitive impairment-focused physical activity guidelines, which will be effective and achievable for older subjects. Walking, as a form of physical activity, is inexpensive, easy to perform and protects from other chronic diseases, such as diabetes, cardiovascular diseases, obesity, and depression. Therefore, a simple physical activity tracker with a mobile application could be a helpful tool in increasing compliance.

Acknowledgements

Contributors

M.J., A.M. and M.W.G. wrote the manuscript. J.B., H.W.W. and Z.P. commented on the manuscript, J.K.N., K.-H.H. and E.M. designed the study and edited the manuscript. J.W. designed the study and commented on the manuscript, as well as supervises and coordinates the study. All authors read and approved the final manuscript.

Conflict of interest statement

J.K.N. reports personal fees from Norsa Pharma, grant support from Biocodex Microbiota Foundation, and non-financial support from Nutricia, outside the submitted work. J.W. received personal fees and non-financial support from Biocodex, BGP Products, Chiesi, Hipp, Humana, Mead Johnson Nutrition, Merck Sharp & Dohme, Nestle, Norsa Pharma, Nutricia, Roche, Sequoia Pharmaceuticals, and Vitis Pharma, as well as research grants, personal fees and non-financial support from Nutricia Research Foundation Poland, outside the submitted work. Other authors declare that they have no competing interests.

Funding

This research was funded by the National Science Centre (Twardowskiego Str. 16, 30–312 Kraków, Poland, https://ncn.gov.pl, telephone: +48 532 082 239, fax: +48 12 341 90 99, e-mail: biuro@ncn.gov.pl), grant number UMO-2017/27/B/NZ7/02924. The sponsor and study participants had no role in the study design and will have no role in the collection, management, analysis, and interpretation of data, writing of the report, and the decision to submit the report for publication.

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