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ABOUT JCTR

Aims and scope

The Journal of Clinical and Translational Research (JCTR) is an open access, peer-reviewed, multidisciplinary scientific journal that publishes studies with at least an ex vivo, in vivo, or clinical component. The published research is centered on any clearly defined clinical problem, which may comprise a disease or the basis of disease, a form of therapy or intervention, and clinical diagnostics or prognostics. Articles (original research, reviews, technical reports, medical hypotheses, commissioned articles, special issue articles, and editorials) are published continuously online and bimonthly in print. Studies performed in cells only will generally not be accepted unless they contain critical data that are in line with the scope of the journal. Some examples of such studies include molecular pathways that lie at the basis of a disease, novel biotechnological approaches for e.g., the production of drugs, or new techniques that improve clinical diagnostics and prognostics. Articles that combine preclinical and clinical data are given priority. Contributions from academic institutions and industry are welcome.

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REVIEW ARTICLE

Effectiveness of health education interventions in patients with fibromyalgia syndrome: an umbrella review

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ABSTRACT

Background: Fibromyalgia syndrome (FMS) is characterized by chronic widespread pain that is accompanied by emotional distress, fatigue, tender points of pain, sympathetic nervous system disturbances, and alterations in the quality of sleep.
Aim: The main aim of this umbrella review was to assess the effectiveness of health education interventions (HEI) in patients with FMS.
Methods: We searched in PubMed, PEDro, EMBASE, CINAHL, Psicodoc, and Google Scholar (August 6, 2022). The outcomes measures were pain intensity, quality of life, functionality, anxiety, and pain catastrophizing. This review was previously registered in the international prospective register of systematic reviews (SR) PROSPERO (CRD42022368068). Methodological quality was analyzed using AMSTAR and ROBIS scale, and the strength of evidence was established according to the guidelines advisory committee grading criteria.
Results: Five SR with and without meta-analysis were included in the study. The results were pooled to assess the effects of HEI in isolation and to assess the effects of HEI in combination with other interventions (multicomponent approach based on therapeutic exercise or pharmacological). The results showed that HEI combined with other interventions was effective in improving pain intensity, quality of life, functionality, and anxiety compared to minimal intervention/usual care or no intervention, although mixed evidence was found improving pain catastrophizing, all with a limited quality of evidence. Regarding HEI in isolation, contradictory evidence was found for pain intensity and quality of life variables with a limited quality of evidence. Finally, no significant results were found in improving functionality, anxiety, and pain catastrophizing variables also with a limited quality of evidence.
Conclusions: Overall, it seems that the addition of HEI to other interventions, mostly therapeutic exercise although we could refer to it in terms of a multimodal approach, leads to greater clinical improvements than HEI in isolation. We have seen this especially in some clinical variables of interest such as pain intensity or quality of life. It seems that the main strength of the HEI is the interaction with other interventions to enhance its efficacy with respect to the outcomes assessed. Further research is needed especially ensuring the correct comparison when combining HEI with other interventions to obtain more consistent results.
Relevance for Patients: Adding therapeutic education programs to the management of patients with FMS seems to have a clinically important effect. However, the application of therapeutic education in isolation does not appear to be effective in the management of these patients. More research is needed in this field.

1. Introduction

Fibromyalgia syndrome (FMS) is characterized by chronic widespread pain that is accompanied by emotional distress, fatigue, tender points of pain, sympathetic nervous

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system disturbances, and alterations in the quality of sleep [1]. Several investigations have suggested that one of the mechanisms that may be involved in the FMS is a process of central hyperexcitability [2,3]. This process involves an amplification of signaling at the neuronal level in the medullary and supramedullary centers, which may lead to increased sensitivity to pain, lowering the excitability threshold of afferent sensory inputs with painful information [4]. On an epidemiological level, FMS has a prevalence in the general population between 0.5% and 5% [5]. The prevalence is higher in women than in men [5]. Regarding mortality, the recent study conducted by Treister-Goltzman and Peleg [6] showed that FMS is associated with an increased mortality rate from all causes, especially suicidal ideation, accidents, and the presence of infections.

Nowadays, there seems to be no objective test that can help clinicians make an accurate pathophysiological diagnosis of FMS [7]. To date, most of the tools and criteria used for the diagnosis of FMS are vaguely specific [8]. This situation, together with the difficulty of subclassifying patients with FMS, poses a huge challenge when treating patients with FMS [8]. Despite this, in 2016 the American College of Rheumatology (ACR) established some criteria [9]. In the revised 2016 ACR criteria, generalized pain (rather than widespread pain) in at least four of five distinct body regions is required for a diagnosis of FMS along with persistent symptoms for more than 3 months, and also high scores on indices of widespread pain and symptom severity [9].

Regarding the treatment of FMS, the effectiveness of some treatments has been evaluated. For example, previous systematic reviews (SR) have assessed the effectiveness of some important interventions such as pharmacological treatment [10,11], psychological therapies [12,13] as well as exercise-based interventions [14,15] to manage the described main symptoms of FMS. However, most of the clinical interventions evaluated do not incorporate educational features in them. Education is fundamental in the management of patients with persistent pain, as it improves the influence of psychosocial variables that can modulate pain perception [16]. Within the biopsychosocial perspective, some health educational interventions (HEI) have been proposed as an alternative, with the aim of reconceptualizing the pain experience, improving coping strategies toward pain, or improving knowledge regarding the disease process to improve some clinical variables of interest such as disability and quality of life in patients with FMS. Educational strategies such as pain neuroscience education (PNE) or pain neurophysiology education (PNpE) are among the most studied educational interventions for patients with persistent pain [17,18]. The number of research studies evaluating the effect of HEI on patients with FMS has grown in recent years [19-23], and so far, no research studies have pooled and analyzed these results. Moreover, the SR published so far are not consistent with the results obtained. We believe that a general overview that encompasses all of them allows us to analyze the effectiveness of these interventions in depth, as well as to analyze and extract possible lines of improvement so that research may continue to be carried out in the near future.

It is therefore that the main aim of this umbrella review was to assess the effectiveness of HEIs in patients with FMS.

2. Methods

This study was conducted in accordance with the Preferred Reporting Items for Overviews of SR including harm checklist (PRIO-harms), which consists of 27 items (56 sub-items), followed by a 5-stage process flow diagram (identification, screening, eligibility, inclusion, and separation of relevant studies) [24]. This review was previously registered in the international prospective register of SR: PROSPERO (CRD42022368068).

2.1. Review inclusion criteria

The inclusion criteria employed in this article were based on methodological and clinical factors such as population, intervention, control, outcomes, and study design [25].

2.1.1. Population

The participants selected for the articles were patients with FMS. Included SR had to explicitly state that they included patients with FMS in their inclusion criteria. We excluded all SR that include patients with other chronic conditions with persistent pain.

2.1.2. Intervention and control

The intervention consisted of HEI (PNE) *(i.e.: Neurophysiology of pain, differences between "pain" and "nociception", factors contributing to the perpetuation of pain, or the influence of thoughts (cognitions) or emotions on pain experience)*, PNpE *(i.e.: neurophysiology of the central nervous system, central/peripheral hyperexcitability or sensitization/habituation concepts)*, and therapeutic education (TE) *(i.e.: FMS symptoms information, active coping strategies, or self-management strategies)* conducted in isolation, in conjunction or combined with other treatments. The education sessions could be individual or group-based and could contain any semantic resources for a better understanding (such as the presence of metaphors). Interventions based on psychological treatment or cognitive behavioral therapy were excluded from the study. The comparator groups used the following interventions: no intervention, minimal interventions in isolation or combined to form a multicomponent approach. (e.g.: information about relaxation, analgesic drugs, therapeutic exercise, or exercises information booklets), or waiting list.

Regarding the intervention studied:

- TE is a therapeutic modality that explicitly involves a non-directional dynamic interaction with the patient, based on a biobehavioral paradigm, which includes educational or training activities that promote learning and acquisition of adaptive skills to improve self-management and knowledge that facilitate changes in beliefs, attitudes, and behaviors associated with disability. TE aims to change maladaptive beliefs, reconceptualize aspects related to pain, implement educational processes on the importance of therapeutic

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exercise, improve adherence, active coping skills training, and sleep regulation strategies. It may also include techniques such as sensory retraining, sensory reinterpretation, experiential motor restructuring, activity and exercise, and graded exercise exposure as part of TE.

- PNE corresponds to educational processes that focus on a broad, multidisciplinary understanding of pain, including neuroanatomical, neurochemical, cognitive emotional, and social aspects that relate to the perception of the pain experience.
- Finally, PNpE corresponds to educational aspects that focus on a more specific understanding of the neurophysiological and neurobiological processes underlying pain perception, also including the transmission of the nociceptive signal, its processing at the central nervous system level and pain modulation systems.

2.1.3. Outcome measures

The outcomes employed to assess the effectiveness of HEI were pain intensity, quality of life, functionality, anxiety, and pain catastrophizing.

2.1.4. Study design

We selected SR (with or without a meta-analysis) of randomized controlled trials (RCTs) or controlled clinical trials (CCTs) and excluded SR that included RCTs or CCTs in combination with non-experimental designs. There were no restrictions for any specific language, as recommended by the international criteria [26].

2.2. Search strategy

We conducted the search for published scientific articles between 1950 and August 6, 2022, in the following databases: MEDLINE (PubMed), EMBASE, PEDro, CINAHL, Psicodoc, and SPORTDiscus. An additional manual search was realized in Google Scholar. The reference sections of the included studies and original studies were screened manually, and the authors were contacted for further information if necessary. The search strategy combined Medical Subjects' Headings (MeSH ["Fibromyalgia"]) or ["Patient Education as topic"], and non-MeSH terms ("fibrositis", "fibromyositis", "rheumatism muscular", "fibromyalgias", "fibromyalgia secondary", "fibromyalgia primary", "PNE", "therapeutic neuroscience education", "pain neurophysiology education", or "patient education") adding a Boolean operator (AND and/or OR) to combine them. Appendix 1 shows the search strategy, which was adapted for each database. The search was conducted by two independent reviewers using the same methodology. Differences that emerged during this phase were resolved by consensus. The reference sections of the original studies were screened manually, and the authors were contacted for further information if necessary.

2.3. Selection criteria and data extraction

Initially, the two independent reviewers conducted a screening assessing the relevance of the SR (with and without a meta-analysis) regarding the studies' questions and objectives. The first screening was based on each study's title information, abstract, and keywords. The full text was reviewed if there was no consensus or if the abstracts contained insufficient information. In the second phase of the screening, the full text was assessed if the studies met all of the inclusion criteria. Differences between the reviewers were resolved by a discussion and consensus process mediated by a third reviewer. The data described in the results section were extracted by means of a structured protocol that ensured that the most relevant information was obtained from each study.

2.4. Methodological quality assessment

The two independent reviewers assessed the methodological quality of the SR (with or without meta-analysis), assessing each of the selected studies based on the Modified Quality Assessment Scale for SR (AMSTAR) developed by Barton *et al.* [27] a scale shown to be a valid and reliable tool for assessing the methodological quality of SR. With a total of 13 items, each worth 2 points (with "yes" scoring 2; "in part" scoring 1; "no" scoring 0), the maximum possible score is 26. A high-quality cutoff of 20 or more points was provided by the developers. The exclusion and keyword criteria were modified to better evaluate the selected SR of this study. In addition, we calculated the kappa coefficient (κ) and percentage (%) agreement scores to assess reliability before any consensus.

2.5. Risk of bias assessment

We assessed the risk of bias with the Risk of bias in SR tool (ROBIS) [28], which consists of three phases: (1) Relevance assessment (optional); (2) identification of concerns with the review process through four domains related to study eligibility criteria, identification and selection of studies, data collection and study appraisal and synthesis and findings; and (3) judgment on the risk of bias.

2.6. Grading of evidence

The physical activity guidelines advisory committee grading criteria (PAGAC) were used to assess the grading of evidence. The criteria used to assess the quality of the evidence were as follows: (1) Applicability of the study sample, exposures, and outcomes to the research question, (2) generalizability to the population of interest, (3) risk of bias/study limitations, (4) quantity and consistency of findings across studies, and (5) magnitude and precision of the effect. With these data, final evidence grades and conclusion statements for each research question were developed [29].

3. Results

3.1. Study selection

The initial search revealed 99 records. Through the title and abstract screening and the full-text assessment, five SRs were eligible according to our criteria. The study screening strategy is shown in the form of a flow chart **(**Figure 1**)**.

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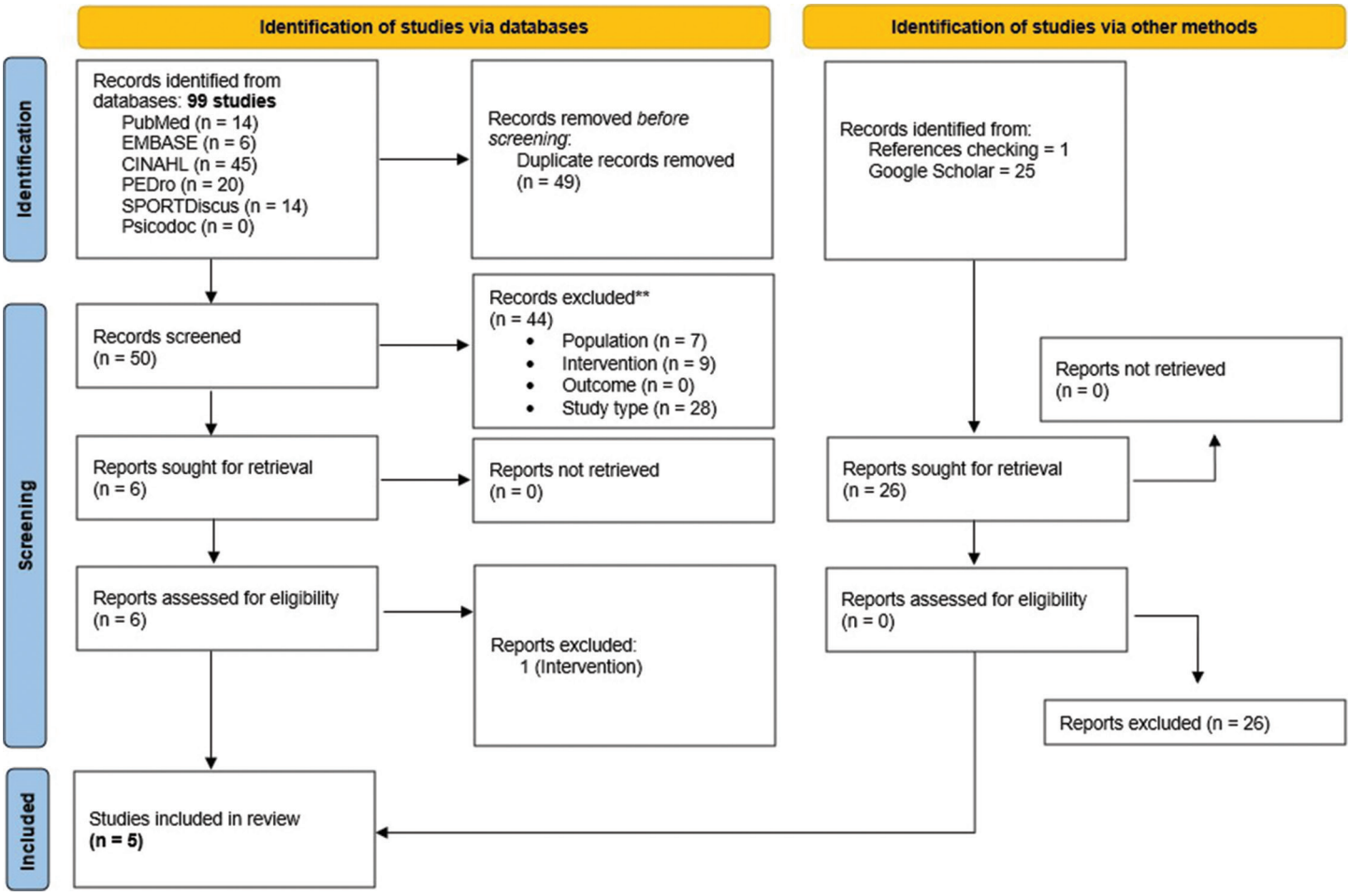

Figure 1. PRISMA Flowchart of studies selection.
*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).
**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.
From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, *et al.* The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org.

3.2. Characteristics of the included SR

Table 1 lists the characteristics of the SR included (study design, original studies included, demographic characteristics, interventions, variables, and results). Antunes *et al.* [19] conducted a SR that included two RCTs only, of which only one primary study evaluated the effect of TE (FMS symptoms information, active coping strategies) as a form of HEI in combination with a multicomponent approach (therapeutic exercise, relaxation techniques or pharmacology) versus no intervention. The study conducted by Elizagaray-García *et al.* [20] analyzed a total of five RCTs. Two of the five primary studies compared HEI (PNE, PNpE, and TE) in isolation against minimal intervention (including information leaflets on stretching, relaxation, or general pain management strategies). Two further studies had at least one study arm that performed some model of HEI in isolation for comparison against no intervention or waiting list. Finally, three RCTs combined HEI with a therapeutic exercise-based approach (aerobic, strengthening, or flexibility exercise) and compared it against waiting list, information leaflet, or no intervention. The study carried out by García-Ríos *et al.* [21] analyzed a total of 12 RCTs. In six studies, FMS patients received HEI as the only form of intervention (including PNE, PNpE, and TE). In the remaining studies, HEI was combined with other interventions such as therapeutic exercise, including pool exercise. Saracoglu *et al.* [22] included only four primary studies where the PNE-based intervention was added to a multicomponent approach (including cognitive-behavioral therapy, mindfulness training, or therapeutic exercise) and compared it to a minimal intervention. Suso-Martí *et al.* [23] analyzed eight RCTs. They included primary studies where the role of HEIs (PNE and PNpE) was assessed in isolation or if combined with an intervention, it had to be in the control group to ensure correct comparison between groups and to be able to attribute clinical differences to HEIs. In fact, only two RCTs combined HEI with therapeutic exercise and relaxation exercises, but these interventions were also in the comparison group.

Table 1. Characteristics of the reviews included in the umbrella review

Study	Studies k (n) Types	Meta-analysis (k)	Population	Intervention	Control	Outcomes (instruments)	Author's conclusions
Antunes *et al*. [19]	2 RCTs (65)	No	FMS FMS (Diagnosis based on 1990/2010ACR criteria)	HEI - TE in FMS (FMS symptoms information and active coping strategies) (+ Multicomponent approach in 1/1 RCT)	Comparator - No intervention	- Pain intensity (NHP subscale) - Quality of life (NHP)	An interdisciplinary health education program can improve pain and quality of life in people with FMS
Elizagaray-García *et al*. [20]	5 RCTs (611)	No	FMS (Diagnosis based on 1990 ACR criteria)	HEI - PNpE - PNE - TE in FMS (FMS symptoms information, SM skills education or active coping strategies) (+TEx in 3/5 RCT)	Comparator - Information about relaxation - Stretching exercises information booklets - No intervention - Waiting list	- Pain intensity (PPT, TS, SSP, CPM, FIQ subscale and number of tender points) - Quality of life (SF-36 and SV-QOLS) - Functionality (FIQ subscale, SF-36 subscale and 6MWT)	HEI, in itself, has not proved to be effective for pain intensity, quality of life or functionality in patients with FMS. However, HEI in combination with TEx showed effectiveness on the variables analyzing.
García-Ríos *et al*. [21]	12 RCTs (1389)	No	FMS (Diagnosis based on 1990 ACR criteria)	HEI - PNpE - PNE - TE in FMS (FMS symptoms information, SM skills education or active coping strategies) (+ Multicomponent approach in 6/12 RCT)	Comparator - Information about relaxation - Relaxation breathing - Stretching exercises information booklets - FMS information booklets - Waiting list - Usual practice - TEx	- Pain intensity (VAS, PPT, SSP, PCI, MPI-S and PVAQ) - Quality of life (FIQ, IPQ-R, EQ-5D, SF-36, NHP and SV-QOLS) - Functionality (FIQ subscale, SF-36 subscale, 6MWT and AIMS) - Anxiety (PGWB and GADS) - Pain Catastrophizing (PCS)	The scientific evidence that supports the effectiveness of HEI in the reduction of pain intensity, quality of life, functionality, anxiety, and pain catastrophizing is limited.
Saracoglu *et al*. [22]	4 RCTs (612)	Yes (4)	FMS (Diagnosis based on 2010 ACR criteria)	HEI - PNE (+ Multicomponent approach in 2/4 RCTs)	Comparator - Minimal intervention (patient information about the disease, recommendations on aerobic exercise, and pharmacological treatment)	- Pain intensity (VAS and NPRS) - Quality of life (FIQ)* - Anxiety (HADs) - Pain Catastrophizing (PCS)	Adding PNE to a multimodal treatment including TEx might be an effective approach for improving functional status, pain-related symptoms, anxiety, and depression for patients with FMS.
Suso-Martí *et al*. [23]	8 RCTs (738)	Yes (8)	FMS (Diagnosis based on 1990/2010/2016 ACR criteria)	HEI - PNE (+TEx in 1/8 RCT)	Comparator - Relaxation - Breathing exercises - Minimal intervention (pharmacological usual care or general advice) - No intervention - TEx	- Pain intensity (VAS, SF-BPI, NPRS) - Quality of life (FIQ) - Anxiety (PASS-20, HAQ, and HADS) - Pain Catastrophizing (PCS)	In patients with FMS, PNE can decrease the pain intensity in the post-intervention period and the quality of life in the follow-up period (3 m). However, it appears that PNE showed no effect on anxiety and pain catastrophizing.

Notes. FMS: Fibromyalgia syndrome; ACR: American college of rheumatology; RCT: Randomized controlled trial; PNpE: Pain Neurophysiology Education; SM: Self-management; PNE: Pain neuroscience education; TE: Therapeutic education; PPT: Pressure pain threshold; TS: Temporal summation; CPM: Conditioned pain modulation (CPM); FIQ: Fibromyalgia impact questionnaire; SF-36: 36-Item Short Form Health Survey; QOLS: Swedish version quality of life scale; 6MW: 6 minutes walking test; HEI: Health education interventions; PT: Physical Therapy; m: months; PCS: Pain catastrophizing scale; VAS: Visual analogue scale; SF-BPI: Short form of brief pain inventory; NPRS: Numeric pain rating scale; HAQ: Health assessment questionnaire; PASS-20: Pain anxiety symptoms scale-20; HADS: Hospital anxiety and depression scale; TEx: Therapeutic exercise; SSP: Spatial summation of pain; PCI: Pain coping inventory; PVAQ: Pain and awareness surveillance questionnaire; MPI-S: Swedish version of the Multidimensional pain inventory; IPQ-R: Revised illness perception questionnaire; EQ-5D: EuroQoL-5D questionnaire; AIMS: Arthritis impact measurement scales; NHP: Nottingham health profile. PGWB: Psychological general well-being and GADS: Goldberg scale of anxiety and depression.

*The quality of life variable was reinterpreted for this study. In the original review, it is found as: severity of FMS

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Table 2. Quality assessment scores (AMSTAR)

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	Score
Antunes *et al*. [19]	2	1	0	1	1	2	2	2	2	0	0	1	0	14
Elizagaray-García *et al*. [20]	2	2	0	2	1	2	2	2	2	0	0	2	1	18
García-Ríos *et al*. [21]	2	2	0	2	1	2	2	2	2	0	0	1	1	17
Saracoglu *et al*. [22]	2	2	2	2	1	2	2	2	2	2	2	2	0	23
Suso-Martí *et al*. [23]	1	2	2	0	2	2	1	2	2	2	2	2	2	22

Notes: 1. Explicitly described to allow replication; 2. Adequate number and range of databases; 3. Alternative searches; 4. Adequate range of key words; 5. Non-English-language papers included in the search; 6. Inclusion criteria explicitly described to allow replication; 7. Excludes reviews which do not adequately address inclusion and exclusion criteria; 8. Two independent reviewers assessing selection bias; 9. Quality assessment explicitly described to allow replication; 10. Meta-analysis conducted on only homogeneous data or limitations to homogeneity discussed; 11. Confidence intervals/effect sizes reported where possible; 12. Conclusions supported by the meta-analysis or other data analysis findings; 13. Conclusions address levels of evidence for each intervention/comparison

Table 3. Summary of findings and quality of evidence (PAGAC)

	2018 PAGAC				Magnitude and precision of effect	Overall grade
Systematic review research questions	Applicability	Generalizability	Risk of bias or study limitations	Quantity and consistency		
Pain intensity	Strong	Limited	Limited	Limited	Not assignable	Limited
Quality of life	Strong	Limited	Limited	Limited	Not assignable	Limited
Functionality	Moderate	Limited	Limited	Limited	Not assignable	Limited
Anxiety	Moderate	Limited	Limited	Limited	Not assignable	Limited
Pain catastrophizing	Moderate	Limited	Limited	Limited	Not assignable	Limited

PAGAC: Physical activity guidelines advisory committee grading criteria

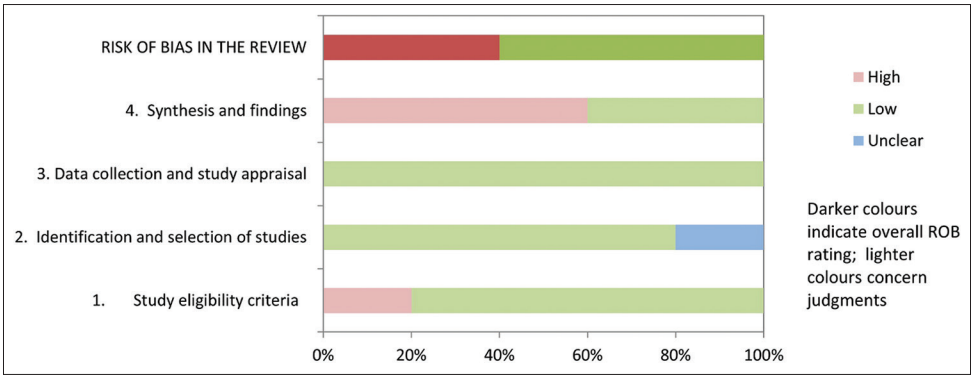

Figure 2. Graphical representation for risk of bias in SR tool results.

Finally, Suso-Martí *et al.* [23] included primary studies that used the ACR criteria from 1990, 2010, and 2016 as the diagnosis for FMS. Antunes *et al.* [19] included studies that used the ACR criteria from 1990 and 2010. Saracoglu *et al.* [22] included only the ACR 2010 diagnosis. García-Ríos *et al.* [21] and Elizagaray-García *et al.* [20] used the ACR 1990 criteria.

3.3. Results of AMSTAR and ROBIS

The scores ranged from 14 to 23 points out of a possible 26, with a mean score of 18.8 points. Only two (40%) study scored above 20 points and were considered high-quality **(**Table 2**)**. The inter-rater reliability of the methodological quality assessment was high ($\kappa = 0.91$). Figure 2 shows the results of the risk of bias assessment using ROBIS. About 60% of studies had a low risk of bias.

3.4. Grading of evidence results (PAGAC)

Table 3 shows the findings regarding the quality of evidence for each outcome of research question. The quality of evidence found for all outcome measures was limited.

3.5. Qualitative synthesis of HEI (in isolation)

3.5.1. HEI (in isolation)

3.5.1.1. Pain intensity

A total of three SR offered at least one outcome for the pain intensity variable [20,21,23]. Elizagaray-García *et al.* [20] found strong evidence ($n = 4$) of HEI, in isolation, did not show significant improvements in reducing pain intensity in the short, medium, or long term. However, García-Ríos *et al.* [21] found statistically significant differences in the pain

intensity variable in favor of HEIs. In addition, Suso-Martí *et al.* [23] found that PNE showed statistically significant differences reducing post-intervention pain intensity with a moderate clinical effect (n = 7, SMD = −0.76; 95% CI: −1.33 – −0.19, P < 0.05, I^2 = 92%) but not at 3 months of follow-up (n = 7, SMD = −0.42; 95% CI: −0.93 – 0.08, P > 0.05, I^2 = 89%).

3.5.1.2. Quality of life

A total of two SRs offered at least one outcome for the quality of life variable [20,23]. Elizagaray-García *et al.* [20] found strong evidence (n = 5) of HEI, in isolation, did not show significant improvements in improving quality of life in the short, medium, or long term. Finally, Suso-Martí *et al.* [23] found that PNE did not show statistically significant post-intervention improvements in quality of life (n = 8, SMD = −0.37; 95% CI: −0.85 – 0.11, P > 0.05, I^2 = 91%). However, Suso-Martí *et al.* [23] found statistically significant improvements in quality of life at 3 months of follow-up with a small clinical effect (n = 8, SMD = −0.44; 95% CI: −0.73 – −0.14, P < 0.05, I^2 = 89%).

3.5.1.3. Functionality

One SR offered at least one outcome for the functionality variable [20]. Elizagaray-García *et al.* [20] found controversial evidence (n = 3) of HEI, in isolation, did not show significant improvements in improving functionality in the short term.

3.5.1.4. Anxiety

One SR offered at least one outcome for the anxiety variable [23]. Suso-Martí *et al.* [23] found no statistically significant differences in anxiety improvement either at post-intervention (n = 5, SMD = −0.06; 95% CI: −0.67 − 0.55, P > 0.05, I^2 = 85%) or at 3-month follow-up (n = 5, SMD = −0.07; 95% CI: −0.69 to 0.82, p>0.05, I^2=85%).

3.5.1.5. Pain catastrophizing

One SR offered at least one outcome for pain catastrophizing variable [23]. Suso-Martí *et al.* [23] found no statistically significant differences in pain catastrophizing improvement either at post-intervention (n = 8, SMD = −0.10; 95% CI: −0.52 – 0.32, P > 0.05, I^2 = 89%) or at 3-month follow-up (n = 8, SMD = −0.16; 95% CI: −0.52 – 0.19, P > 0.05, I^2 = 86%).

3.5.2. HEI (in combination with other interventions)

3.5.2.1. Pain intensity

A total of four SR offered at least one outcome for the pain intensity variable [19-22]. Antunes *et al.* [19] found in one primary study that HEI plus multicomponent approach significantly reduced pain intensity. Elizagaray-García *et al.* [20] found moderate evidence (n = 2) of HEI plus therapeutic exercise showed significant improvements in reducing pain intensity in the medium term although mixed results were found in the short term. García-Ríos *et al.* [21] found that studies analyzing the impact of HEI, in combination with other approaches, showed a significant improvement in pain intensity variable (n = 8). Finally, Saracoglu *et al.* [22] also found that adding PNE to a multicomponent approach resulted in a statistically significant decrease in pain intensity with a moderate clinical effect (n = 3, standardized mean differences (SMD) = −1.05; 95% confidence interval (CI): −1.4 – −0.69, P < 0.001, I^2=37.7%).

3.5.2.2. Quality of life

A total of four SRs offered at least one outcome for the quality of life variable [19-22]. Antunes *et al.* [19] found in one primary study that HEI plus multicomponent approach significantly improved quality of life. Elizagaray-García *et al.* [20] found strong evidence (n = 4) of HEI plus therapeutic exercise significantly improved quality of life in the short, medium, and long term. García-Ríos *et al.* [21] reported that the best results in improving quality of life were found when a multicomponent approach was added to HEIs. Finally, Saracoglu *et al.* [22] found that adding PNE to a multicomponent approach resulted in a statistically significant improve in quality of life with a moderate clinical effect (n = 4, SMD = −1.05; 95% CI: −1.3 – −0.79, P < 0.001, I^2 = 86%).

3.5.2.3. Functionality

A total of two SRs offered at least one outcome for the functionality variable [20,21]. Elizagaray-García *et al.* [20] found strong evidence (n = 3) of HEI plus therapeutic exercise significantly improved functionality in the short and the medium term. Finally, García-Ríos *et al.* [21] found that adding HEI to a multicomponent approach resulted in a statistically significant improve in functionality (n = 3).

3.5.2.4. Anxiety

A total of two SRs offered at least one outcome for the anxiety variable [21,22]. García-Ríos *et al.* [21] found that adding HEI to a multicomponent approach resulted in a statistically significant improve in anxiety (n = 4). Finally, Saracoglu *et al.* [22] found that adding PNE to a multicomponent approach resulted in a statistically significant improve in anxiety with a moderate clinical effect (n = 4, SMD = −0.711; 95% CI: −0.86 – −0.55, P < 0.001, I^2 = 51.6%).

3.5.2.5. Pain catastrophizing

A total of two SRs offered at least one outcome for pain catastrophizing variable [21,22]. García-Ríos *et al.* [21] showed contradictory results with regard to the improvement of pain catastrophizing variable (n = 2). Finally, Saracoglu *et al.* [22] found that adding PNE to a multicomponent approach resulted in a statistically significant improve in pain catastrophizing with a moderate clinical effect (n = 3, SMD = −0.89; 95% CI: −1.43 – −0.34, P = 0.001, I^2 = 70.5%).

4. Discussion

The main aim of this review was to assess the effectiveness of HEI in patients with FMS. We divided the results into two groups: When HEI were evaluated in isolation and when HEI

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were evaluated in combination with other interventions, which were not present in the comparator group.

4.1. Summary results

Analyzing the outcome for each variable, for pain intensity, we found mixed evidence in favor of HEI alone, as we found significant and non-significant post-intervention results. However, in the short- to medium-term, no significant differences were found in favor of HEI. When HEI was combined with other interventions, the results showed significant effects on the reduction of pain intensity in the short and even in the medium term. With respect to quality of life, HEI in isolation did not lead to significant improvements in the short term; however, mixed evidence was found in the short- to medium-term. When HEI was combined with other interventions, the results showed significant effects on improving quality of life in the short, medium, and even long term. On the variables functionality and anxiety, the HEI alone did not show any significant effect on the improvement of these variables. However, when analyzing the combination of HEI with other interventions, we found significant improvements in both functionality and anxiety symptoms in favor of HEI combined with other interventions. Finally, with regard to the pain catastrophizing variable, the results showed that the HEI alone did not lead to any significant improvement. When evaluating the combination of the HEI with other interventions, the evidence found was mixed.

4.2. Strengths and weaknesses of HEIs

Overall, it seems that the addition of HEI to other interventions, mostly therapeutic exercise although we could refer to it in terms of a multimodal approach, leads to greater clinical improvements than HEI in isolation. We have seen this especially in some clinical variables of interest such as pain intensity or quality of life. It seems that the main strength of the HEI is the interaction with other interventions to enhance its efficacy with respect to the outcomes assessed. HEI are clinical interventions that has the communication process as a key point of its application and where the patient feels listened to, cared for and, in addition, allows patients to better understand their clinical condition process [30]. This increased knowledge from a patient perspective, together with an adequate context promoted by empathy, shared understanding between health professional and patient, and increasing social support, seems to help improve the influence of psychological variables that are widely present in chronic pain processes. However, despite this, a clinical approach based on HEI in isolation may be insufficient to provide clinically relevant and meaningful outcomes in patients with FMS, and we believe that HEI should be combined with an active and/or passive intervention (such as exercise-based interventions, manual neuro-orthopedic physiotherapy, or pharmacological) to improve its efficacy. Positive effects on decreasing pain intensity, disability levels, or catastrophic thoughts have been described when researchers combined PNE together with an exercise-based intervention compared to exercise-based intervention alone in patients with chronic musculoskeletal pain [31] or in patients with chronic non-specific spinal pain [32]. Given that exercise has already shown positive results in pain patients such as FMS [33,34] or chronic non-specific low back pain [35,36] in the scientific literature, it seems that future studies should address whether HEI could improve the efficacy of therapeutic exercise-based interventions. It is important to highlight at the clinical level the dosage of HEIs in the patient with persistent pain, in this case, applied to FMS. Recently, the study conducted by Salazar-Méndez *et al.* [37] aimed to evaluate how long it is necessary to perform PNE and PNpE in patients with chronic pain to obtain a clinical change in psychosocial variables. The authors found very interesting results. For example, they found that the longer the HEIs time, the greater improvements were found in variables such as anxiety, catastrophizing, or movement-related fear. In fact, it was estimated that a dose of 100, 200, and 400 min of HEIs exceeded the clinically relevant difference in the improvement of the three variables mentioned above.

Finally, as a practical recommendation for implementing HEI in patients with FMS, the authors of this article propose that HEI should be implemented in combination with other clinical interventions (such as therapeutic exercise) to achieve a stronger clinical effect. The application should be individualized and person-centered. Consideration should be given to the application of not only educational aspects but also processes focused on changing dysfunctional behaviors to have a greater impact on the person and be applicable to the person's daily life. Finally, dosage matters and clinicians must deliver the number of sessions (or intervention time) necessary to have an influence on the clinical variables of interest in FMS patients.

4.3. Study limitations

This review has some limitations that need to be taken into consideration. First, a great deal of heterogeneity has been found with education models, which makes it difficult to draw solid conclusions. Studies are needed to define well what each intervention is and how to implement it so that it has its own name. Second, the results were categorized into "HEI in isolation" and "HEI combined with other interventions". We included in the first those studies where only the role of HEI was evaluated or if HEI was combined with an intervention, the latter should also be in the comparator group to ensure correct comparability between the groups. The group "HEI combined with other interventions" was created when HEI was combined with other interventions which were not found in the comparison group. This is a relevant methodological problem because the clinical effect cannot be attributed to HEIs completely. In addition, the quality of evidence was low for most of the included studies. This is an issue to be considered, as more studies in this field could probably change the results of the outcome measures. Future studies should ensure proper comparability to draw more robust conclusions. Finally, as no statistical aggregation could be performed due to the low number

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of included studies, the conclusions are somewhat ambiguous as they satisfy a qualitative analysis, and not a quantitative one (which would be more robust).

5. Conclusion

Overall, it seems that the addition of HEI to other interventions leads to greater clinical improvements than HEI in isolation. We have seen this especially in some clinical variables of interest such as pain intensity or quality of life. It seems that the main strength of the HEI is the interaction with other interventions to enhance its efficacy with respect to the outcomes assessed. Further research is needed especially ensuring the correct comparison when combining HEI with other interventions to obtain more consistent results.

Finally, we could raise public awareness through informative campaigns, and on the health-care front, we can implement educational programs for health-care professionals with the aim of improving the understanding and management of FMS.

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Conflicts of Interest

None declared.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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Appendix

Appendix 1. Database search strategies

-PubMed (Medline) (14 articles retrieved)

((“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR “fibromyalgias”[All Fields] OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“fibromyalgia”[All Fields] AND “fibromyositis”[All Fields] AND “syndrome”[All Fields])) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“syndrome”[All Fields] AND “fibromyalgia”[All Fields] AND “fibromyositis”[All Fields])) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“rheumatism”[All Fields] AND “muscular”[All Fields]) OR “rheumatism muscular”[All Fields]) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR “fibrositis”[All Fields]) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“diffuse”[All Fields] AND “myofascial”[All Fields] AND “pain”[All Fields] AND “syndrome”[All Fields])) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“fibromyositis”[All Fields] AND “fibromyalgia”[All Fields] AND “syndrome”[All Fields])) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“fibromyalgia”[All Fields] AND “secondary”[All Fields]) OR “fibromyalgia secondary”[All Fields]) OR (“fibromyalgia”[MeSH Terms] OR “fibromyalgia”[All Fields] OR (“fibromyalgia”[All Fields] AND “primary”[All Fields]) OR “fibromyalgia primary”[All Fields])) AND (((“pain”[MeSH Terms] OR “pain”[All Fields]) AND (“neuroscience s”[All Fields] OR “neurosciences”[MeSH Terms] OR “neurosciences”[All Fields] OR “neuroscience”[All Fields]) AND (“educability”[All Fields] OR “educable”[All Fields] OR “educates”[All Fields] OR “education”[MeSH Subheading] OR “education”[All Fields] OR “educational status”[MeSH Terms] OR (“educational”[All Fields] AND “status”[All Fields]) OR “educational status”[All Fields] OR “education”[MeSH Terms] OR “education s”[All Fields] OR “educational”[All Fields] OR “educative”[All Fields] OR “educator”[All Fields] OR “educator s”[All Fields] OR “educators”[All Fields] OR “teaching”[MeSH Terms] OR “teaching”[All Fields] OR “educate”[All Fields] OR “educated”[All Fields] OR “educating”[All Fields] OR “educations”[All Fields])) OR (“patient education handout”[Publication Type] OR “patient education as topic”[MeSH Terms] OR “patient education”[All Fields]))) AND (meta-analysis[Filter] OR systematicreview[Filter])

-EMBASE (6 articles retrieved)

‘fibromyalgia’/exp AND (‘pain education’/exp OR ‘pain neuroscience education’/exp) AND (‘systematic review’/exp OR ‘review, systematic’ OR ‘systematic review’ OR ‘meta analysis’/exp OR ‘analysis, meta’ OR ‘meta analysis’ OR ‘meta-analysis’ OR ‘metaanalysis’)

Mapped terms ‘’meta analysis” mapped to ‘meta analysis’, term is exploded

-PEDro (20 articles retrieved)

1. Abstract and Title: Pain Neuroscience Education AND Fibromyalgia. (seven articles retrieved)
2. Abstract and Title: Pain Neuroscience Education AND Fibromyalgia. Method: systematic review (one article retrieved)
3. Abstract and Title: Pain Neurophysiology Education AND Fibromyalgia. Method: systematic review (0 articles retrieved)
4. Abstract and Title: Pain Education AND Fibromyalgia. Method: systematic review (12 articles retrieved)

-CINAHL (45 articles retrieved)

-(pain neuroscience education or pain neurophysiology education) AND (fibromyalgia or fibromyalgia syndrome or fms or fm)
-(pain neuroscience education or pain neurophysiology education) AND (fibromyalgia [mesh] or fibromyalgia or fibromyalgia syndrome or fms or fm)
-(pain neuroscience education’ or ‘pain education’ or ‘pain neurophysiology education’ or ‘therapeutic neuroscience education) AND fibromyalgia syndrome)
-(pain neuroscience education’ or ‘pain education’ or ‘pain neurophysiology education’ or ‘therapeutic neuroscience education) AND fibromyalgia syndrome AND (systematic review or meta-analysis)

-Psicodoc (0 articles retrieved)

Terms employed:
-Pain neuroscience education
-Pain education
-Pain neurophysiology education
-Therapeutic neuroscience education
-Fibromyalgia

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-SPORTDiscus (14 articles retrieved)

-pain neuroscience education' or 'pain education' or 'pain neurophysiology education' or 'therapeutic neuroscience education) AND (fibromyalgia [mesh] or fibromyalgia or fibromyalgia syndrome or fms or fm) AND (systematic review or meta-analysis
-pain neuroscience education' or 'pain education' or 'pain neurophysiology education' or 'therapeutic neuroscience education) AND (fibromyalgia [mesh] or fibromyalgia or fibromyalgia syndrome or fms or fm)

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ORIGINAL ARTICLE

Association between response to neoadjuvant chemotherapy and survival outcome after radical surgery in patients with yielding pathological T2≤ and/or N+ urothelial carcinoma

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ABSTRACT

Background and Aim: In early 2022, the use of adjuvant nivolumab for patients with high-risk muscle-invasive urothelial carcinoma (MIUC) was approved in Japan, European countries, and USA based on the positive results of CheckMate 274 trial, which included participants who received neoadjuvant chemotherapy (NAC). Subgroup analyses of CheckMate 274 trial do not report response to NAC and benefit from adjuvant nivolumab. Herein, we investigated the association between response to NAC and survival outcomes after radical surgery in patients with residual MIUC and/or lymph node disease.
Methods: This multicenter retrospective study included a total of 95 NAC-treated patients with yielding pathological (yp) T2≤ and/or ypN+ UC on radical surgery specimens. Based on the comparison of clinical T and N category with yp T and N category, the patients were categorized into three groups: Down-staged ypT2≤ (n = 14), no-changed ypT2≤ (n = 39), and up-staged ypT2 ≤ groups (n = 42).
Results: There was no significant difference in extraurinary tract recurrence-free survival, cancer-specific survival, and overall survival after the radical surgery among three groups. Subgroup analysis of a bladder cancer cohort showed a marginal association between better response and longer cancer-specific survival (P = 0.073).
Conclusion: Our finding suggested that adjuvant nivolumab should be considered for all the patients with pathological ypT2≤ or ypN+ UC regardless of response to NAC. Further research is mandatory in finding predictive factors that serve in decision-making for NAC-treated patients who are likely to benefit from adjuvant nivolumab.
Relevance for Patients: To develop a decision-making tool for adjuvant nivolumab, we investigated the association between response to NAC and survival after radical surgery. Further research is mandatory in finding predictive factors that serve in decision-making for NAC-treated patients who are likely to benefit from adjuvant nivolumab.

1. Introduction

Urothelial carcinoma (UC) arises from the urinary tract mucosa in the renal pelvis, ureters, bladder, or urethra. Particularly, muscle-invasive UC (MIUC) is aggressive and associated with a poor clinical outcome, requiring multidisciplinary management. Radical nephroureterectomy (RNU) with bladder cuff remains the standard care for localized upper

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urinary tract UC (UTUC) [1]. According to reports of muscle-invasive UTUC in the 2000s, 5-year cancer-specific survival (CSS) rates of pT2, pT3, and pT4 were 75 – 84%, 54 – 56%, and 0 – 12%, respectively [2-4]. A randomized control trial (RCT) [5] and recent meta-analyses of 11 retrospective studies [6] revealed that for high-risk UTUC, RNU with both neoadjuvant chemotherapy (NAC) and adjuvant chemotherapy (AC) provides better survival than RNU alone. In the latest European Association of Urology Guidelines on UTUC, the evidence level of AC was positive level 1b, and platinum-based AC was recommended for patients having muscle-invasive UTUC and/or pN + disease without NAC [7]. In muscle-invasive bladder cancer (MIBC), cisplatin-based NAC followed by radical cystectomy (RC) is the current standard care based on level 1 evidences [7-9]. A systematic review and meta-analysis including 15 RCTs with >3000 patients demonstrated that cisplatin-based NAC decreased the risk of mortality by approximately 20% compared to RC without NAC [10].

The pathologic response to NAC, frequently defined as ≤ yielding pathological (yp) T1 and ypN0 was associated with favorable survival outcome after RC or RNU for patients with MIUC [11-13]. In contrast, residual MIUC disease, *that is*, ypT2≤ and/or ypN+ after NAC, was a strong poor prognostic factor for disease recurrence and death. Recently, the CheckMate 274 trial demonstrated that adjuvant nivolumab provided significant benefit on disease-free survival in NAC-treated patients with residual MIUC disease and/or ypN+ [14]. Although adjuvant nivolumab is recommended for the disease subset in several guidelines [7-9], many patients having UC are elderly and vulnerable, and immune checkpoint inhibitors (ICIs) can cause divergent immune-related adverse events, which are sometimes serious and lethal, requiring high-dose steroids [14-18]. Moreover, updated data of the CheckMate 274 trial demonstrated that Grade 3 – 4 treatment-related adverse events occurred in 18.2% and 7.2% of patients in the nivolumab and placebo arms, respectively [19]. Because the patient subset indicated for adjuvant nivolumab in the guidelines is heterogeneous, it would be vital to select patients who are likely to benefit from this treatment.

The association between response to NAC and survival outcomes after RC or RNU remains unclear. We hypothesized that patients with pre-NAC cT3 and post-NAC ypT2 (down-staged) could have better prognosis compared to those with pre-NAC cT2 and post-NAC ypT2 (no-changed). This study investigated the potential association by stratifying NAC-treated patients with MIUC into three groups: Down-staged ypT2≤, no-changed ypT2≤, and up-staged ypT2≤ groups.

2. Methods

2.1. Study cohorts of NAC-treated MIUC patients and data collection

This retrospective multicenter study was approved by the ethics committee of each participating institute (reference ID: 1298, 1958, 2891, H30-048, and 2018-036) of the Nishinihon Uro-Oncology Collaborative Group framework. Informed consent was obtained from the participants or bereaved families through posters and/or websites using the opt-out method [20]. We reviewed the medical charts of 214 consecutive patients with bladder cancer who underwent RC between 2000 and 2021 at the Nara Medical University Hospital and 1,775 patients with UTUC who underwent RNU between 1995 and 2018 at four hospitals across Western Japan (Figure 1). Inclusion criteria were as follows: (1) Patients receiving NAC for invasive UC before radical surgery and (2) pathologically diagnosed ypT2≤ and/or ypN+ UC in the radical surgery specimens. Exclusion criteria were as follows: Patients with critical data missing. Of 1989 patients, 95 (4.8%) who received NAC followed by radical surgery, RC, or RNU and diagnosed with ypT2≤ tumors and/or ypN+ were eligible for the analysis (Figure 1A).

2.2. Image interpretation for MIUC

All radiographic data of computed tomography (CT), CT urography, and/or magnetic imaging resonance (MRI) taken before the initiation of NAC were uploaded in a cloud medical imaging platform (Ambra Health, New York, NY, USA). The images were reevaluated and interpreted by a radiologist (Marugami N.) with special expertise in urogenital imaging, who was blinded to any other clinicopathological variables. Tumor stage (according to the Eighth Edition American Joint Committee on Cancer tumor-node-metastasis staging system) was determined based on multiplanar reconstruction, including axial, sagittal, and coronal CT images. To determine the clinical T stage (≤cT2, cT3, or cT4) of UTUC, the investigator performed comprehensive assessment using tumor appearance (filling defect/mass or wall thickening/stricture), margin (smooth or spiculated/irregular), texture (homogeneous, heterogeneous), hydronephrosis, and calcification [21,22].

2.3. Radical surgery and pathologic response to NAC

RC was performed with open surgery, standard laparoscopic surgery, and robotic surgery with lymph node dissection (LND) and urinary diversion. The LND procedures, including removal of the obturator, external iliac, common iliac, and parasacral lymph node chains, were performed basically according to the extended template [23]. RNU was performed through open or laparoscopic retroperitoneal access using a standard procedure consisting of whole kidney dissection, including the perirenal fat with the ureter and adjacent segment of the bladder cuff [24]. The methods used for the LND were inconsistent among surgeons and hospitals, which changed over time. In general, a template-based dissection that was dependent on the tumor location was performed in our collaborative academic hospitals for patients with UTUC [25].

We focused on pathologic response to NAC by comparing pre-NAC cT and post-NAC ypT categories. Patents with ypT less than cT and ypN0 were categorized into the down-staged ypT2≤ group, irrespective of their cN status. Patents with ypT more than cT and those with cTany cN- and ypTany ypN+ were categorized into an up-staged ypT2≤ group. Patients who met neither the down-staged ypT2≤ group nor the up-staged ypT2≤ group were categorized into a no-changed ypT2≤ group.

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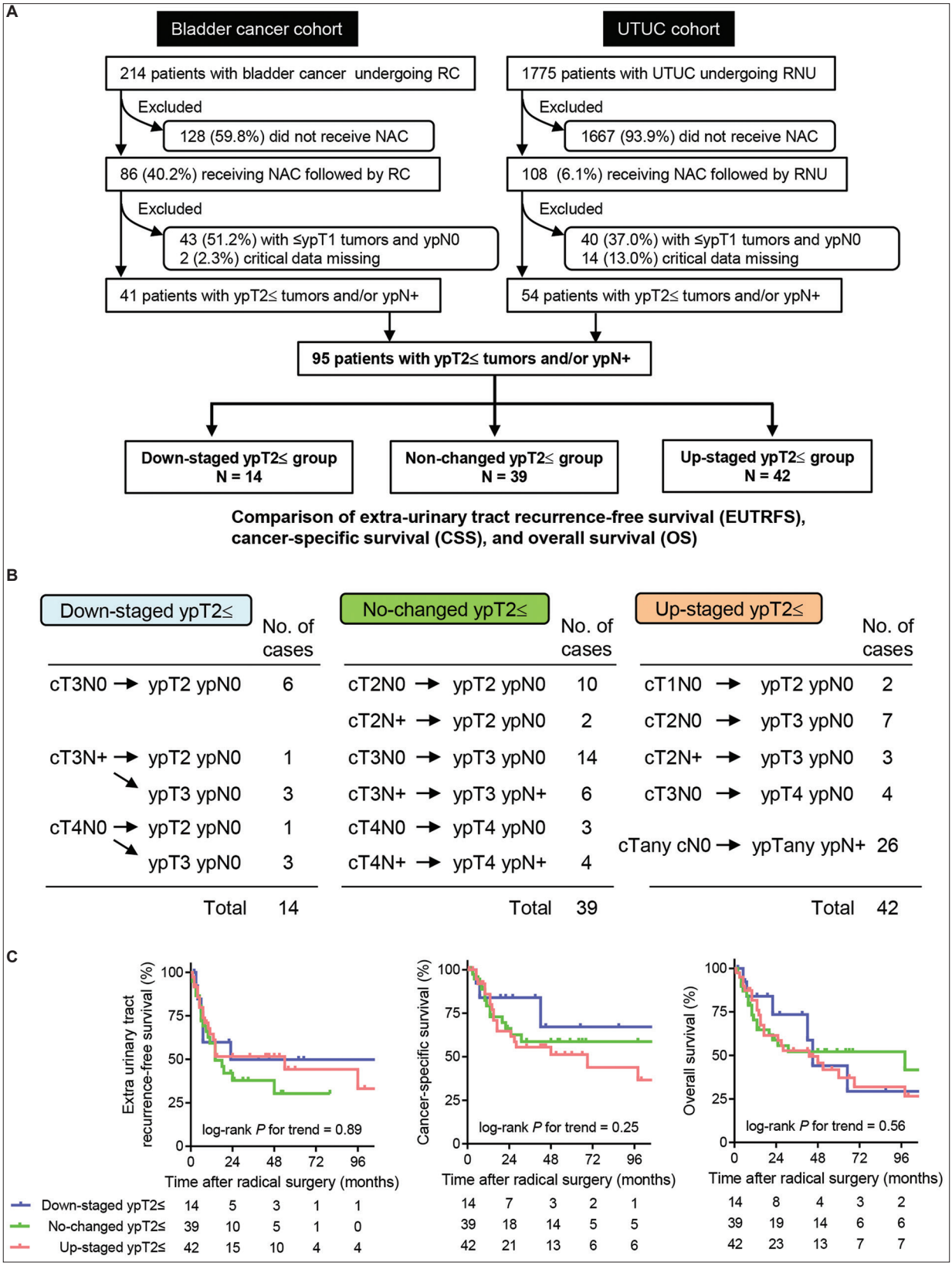

Figure 1. Flow chart for the patient's cohort data sets and schematic design of the study (A). Patterns of pathological response to NAC (B). Event-free survival curves were obtained from the day of radical surgery using the Kaplan–Meier method and compared using the log-rank test for trend (C). This study evaluated three endpoints: Extra-urinary tract recurrence-free survival, cancer-specific survival, and overall survival. Extra-urinary tract recurrence was defined as any recurrence, excluding bladder, upper urinary tract, and urethral recurrences.

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2.4. Follow-up and endpoints

A standard protocol was generally used for the follow-up after RC or RNU: Cystoscopy only for patients undergoing RNU, urinary cytology if needed, and abdominopelvic and chest CT or MRI are performed every 3 months for 2 years, every 6 months until 5 years, and then yearly [1,9]. This study evaluated three endpoints: Extra-urinary tract recurrence-free survival (EUTRFS), CSS, and overall survival (OS). Extra-urinary tract recurrence was defined as any recurrence, excluding bladder, upper urinary tract, and urethral recurrences. While urinary tract recurrence is generally considered non-life-threatening, EUTR includes life-threatening events, such as local recurrence in soft tissue, regional lymph node, or distant organs. Patients who were alive without events were censored at the date of the last follow-up, including the last imaging examination for EUTR and the last visit for cancer-specific death.

2.5. Statistical analysis

Data visualization and statistical analyses were performed using PRISM software version 9 (GraphPad Software, Inc., San Diego, CA, USA). Event-free survival curves from the day of radical surgery were obtained using the Kaplan–Meier method and compared by log-rank test for trend. Variables that potentially affected prognosis ($P < 0.05$) in univariate analysis were included in a step-wise Cox proportional hazards regression model. Regression model. Hazard ratio (HR) with 95% confidence interval (CI) was calculated to identify independent prognostic variables. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Patient characteristics and pathological response to NAC

Clinicopathological characteristics of the 95 patients consisting 41 with bladder cancer and 54 with UTUC are depicted in Table 1. Of note, the number of NAC cycles was 2 or less in 78% of patients with UTUC, while 56% of bladder cancer received three cycles of NAC. According to the pathological response to NAC, 14 (15%), 39 (41%), and 42 (44%) patients were categorized into down-staged ≥ypT2, no-changed ≥ypT2, and up-staged ≥ypT2 groups, respectively. The patterns of pathological response to NAC are shown in Figure 1B. The two most common patterns were cTany cN0 to ypTany ypN+ in 26 patients (up-staged group) and cT3N0 to ypT3 ypN0 in 14 patients (no-changed group).

To investigate possible factors associated with pathological response to NAC, we compared patient characteristics among down-staged ≥ypT2, no-changed ≥ypT2, and up-staged ≥ypT2 groups (Table 2). Sex, clinical T category, and clinical N category were found to be different among groups. More than half of male patients were categorized into the up-staged ypT2≤ group, while more than half of female patients were the no-changed ypT2≤ group. Majority of the patients with clinical N- tumor were categorized into the up-staged ≥ypT2 group. The regimen and cycles of NAC were not different among three groups.

3.2. Association between response to NAC and survival outcomes

There was no significant difference in EUTRFS, CSS, and OS among the three groups (Figure 1C). We performed univariate and multivariate analyses using the Cox proportional hazards regression model to found prognostic factors for EUTRFS, CSS, and OS in patients with ypT2 and/or ypN+ UC after NAC (Table 3). The univariate analysis of EUTRFS showed advanced tumor such as cT4 and ypT4 (*vs.* ypT2; HR = 3.33, $P = 0.009$) were significantly associated with a high risk of disease recurrence, whereas no independent prognostic factor was found in the multivariate analysis. Similar results were seen in the univariate analysis of CSS (ypT4 vs. ypT2; HR = 3.74, $P = 0.02$), and OS (ypT4 vs. ypT2; HR = 2.55, $P = 0.03$). Multivariate analysis was not performed in CSS and OS because the univariate analysis did not show multiple prognostic factors.

Table 1. Characteristic of patients with yielding pathological T2≤ and/or N+ urothelial carcinoma after neoadjuvant chemotherapy

Variables	Overall	Bladder cancer cohort	UTUC cohort
N (%)	95 (100%)	41 (100%)	54 (100%)
Age (years-old), mean±standard deviation	69.3±9.5	69.7±8.8	69.0±10.1
Sex			
Male	73 (77%)	31 (76%)	42 (78%)
Female	22 (23%)	10 (24%)	12 (22%)
ECOG-PS			
0	77 (81%)	37 (90%)	40 (74%)
1	12 (13%)	4 (10%)	8 (15%)
2	2 (2.1%)	0	2 (3.7%)
Unknown	4 (4.2%)	0	4 (7.4%)
Tumor multifocality			
Single	65 (68%)	31 (76%)	34 (63%)
Multiple	25 (26%)	10 (24%)	15 (28%)
Unknown	5 (5.3%)	0	5 (9.3%)

(Contd...)

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Table 1. *(Continued)*

Variables	Overall	Bladder cancer cohort	UTUC cohort
Clinical T category			
cT1	6 (6.3%)	0	6 (11%) [#]
cT2	25 (27%)	15 (37%)	11 (20%)
cT3	44 (46%)	14 (34%)	30 (56%)
cT4	14 (15%)	12 (29%)	2 (3.7%)
Unknown	6 (6.3%)	0	6 (11%) [##]
Clinical N category			
cN0	75 (79%)	31 (76%)	44 (82%)
cN+	20 (21%)	10 (24%)	10 (18%)
NAC regimen			
GC	60 (63%)	25 (61%)	35 (65%)
MVAC	11 (12%)	5 (12%)	6 (11%)
Others	24 (25%)	11 (27%)	13 (24%)
The number of NAC cycles			
2 or less	53 (56%)	11 (27%)	42 (78%)
3	28 (29%)	23 (56%)	5 (9.2%)
4	6 (6.3%)	4 (9.7%)	2 (3.7%)
5 or more	4 (4.2%)	0	4 (7.4%)
Unknown	4 (4.2%)	3 (7.3%)	1 (1.8%)
Pathological T category			
ypTis	1 (1.1%)	0	1 (1.9%)
ypT1	5 (5.3%)	2 (4.9%)	3 (5.6%)
ypT2	28 (30%)	13 (32%)	15 (28%)
ypT3	47 (50%)	17 (42%)	30 (56%)
ypT4	14 (15%)	9 (22%)	5 (9.3%)
Pathological N category			
ypN0	57 (60%)	24 (59%)	33 (61%)
ypN+	38 (40%)	17 (41%)	21 (39%)
CIS			
No	80 (84%)	35 (85%)	45 (83%)
Yes	14 (15%)	6 (15%)	8 (15%)
Unknown	1 (1.1%)	0	1 (1.9%)
LVI			
No	41 (43%)	15 (37%)	26 (48%)
Yes	53 (56%)	26 (63%)	27 (50%)
Unknown	1 (1.1%)	0	1 (1.9%)
Variant histology			
No	90 (95%)	38 (93%)	52 (96%)
Yes	5 (5.3%)	3 (7.3%)	2 (3.7%)
Pathological response to NAC			
Down-staged ypT2≤	14 (15%)	6 (15%)	8 (15%)
No-changed ypT2≤	39 (41%)	20 (49%)	19 (35%)
Up-staged ypT2≤	42 (44%)	15 (37%)	27 (50%)

CIS: Carcinoma *in situ*; ECOG-PS: Eastern Cooperative Oncology Group-performance status; LVI: Lymphovascular invasion; GC: Gemcitabine and cisplatin combination chemotherapy; MVAC: Methotrexate, vinblastin, doxorubicin, and cisplatin combination chemotherapy; NAC: Neoadjuvant chemotherapy.
[#]Of six patients with cT1 UTUC, two had ypT2 ypN0 and the remaining four had ypN+ in the nephroureterectomy specimens after NAC;
[##]All six patients with unknown cT UTUC had ypN+ in the nephroureterectomy specimens after NAC

In addition, we conducted a subgroup analysis of the bladder cancer and UTUC cohorts. In 41 patients with bladder cancer (Figure 2), there was a marginal association between better response and longer CSS ($P = 0.073$), not EUTRFS and OS (Figure 2). In the analysis of the UTUC cohort, no difference was observed in EUTRFS, CSS, and OS among the three groups (Figure 3).

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Table 2. Comparison of baseline characteristics according to response to neoadjuvant chemotherapy in patients with yielding pathological T2≤ and/or N+ urothelial carcinoma

Variables	Down-staged ypT2≤	No-changed ypT2≤	Up-staged ypT2≤	*P*-value
N (%)	14 (100%)	39 (100%)	42 (100%)	-
Age (years-old), mean±standard deviation	69.4±6.8	68.3±10.5	70.2±9.4	0.66
Sex				
Male	13 (93%)	25 (64%)	35 (83%)	0.037
Female	1 (7.1%)	14 (36%)	7 (17%)	
ECOG-PS				
0	13 (93%)	32 (82%)	32 (76%)	0.52
1	1 (7.1%)	6 (15%)	5 (12%)	
2	0	0	2 (4.8%)	
Unknown	0	1 (2.6%)	3 (7.1%)	
Multiplicity				
Single	12 (86%)	28 (72%)	25 (60%)	0.29
Multiple	2 (14%)	10 (26%)	13 (31%)	
Unknown	0	1 (2.6%)	4 (9.5%)	
Clinical T category				
cT1	0	0	6 (14%)[#]	0.004
cT2	0	12 (31%)	13 (31%)	
cT3	10 (71%)	20 (51%)	14 (33%)	
cT4	4 (29%)	7 (18%)	3 (7.1%)	
Unknown	0	0	6 (14%)[##]	
Clinical N category				
cN0	13 (93%)	23 (59%)	39 (93%)	0.003
cN+	1 (7.1)	16 (31%)	3 (7.1%)	
NAC regimen				
GC	9 (64%)	30 (77%)	21 (50%)	0.16
MVAC	2 (14%)	3 (7.7%)	6 (14%)	
Others	3 (21%)	6 (15%)	15 (36%)	
The number of NAC cycles				
2 or less	8 (57%)	22 (56%)	23 (55%)	0.72
3	4 (29%)	14 (36%)	10 (24%)	
4	1 (7.1%)	1 (2.6%)	4 (9.5%)	
5 or more	0	1 (2.6%)	3 (7.1%)	
Unknown	1 (7.1)	1 (2.6%)	2 (4.8%)	
Pathological T category				
ypTis	0	0	1 (2.4%)	<0.001
ypT1	0	0	5 (12%)	
ypT2	11 (79%)	12 (31%)	5 (12%)	
ypT3	3 (21%)	20 (51%)	24 (57%)	
ypT4	0	7 (18%)	7 (17%)	
Pathological N category				
ypN0	14 (100%)	28 (72%)	15 (36%)	<0.001
ypN+	0	11 (28%)	27 (64%)	
CIS				
No	11 (79%)	31 (80%)	38 (91%)	0.1
Yes	2 (14%)	8 (20%)	4 (9.5)	
Unknown	1 (7.1%)	0	0	
LVI				
No	6 (42.9)	17 (43.6)	18 (42.9)	0.87
Yes	8 (57.1)	22 (56.4)	23 (54.8)	
Unknown	0	0	1 (2.4)	0.24
Variant histology				
No	14 (100%)	38 (97%)	38 (91%)	
Yes	0	1 (2.6%)	4 (9.5%)	

CIS: Carcinoma *in situ*; ECOG-PS, Eastern Cooperative Oncology Group-performance status; LVI, lymphovascular invasion; NAC, neoadjuvant chemotherapy [#]Of six patients with cT1 UTUC, two had ypT2 ypN0 and the remaining four had ypN+in the nephroureterectomy specimens after NAC; [##]All six patients with unknown cT UTUC had ypN+in the nephroureterectomy specimens after NAC

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Table 3. Prognostic analyses for survival outcomes after neoadjuvant chemotherapy followed by radical surgery in patients with ypT2 and/or ypN+ urothelial carcinoma using Cox proportional hazards regression model.

Variables	EUTRFS, univariate			EUTRFS, multivariate			CSS, univariate			OS, univariate		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age, years												
<70	1						1			1		
≥70	1.40	0.77 – 2.53	0.27				1.47	0.73 – 2.99	0.28	1.59	0.86 – 2.91	0.14
Sex												
Male	1						1			1		
Female	1.04	0.54 – 2.00	0.92				0.74	0.32 – 1.70	0.48	0.56	0.26 – 1.21	0.14
Tumor multifocality												
Single	1						1			1		
Multiple	0.92	0.67 – 1.27	0.61				1.01	0.70 – 1.46	0.95	1.07	0.80 – 1.43	0.65
Clinical T category												
cT1	1			1			1			1		
cT2	1.32	0.36 – 4.89	0.68	1.12	0.30 – 4.2	0.87	0.84	0.21 – 3.38	0.81	0.70	0.24 – 2.11	0.53
cT3	2.42	0.73 – 8.05	0.15	1.73	0.52 – 5.8	0.38	1.43	0.42 – 4.89	0.57	1.02	0.38 – 2.70	0.97
cT4	3.78	1.03 – 13.94	0.046	1.76	0.37 – 8.4	0.48	1.75	0.45 – 6.78	0.42	1.51	0.51 – 4.43	0.45
Clinical N category												
cN0	1						1			1		
cN+	1.36	0.85 – 2.16	0.20				0.90	0.49 – 1.65	0.73	0.80	0.46 – 1.38	0.42
NAC regimen												
GC	1						1			1		
MVAC	1.06	0.76 – 1.49	0.72				1.16	0.78 – 1.72	0.45	1.01	0.71 – 1.43	0.97
The number of NAC cycles												
2 or less	1						1			1		
3 or more	1.14	0.64 – 2.04	0.65				0.79	0.40 – 1.57	0.50	0.75	0.42 – 1.34	0.33
Pathological T category												
ypT2	1			1			1			1		
ypT3	1.60	0.79 – 3.21	0.19	1.49	0.73 – 3.1	0.28	2.35	0.95 – 5.85	0.07	1.25	0.63 – 2.49	0.52
ypT4	3.33	1.36 – 8.18	0.009	2.53	0.71 – 9.0	0.15	3.74	1.25 – 11.17	0.02	2.55	1.11 – 5.86	0.03
Pathological N category												
ypN0	1						1			1		
ypN+	1.18	0.82 – 1.69	0.37				1.17	0.77 – 1.76	0.46	1.04	0.72 – 1.49	0.83
CIS												
Negative	1						1			1		
Positive	0.57	0.24 – 1.35	0.20				0.68	0.26 – 1.80	0.44	1.20	0.65 – 2.22	0.56
LVI												
Negative	1						1			1		
Positive	1.49	0.88 – 2.52	0.14				1.36	0.72 – 2.55	0.34	1.68	0.98 – 2.87	0.057
Variant histology												
Negative	1						1			1		
Positive	1.12	0.35 – 3.61	0.85				1.63	0.50 – 5.36	0.42	1.17	0.36 – 3.77	0.79
Response to NAC												
Down-staged ypT2≤	1						1			1		
No-changed ypT2≤	1.51	0.61 – 3.74	0.38				1.59	0.45 – 5.57	0.47	1.07	0.42 – 2.69	0.89
Up-staged ypT2≤	1.09	0.44 – 2.71	0.86				1.98	0.58 – 6.74	0.27	1.25	0.51 – 3.07	0.63

CIS: Carcinoma in situ; ECOG-PS: Eastern Cooperative Oncology Group-performance status; LVI: Lymphovascular invasion; NAC: Neoadjuvant chemotherapy, #Of six patients with cT1 UTUC, two had ypT2 ypN0 and the remaining four had ypN+ in the nephroureterectomy specimens after NAC; ##All six patietns with unknown cT UTUC had ypN+ in the nephroureterectomy specimens after NAC.

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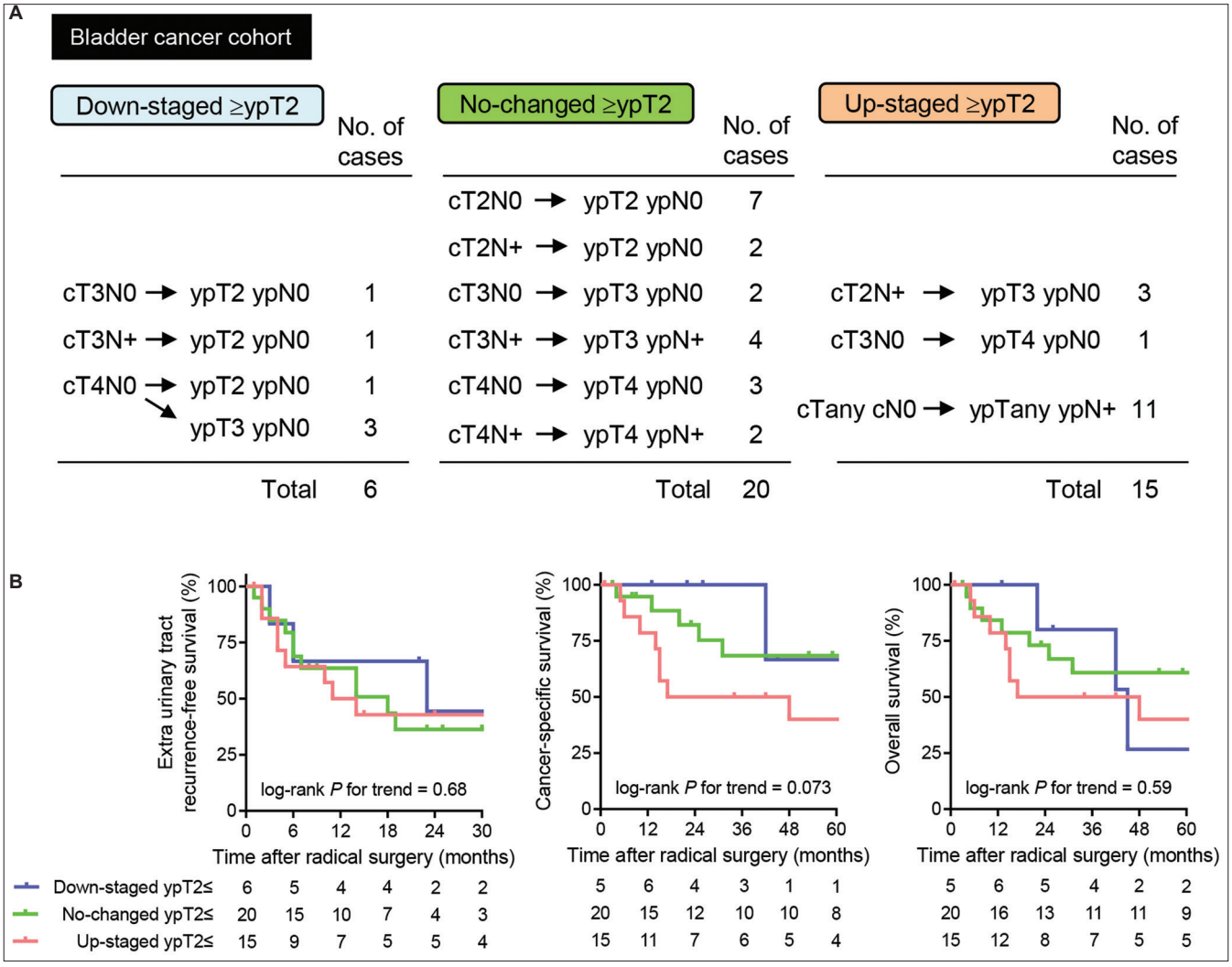

Figure 2. The subgroup analysis of bladder cancer cohort. The patterns of pathological response to neoadjuvant chemotherapy (A). Event-free survival curves were obtained from the day of radical surgery using the Kaplan–Meier method and compared using the log-rank test for trend (B). This study evaluated three endpoints: Extra-urinary tract recurrence-free survival, cancer-specific survival, and overall survival. Extra-urinary tract recurrence was defined as any recurrence, excluding bladder, upper urinary tract, and urethral recurrences.

4. Discussion

In this study, we investigated the potential association between response to NAC and survival after radical surgery in NAC-treated patients with residual MIUC disease and/or ypN+ disease. In contrast to our hypothesis, response to NAC was not significantly associated with favorable outcomes in this subset. However, in the subgroup analysis of the bladder cancer cohort, there was a marginal association between better response and longer CSS ($P = 0.073$). Our finding supports the guideline recommendation (7–9) in which all patients with residual MIUC and/or lymph node tumor are indicated for adjuvant nivolumab therapy. Our finding suggested that adjuvant nivolumab should be considered for all the patients with pathological ypT2≤ or ypN+ UC regardless of response to NAC.

The rationale for prior chemotherapy approach following ICI in the management of UC has been reported to date [26]. The prior chemotherapy can sensitize the tumor cells to ICIs through potential molecular mechanisms, including (i) enhancement of neo-antigen release; (ii) alteration of cytokine composition of the immunogenic tumor microenvironment toward antigen presentation and cytotoxic T cell infiltration; (iii) downregulation of immune-suppressing cells, such as myeloid-derived suppressor cells; and (iv) upregulation of PD-L1 expression on tumor cells [25]. This process is essential to prime tumor cells for an immune response, and it enhances anti-tumor activity of ICI drugs. Unfortunately, the CheckMate 274 trial has not yet updated data regarding response to NAC and benefit of adjuvant nivolumab (14). One of the biggest limitations of this study is that the cohorts did not include any patients who received adjuvant nivolumab. However, our group [27] and the Japanese Urological Oncology Research Group demonstrated a positive correlation between response to the following ICI (pembrolizumab) and response to previous chemotherapy in patients with advanced/metastatic

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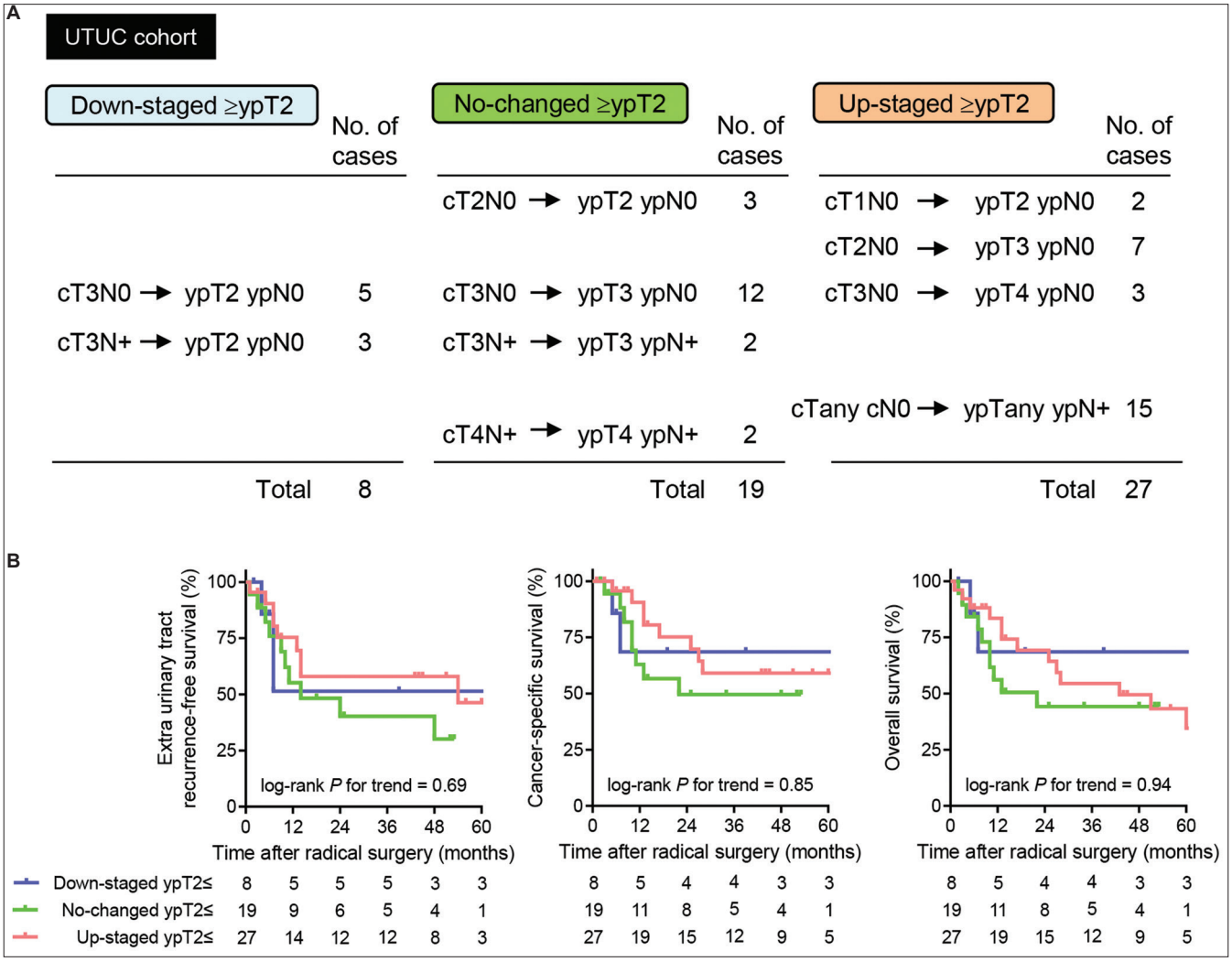

Figure 3. The subgroup analysis of the upper urinary tract urothelial carcinoma cohort. The patterns of pathological response to neoadjuvant chemotherapy (A). Event-free survival curves were obtained from the day of radical surgery using the Kaplan–Meier method and compared using the log-rank test for trend (B). This study evaluated three endpoints: Extra-urinary tract recurrence-free survival, cancer-specific survival, and overall survival. Extra-urinary tract recurrence was defined as any recurrence, excluding bladder, upper urinary tract, and urethral recurrences.

UC [27,28]. Considering these findings, response to NAC may provide a positive effect on adjuvant nivolumab.

The previous study showed that patients aged 70-year-old or more who underwent RNU for localized UTUC had worse outcomes compared to younger patients, concluding that older patients need an improved care and management to improve their outcomes [29]. Similarly, our cohort showed that patients aged 70-year-old or more had worse EUTRFS, CSS, and OS as compared to patients aged less than 70-year-old (Table 3). Substantial population of patients with UC are elderly and vulnerable, and ICIs can cause divergent immune-related adverse events, which are sometimes serious and lethal, requiring high-dose steroids [14-18]. Therefore, predicting positive efficacy of ICI before start of the treatment is vital to develop precision medicine in this medical field. Ferro *et al.* performed a large-scale systematic review and meta-analysis to find predictors of efficacy of ICIs in patients with advanced UC [30]. The quantitative analysis of 6524 patients demonstrated that no visceral metastatic lesion (HR = 0.67; 95% CI, 0.76 − 0.90) and high PD-L1 expression (HR = 0.74; 95% CI, 0.64 − 0.87) were significantly associated with favorable prognosis in risk of death. According to the subgroup analysis of CheckMate 274 trial, PD-L1 expression level at baseline associated with better disease-free survival in patients treated with adjuvant nivolumab as compared to the placebo as follow: HR 0.67 (95% CI, 0.40 − 0.80) in 1% ≤ PD-L1 tumor expression and HR 0.82 (95% CI, 0.63 − 1.06) in 1% > PD-L1 tumor expression [14]. The usefulness of PD-L1 expression level could not be validated in our study because PD-L1 expression level was not available and no patient was treated with nivolumab.

This study has other limitations. Accurate clinical staging before NAC is vital to determine the pathological response to NAC, especially in the UTUC cohort. The previous report evaluated the concordance between the ureteroscopy-based clinical T category and pathological T category, concluding concordant rate was 34.5%

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(208 out of 603 patients with UTUC) [31]. Discordance between the clinical TN category and pathological TN category was not avoidable in this study design. The retrospective study design has an inherent potential for selection bias, and the decision criteria for the implementation of NAC, chemotherapy regimen, timing of changing the treatment, and interval of radiographic evaluation were dependent on the institutional protocol and physician's discretion. The cohort was derived from multiple institutions, which may have introduced inconsistencies in surgical skills, clinical interpretation, and pathological diagnoses. The treatment strategy, modality, especially approval of gemcitabine plus platinum combination chemotherapy and advent of ICIs, and surgical skill change over time may have influenced outcomes. We did not include NAC-induced histological changes in the analysis, because only one patient with MIBC showed downgrading from high-grade UC in the transurethral resection specimens to low-grade UC in the radical surgery specimen. Lastly, statistical power may be limited due to the small number of patients and events in some subgroups.

We suggest that it is vital to select NAC-treated patients with residual MICU and/or lymph node disease who have a low risk of EUTR and a high risk of adverse events and financial toxicity for adjuvant nivolumab. The transurethral resection specimens and radical surgery specimens are easy to access after surgery. Based on the subgroup analysis of CheckMate 274 [14], the tumor positive score (cutoff, ≥1% or <1%) evaluated with anti-PD-L1 antibody (28-8 pharmDx, DAKO) can be a predictive biomarker. Not only assessment of tumor immune microenvironment including the extent of pro-tumoral inflammation and anti-tumoral inflammation but also molecular subtyping would be helpful to determine the accurate phenotyping and genotyping of MIUC. Routine clinical testing of immune checkpoint molecules, *for example*, PD-1 and PD-L1, and molecular subtyping with luminal markers such as GATA3, CK20, and p16 and basal type markers such as CK5/6 and CK20 should be considered for making decisions on perioperative systemic therapy in ICI era. Therefore, data accumulation is mandatory in finding predictive factors that are useful in decision-making for NAC-treated patients who are likely to benefit from adjuvant nivolumab.

Acknowledgments

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This research has received no external funding.

Conflicts of Interest

Nothing to declare.

Ethics Approval and Consent to Participate

This retrospective multicenter study was approved by the Ethics Committee of each participating institute (reference ID: 1298, 1958, 2891, H30-048, and 2018-036) of the Nishinihon Uro-Oncology Collaborative Group framework. Informed consent was obtained from the participants or bereaved families through posters and/or websites using opt-out method.

Consent for Publication

Not applicable.

Availability of Data

The data underlying this article will be shared on reasonable request to the corresponding author.

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SPECIAL ISSUE ARTICLE

How do the characteristics of juvenile idiopathic arthritis affect the continuation or refusal of vaccination against diphtheria? A cross-sectional study data

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ABSTRACT

Introduction: Patients with juvenile idiopathic arthritis (JIA) often stop being vaccinated after the onset of the disease due to fear of disease flare, although the effectiveness and safety of vaccination in immune compromised patients have been demonstrated.
Aim: The objective of this study was to evaluate the JIA characteristics associated with the refusal to continue to be vaccinated against diphtheria.
Methods: In a cross-sectional study, we included data about patients who continued (n = 25) or refused (n = 51) vaccination against diphtheria after the development of JIA. In all patients, the levels of anti-diphtheria vaccine antibodies (immunoglobulin G) were determined with the enzyme-linked immunosorbent assay. The data are presented with a median and 25 – 75%.
Results: The age of disease onset, JIA duration, and JIA categories were similar between groups. Patients who declined the following vaccination often received methotrexate and biologics and switched at least one biologic. Methotrexate (odds ratio [OR] = 9.5 [95% confidence interval (CI): 1,004; 90.3]) and biologics (OR = 4.4 [95% CI: 1.6; 12.1]) were predictors of refusal of revaccination against diphtheria. Vaccination against diphtheria was effective, as evidenced by the almost two-fold prevalence of patients with a protective antibody titer compared to those who refused revaccination. Serious adverse events, as well as JIA flares in 3 months after vaccination were not observed.
Conclusion: The continuation of vaccination against diphtheria in children with JIA was effective and safe. The treatment with methotrexate and biologics was a predictor of refusal of revaccination against diphtheria. Further studies are needed to confirm the safety and efficacy of vaccination against diphtheria in children with JIA and can increase the level of confidence of physicians in the vaccination of children with rheumatic diseases.
Relevance for Patients: Patients with JIA should know the necessity, efficacy, and safety of vaccination against diphtheria. There are no contraindications from the disease side to vaccination against diphtheria. Health-care providers should discuss and encourage any vaccination in immune-mediated children.

1. Introduction

Vaccination is an important tool for infection prevention, especially for immunocompromised patients [1]. Immunocompromised patients are at risk for frequent and severe infections due to immune system dysfunction, treatment with immunosuppressive medications, and incomplete vaccination [2-6]. High-risk groups include patients with rheumatic diseases who may have vaccinations according to national schedules but do not

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have protective levels of post-vaccination antibodies [7]. It is known that younger children (≤4 years) with juvenile idiopathic arthritis (JIA) and patients with higher disease activity have an increased incidence of infection [8,9]. The majority of vaccinations against vaccine-controlled infections occur at the age of 4 years. Immune-mediated disease and immunosuppressive treatment (corticosteroids, tumor necrosis factor-alpha (TNF-α) inhibitors, and anti-B cell therapy) may influence the protective level of anti-vaccine antibodies, and memory B-cell function [10-12]. Many national and international professional associations are calling for the commitment of physicians and patients to the vaccination process [10]. However, despite this, many patients, parents, and physicians are opposed to vaccinations due to fear of flare of the disease or low efficacy of the vaccination [9,13,14]. In the Russian Federation, the vaccination coverage of children with JIA in the last decade against major vaccine-controlled infections remains at a low level and amounts to 50 – 58% [9].

In recent years, the number of cases of diphtheria reported worldwide has been gradually increasing. In 2018, 16,651 cases were registered, which is more than twice the annual average for 1996 – 2017 (8105 cases) [15]. The coronavirus disease 2019 pandemic caused the largest decline in vaccination in the past three decades [16,17]. In 2021, according to the World Health Organization, 25 million children did not receive a vaccine against measles, diphtheria, and tetanus [18]. It is known that diphtheria is a life-threatening disease, with high mortality associated with asphyxia due to obstruction of the respiratory tract by edema and patches, as well as myocardial and nervous system involvement resulting from exposure to bacterial toxins [19]. In the early stages, anti-diphtheria serum and antibacterial therapy are used for treatment, which may eventually become ineffective due to the development of resistance of C. diphtheriae to antibiotics [15]. The schedule of primary vaccinations against diphtheria and tetanus is the same in different countries, with differences mainly in the number and timing of booster doses [20]. In the Russian Federation, vaccination against diphtheria is carried out from the age of 3 months. The primary vaccination consists of three doses, starting from 3 months, performed at intervals of 45 days. Revaccination is carried out 1 year after the initial vaccination, then at 7 and 14 years, for adults – every 10 years throughout life [21]. Vaccination with three doses of diphtheria toxoid vaccine is highly (87%) effective against symptomatic disease and reduces transmission by 60% [19].

Despite the developed immunization schedules, the global suboptimal vaccination coverage is typical for many countries. It may lead to outbreaks of vaccine-controlled infections, including diphtheria, due to a lack of collective immunity. To reduce morbidity, it is necessary to achieve high vaccination coverage and introduce recommended booster doses, and it is also important to identify persons, who require routine assessment of the level of anti-vaccine antibody over the safety of post-vaccination immunity. The study aimed to evaluate the characteristics of JIA associated with the refusal of the following vaccinations against diphtheria.

2. Methods

In the cross-sectional study, we included information about patients, who continued (n = 25) or declined (n = 51) vaccination against diphtheria due to the onset of JIA. Study inclusion lasted from 2019 to 2020 years and was conducted in the Department of Pediatric Rheumatology of Saint-Petersburg State Pediatric Medical University.

Inclusion criteria were as follows: (i) Diagnosis of JIA was established according to International League of Associations for Rheumatology criteria [21]; (ii) age <18 years; (iii) done initial vaccination against diphtheria in the 1st year of life; and (iv) similar demographic characteristics of the patients with a similar number of vaccines before the onset of the JIA.

Exclusion criteria were as follows: (i) Incomplete initial vaccination in the 1st year of life and (ii) patients, whose demographic characteristics and number of vaccines differed between groups.

We evaluated demography, JIA category, treatment, vaccination status, and levels of antibodies against the diphtheria vaccine.

The data about the JIA course and treatment were obtained from the patient's charts. We selected the oligoarticular course (<5 active joints), polyarticular course (extended oligoarthritis, rheumatoid factor [RF]-positive, and RF-negative polyarthritis), and systemic arthritis. The following classes of immunosuppressive medications, which were used by the patients during study recruitment, were taken into account: corticosteroids, methotrexate, and biologics.

2.1. National vaccine schedule

Russian national vaccine schedule supposes diphtheria-tetanus-pertussis vaccination in 3, 4½, 6, and 18 months and further diphtheria-tetanus vaccination in 6 – 7 and 14 years. Vaccination is mandatory in the Russian Federation. According to national recommendations, immunosuppressive drugs were not discontinued before/during diphtheria vaccination.

2.2. Assessment of the levels of antibodies against diphtheria

In all patients, the levels of post-vaccination antibodies (immunoglobulin G [IgG]) for diphtheria were measured with enzyme-linked immunosorbent assay during study inclusion. IgG concentrations were determined from calibration curves constructed using Dynex Technologies Inc. Software (USA). The protective level of antibodies was established by the criteria specified in the manufacturer's instructions for diphtheria – 0.09 IU/ml (7, 5%; 0.004 IU/ml). To detect diphtheria antibodies, we used the commercial kit, created by IBL International GMBH (Germany). Information about the scheduled vaccination against diphtheria was obtained from the personal vaccine certificates.

2.3. Statistical analysis

Statistical analysis was performed with the software STATISTICA, version 10.0 (StatSoft Inc., USA) and MedCalc (MedCalc Software, Belgium). The sample size was not calculated, the power was 0.409. All continuous variables were checked by the

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Kolmogorov–Smirnov test, with no normal distribution identified. Description of quantitative variables was done with median and interquartile range (25%; 75%) for continuous variables and in terms of absolute meanings and percentages for categorical variables. For comparison, the categorical variables Pearson's χ^2 test or Fisher's exact test in case of expected frequencies <5 were used, and a comparison of two quantitative variables was carried out using the Mann–Whitney test. $P < 0.05$ was considered statistically significant.

2.4. Ethics

Written consent was obtained according to the Declaration of Helsinki. The Ethics Committee of Saint Petersburg State Pediatric Medical University (protocol number 9/2 from September 2, 2019) approved this retrospective study's protocol. All patients or patients' representatives (for patients under the age of 15) gave their consent in their case report forms authorizing the anonymous use of their medical information. All patients were appropriately anonymized.

3. Results

3.1. Patients' demography

The studied population was presented with girl predominance (n = 48; 63%), oligoarticular (n = 31; 41%), and polyarticular (n = 30; 40%) predominance. Treatment modalities included corticosteroids (n = 33; 43%), methotrexate (n = 71; 93%), and biologics (n = 48; 63%). Ten patients (13%) received two biologics and more, consequently.

3.1.1. The predictors of the refused vaccination against diphtheria

The protective level against diphtheria was in 33 (%) of patients. The following differences were found among patients with JIA who continued to be vaccinated against diphtheria. Patients with a less severe course of JIA, who received methotrexate less often, who needed less both primary administration of biologics and switching between biologics more often continued to be vaccinated. We did not observe the association of vaccination refusal, depending on the type of biologic. Vaccination against diphtheria was effective, as evidenced by the almost two-fold prevalence of patients with a protective antibody titer compared to those who refused revaccination. Data are in Table 1.

4. Discussion

Many children with rheumatic diseases in the Russian Federation stop being vaccinated after diagnosis [21]. A lot of practicing pediatricians and pediatric rheumatologists are unreasonably afraid of post-vaccination complications, and flares of rheumatic disease, and also consider vaccination ineffective when using immunosuppressive therapy. Often they do not take into account the fact that prolonged use of immunosuppressive drugs, escalation of treatment regimens, and the presence of signs of immune dysfunction leads to an altered "protective"

Table 1. Patients demography and post-vaccination immunity against diphtheria in children with JIA

Parameter	Vaccination against diphtheria		*P*
	Continued (n=25)	Declined (n=51)	
Demography			
Sex, males, n (%)	12 (48)	16 (31)	0.158
JIA onset age, years, Me (25%; 75%)	5.0 (4.1; 6.5)	5.1 (3.0; 11.4)	0.820
Age of inclusion in the study, years, Me (25%; 75%)	11.6 (9.8; 16.0)	13.8 (11.0; 15.8)	0.670
JIA duration, years, Me (25%; 75%)	6.5 (4.9; 8.2)	6.2 (3.6; 9.5)	0.699
JIA courses			
Oligoarthritis, n (%)	11 (44)	20 (39)	0.678
Polyarthritis, n (%)	8 (32)	22 (43)	
Systemic arthritis, n (%)	1 (4)	3 (6)	
Vaccine diphtheria status			
Antibodies against diphtheria, IgG, IU/ml, Me (25%; 75%)	0.14 (0.07; 0.34)	0.06 (0.02; 0.22)	0.695
Patients with protective levels of antibodies against diphtheria, n (%)	15 (60)	18 (35)	0.041
Time since the last diphtheria vaccination, years, Me (25%; 75%)	5.6 (3.6; 10.3)	6.7 (4.0; 10.7)	0.025
Treatment			
Corticosteroids, n (%)	4 (16)	16 (31)	0.153
Methotrexate, n (%)	21 (84)	50 (98)	0.020
Methotrexate duration treatment, years, Me (25%; 75%)	3.1 (1.6; 5.8)	5.1 (2.6; 8.7)	0.027
Biologics, n (%)	10 (40)	38 (75)	0.003
Biologics duration treatment, years, Me (25%; 75%)	1.9 (1.0; 4.1)	3.4 (2.6; 8.7)	0.674
Using > one biologics, sequentially, n (%)	1/9 (11)	9/29 (31)	0.009

Abbreviations: JIA: Juvenile idiopathic arthritis; Me: Median

Serious adverse events, as well as JIA flares in 3 months after vaccination were not observed. We found that methotrexate odds ratio [OR]=9.5 (95% confidence interval [CI]: 1,004; 90.3) and biologics OR=4.4 (95% CI: 1.6; 12.1) were predictors of refusal of revaccination against diphtheria.

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immune response and an increase in the risk of infectious diseases [13,22].

Intercurrent infections not only lead to flares of JIA but also require treatment discontinuation, which negatively affects the achievement or maintenance of the inactive status of the disease and significantly increases the financial costs associated with treatment [2]. The disrupted vaccination schedule in patients with rheumatological diseases is typical even for developed countries, the share of missed vaccines from the national calendar, for example, in Slovenia is 35%, and in Canada 39% [23,24]. It was found that the proportion of missed vaccines is proportional to age, as well as the severity of arthritis (polyarthritis, systemic arthritis), and depends on the type of vaccines. In the above-described studies, revaccinations against measles, hepatitis B, diphtheria, rubella, and mumps were most often missed [23-25].

According to research, the fear of parents and physicians was often the reason for the refusal of subsequent vaccinations in children with rheumatic diseases [23,26-29]. Many physicians postpone vaccination until the inactive stage of the disease is reached or long-term remission of JIA, which affects the presence of a protective antibody titer [23,26].

4.1. Is vaccination against diphtheria safe and effective?

There is little international experience in the safety and efficacy of vaccination of children with rheumatic diseases against diphtheria. In a cohort of 29 patients previously vaccinated against diphtheria and tetanus, aged 2 – 5 years, with polyarticular JIA, who received subcutaneous abatacept, the protective level of antibodies against diphtheria was detected in 26 (89.7%) children. Methotrexate and low doses of corticosteroids did not affect the level of antibodies [30].

In our study, the protective level of antibodies against diphtheria was detected in 51.8% of patients with JIA. However, it should be borne in mind that in the study by Brunner *et al.*, children at the time of inclusion were younger or preschool age [30]. It should be noted that the more time passes since the last vaccination, the more likely it is to have a low level of antibody. According to the study of Heijstek *et al.*, patients with different JIA categories had equal levels of antibodies against diphtheria similar to the results of the previous study [30,31]. In this study, incomplete vaccination, methotrexate treatment duration, and biologics affected the level of antibodies against diphtheria, which is also observed in our study [31]. Methotrexate disturbed the production of antibodies against diphtheria in a prospective multicenter study by Bühler *et al.* There were no flares of rheumatic disease after vaccination. These data also coincide with our results [32]. In a multicenter study on the duration of antibody persistence after vaccination against diphtheria/tetanus in patients with rheumatic diseases undergoing immunosuppressive therapy after vaccination, median concentrations of antibodies against diphtheria were lower in patients with rheumatic diseases than in the control group (0.05 vs. 0.22; $P = 0.002$). Patients with rheumatic diseases had lower proportions of short-term tetanus and diphtheria protection as demonstrated by crude OR of 0.30 ($P = 0.017$) and OR: 0.52 ($P = 0.004$), respectively [33].

Recently, the European League Against Rheumatism published updated recommendations on the vaccination of children with rheumatic diseases. This update takes into account new studies on the safety of live attenuated vaccines and the immunogenicity of vaccines in patients receiving new anti-rheumatic drugs. This update addresses three important aspects of vaccine safety: no serious side effects, no flare of the underlying disease, and no triggering of infections in the case of live attenuated vaccines [10].

According to these recommendations, non-live vaccines can be prescribed to children receiving glucocorticosteroids, disease-modifying anti-rheumatic drugs. Patients with rheumatic diseases may have lower antibody titers compared to healthy peers, but in general, vaccination is effective and safe [31,34-37]. Several studies confirmed the safety of vaccines in pediatric rheumatic diseases [35,38,39].

No increased frequency of JIA flares after vaccination against chickenpox, PCP, diphtheria, or poliovirus (inactivated) was observed [35,38,39].

The group of patients who need to monitor antibodies against vaccines includes patients who also receive any biological drugs, as well as those who have an incomplete set of vaccines [40]. Personalized vaccination is recommended for patients suffering from rheumatic diseases based on the presence of risk factors, as well as determining the level of the protective titer of antibodies [10].

Educational work with physicians and health-care providers reduces fears of vaccination and encourages vaccination in children with immunocompromised conditions [41,42].

Our study is not without the limitations. JIA is a rare disease and a small sample size, specific selection of the patients, and different times between and after vaccination could affect the study results.

5. Conclusion

Treatment with methotrexate and biological drugs is a predictor of refusal of subsequent vaccination against diphtheria after the onset of JIA. Vaccination against diphtheria in children with JIA is a safe and effective tool for controlling incidence in this group of patients. It is necessary to increase the level of confidence of doctors in the vaccination of children with rheumatic diseases.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethics Approval and Consent to Participate

The Ethics Committee of Saint Petersburg State Pediatric Medical University (protocol number 9/2 from September 2, 2019) approved this retrospective study's protocol. All patients or patients' representatives (for patients under the age of 15) gave their consent in their case report forms authorizing the anonymous use of their medical information. All patients were appropriately anonymized. Written consent was obtained according to the Declaration of Helsinki.

Consent for Publication

All participants and/or their legal representatives had given their consents for publication of the materials. The consents were obtained in the patients' case histories.

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ORIGINAL ARTICLE

Frequency of consumption of green leafy vegetables and prevalence of hyperglycemia in Ankole and Teso sub-regions of Uganda

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ABSTRACT

Background: Type 2 diabetes-related hyperglycemia is a global health problem, with developing countries like Uganda currently experiencing substantial rises in the metabolic disorder. Current hyperglycemia therapies can bring a patient to glycemic target; however, they are costly and have other limitations. Vegetable extracts have health-protecting effects and contain thousands of components with putative hypoglycemic effects, rendering them a cheaper alternative toward prevention and management of hyperglycemia.
Aim: The goal of this study was to determine the frequency and patterns of consumption of green leafy vegetables, and their relationship with the prevalence of hyperglycemia in two sub-regions of Uganda.
Methods: A cross-sectional household survey was conducted in Ankole and Teso sub-regions of Uganda. Using a questionnaire for both face-to-face interviews and focus group discussions, the frequently eaten vegetables and their consumption were documented, and fasting blood glucose levels measured to determine the prevalence of hyperglycemia.
Results: The most frequently eaten vegetables in both sub-regions were *Amaranthus* species. *Brassica species, Cucurbita maxima* L., *Solanum nigrum* sensu lato, and *Phaseolus vulgaris* L. were eaten mostly in Ankole sub-region while *Vigna unguiculata* (L.) Walp. and *Hibiscus sabdariffa* L. were eaten mostly in Teso sub-region. In Ankole sub-region, the vegetables were steamed, while boiling and adding peanut/simsim butter was preferred in Teso sub-region. Consumption of leafy vegetables was higher in Teso sub-region than in Ankole sub-region. The overall prevalence of hyperglycemia was 29.15%; it was higher in Ankole at 35.5% and lower in Teso at 19.5% (95% CI: 0.27 – 0.69).
Conclusion: The difference in prevalence of hyperglycemia is relatively high in these sub-regions. Consumption of different leafy vegetable species and their various preparation methods likely contributes to this prevalence; however, factors such as phytochemical constituents, genetics, and social-economic status could help explain this difference further.
Relevance for Patients: This study reveals that when hyperglycemic patients incorporate the consumption of appropriate vegetables (in the recommended amount) and prepared using methods that preserve and/or augment the nutrients and phytonutrients therein, in their diet, they could control and prevent high blood glucose levels.

1. Introduction

Hyperglycemia is a technical term for blood glucose level higher than optimum [1]. It can be due to either a defect in insulin secretion, insulin action, or both [2,3]. This high blood glucose level, if not managed, increases the risk of developing diabetes, and

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microvascular and macrovascular complications, which could lead to poor quality of life or/and death. [4]. In 2016, the World Health Organization (WHO) reported that 2.2 million deaths registered worldwide were attributed to high blood glucose levels and the largest number occurred in middle-income countries. The most worrying part is that, more than half of these deaths are premature [5]. Globally, as of 2019, hyperglycemia was prevalent by an estimate of 7.5% (374 million) and was projected to increase to 8% (444 million) by 2030 and 8.6% (548 million) by 2045 [6]. In Africa, the prevalence of hyperglycemia (measured by impaired glucose tolerance) was estimated to rise from 42.9 million (8.3%) in 2017 to 108.6 million (9.5%) in 2045 [7]. Uganda has been reported to have prevalence of diabetes mellitus at 2%, and it is higher in urban areas and in the male gender. Considering sub-regions of the country, the western sub-region was reported to have the highest prevalence (3.3%) of hyperglycemia compared to the eastern (0.8%) and central sub-region (1.6%) [8]. The national prevalence is relatively low, therefore, presenting an opportunity for prevention and management of hyperglycemia. The discovery of insulin and other hypoglycemic drugs have certainly not only reduced mortality from complications originating from hyperglycemia but also reduced morbidity. However, these drugs have been shown to possess inherent limitations and side effects which have on their own claimed lives of the diabetic patients [9-11]. Having long seen that hyperglycemia is a result of abnormal metabolism [12], which in itself, is a reflection from defects in insulin secretion or/and insulin action [3], the causal factors are a heterogeneous group of genetic and environmental factors including diet, endocrine, and autonomic nervous system dysfunction [2]. The diet factor implicated in the cause of hyperglycemia presents an opportunity through consumption of appropriate vegetables, as both secondary prevention measure (to make up for the down side of the current anti-hyperglycemia therapies), and a primary prevention measure (to reduce the risk of its onset), and altogether prevent and control hyperglycemia [13-16]. The plant-based food we consume often contains many sterol-based bioactive compounds [17]. It is well-documented that these compounds could effectively manage the processes of insulin metabolism and cholesterol regulation. Insulin resistance followed by hyperglycemia often results in oxidative stress level enhancement and increased reactive oxygen species production. At the molecular level, these changes induce apoptosis in pancreatic cells and, hence, lead to insulin insufficiency [17]. Globally, vegetables are among the numerous plant adjuncts indispensable for a balanced diet since they charge dietary fiber, phytochemicals, vitamins, and minerals that are all correlated with improved gastrointestinal health and reduced risk of ailments such as hyperglycemia [13,18,19]. *Gymnema inodorum* (GI) is a leafy green vegetable found in the northern region of Thailand. A GI leaf extract has been developed as a dietary supplement for metabolic diabetic control [20]. However, the active compounds in the GI leaf extract are relatively non-polar [20]. The phospholipid component of phytosomes slightly interfered with the anti-insulin-resistant effects of the GI extract by decreasing the glucose uptake activity and increasing the lipid degradation of adipocytes. Altogether, the nanophytosome is a potent carrier for transporting GI phytochemicals to prevent an early stage of type 2 diabetes mellitus (T2DM) [20]. In Africa, leafy vegetables are not just an important component in the traditional diet, they also make the greatest proportion of it due to their abundance and the fact that the leaves are the first vegetable plant part to mature and harvested compared to the flowers, fruits, and seeds [21-24]. For the last 15 years, Solanaceae, Amaranthaceae, and Malvaceae have been and are still the most predominant families that contain indigenous leafy vegetables in Uganda (43.4%, 15.5%, and 11.6%, respectively). Species such as *Amaranthus dubius* Mart. Ex Thell., *Phaseolus vulgaris* L., *Cucurbita maxima* D., *Vigna unguiculata* (L.) Walp., and *Cleome gynandra* L. were grown for majorly food security, income generation, and nutrition purposes [22,24,25] till recently. In Uganda, studies to investigate their chemical composition for the acclaimed disease prevention and treatment purposes are optimistically on a rise [24,26,27]. However, a direct influence of consumption of these vegetables (as part of the traditional diet) on management and treatment of high blood sugar has scantly been looked at. Therefore, this research contributes to this gap by documenting the regional (Ankole and Teso sub-regions) consumption of the most frequently eaten vegetables and prevalence of hyperglycemia in these sub-regions of Uganda.

2. Methods

2.1. Study area

The study was carried out in Ankole and Teso sub-regions of Uganda (Figure 1). The community survey was done in sampled sub-counties of the sampled districts in the sub-regions. There were five districts sampled from the north, east, south, west, and central parts in each sub-region, and four sub-counties from each district sampled from the northern, eastern, southern, and western parts in each districts. During the survey, information on the frequently eaten vegetables, their consumption, and fasting blood glucose (FBG) levels were collected.

2.2. Ankole sub-region of Uganda

Ankole sub-region is located in south-western part of Uganda, with geographical coordinates of latitude: 0° 29' 59.99'' N and longitude: 30° 29' 59.99' E. Most of its ten districts (based on the 2014 national census enumerated areas) lie at about 1806 m above sea level and they are: Bushenyi, Buhweju, Mitooma *https://en.wikipedia.org/wiki/Bushenyi_District*, Rubirizi, Sheema, Ntungamo, Mbarara, Kiruhura, Ibanda, and Isingiro district. However, the selected representative study sub-counties in the representative districts of the region are as follows; Ibanda district (Nyamarebe, Rukiri, Kizuzi, and Bisheshe sub-counties), Kiruhura district (Buremba, Sanga, Kashongi, and Kinoni sub-counties), Mbarara district (Rubindi, Bagumba, Rubayo and Bubaare sub-counties), Rubirizi district (Rutoto, Magambo, Kirugu, and Kyabakara sub-counties), and Ntugamo district (Ngoma, Rwekiniro, Itojo, and Rukoni sub-counties).

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Figure 1. Map of Uganda showing the study areas (Drawn from GPS coordinates from the survey Sept-Nov 2021 using Arc GIS Version 10.5 on 19/7/22).

2.3. Teso sub-region of Uganda

Teso sub-region is in the eastern part of Uganda with coordinates of latitude: 1.7159° N and longitudes: 33.6111° E. Most of its eight districts (based on the 2014 national census enumerated areas) (Amuria, Bukedea, Kaberamaido, Katakwi, Kumi, Ngora, Serere, and Soroti district) lie about 1129 m above sea level. The selected representative sub-counties in the representative districts of Teso sub-region were as follows; Soroti district (Gweri, Asuret, Tubur and Kamuda sub-counties), Ngora district (Mukura, Kobwin, Ngora, and Kapir sub-counties), Amuria district (Obalanga, Acuwa, Orungo, and Wera sub-counties), Kaberamaido district (Ochero, Kaberamaido, Anyara, and Bululu sub-counties), and Katakwi district (Ongongoja, Kapujan, Ngariam, and Katakwi sub-counties).

2.4. Ethics approval and consent to participate

The study was approved by Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) under Protocol number MUST-2021-52 and registered with the Uganda National Council for Science and Technology (UNCST) under registration number HS1840ES. Before going to collect data, written permission was first sought from the District Health Officers (DHOs) of the study districts in the two sub-regions. A copy of the approval letter from the DHOs was presented to the in-charge at Health Center III in the study sub-counties, who permitted a nurse and a Village Health Team leader to be recruited as research assistants to collect both data and vegetable samples for identification. The purpose and nature of the study was explained to the participants to allow them to make informed decisions on whether to participate in the study or not. The participants were then requested to sign a consent form to confirm their approval to participate in the study. Both the questionnaire and informed consent form were translated into *Runyankole/Rukiga* and *Ateso* which are the most commonly used dialects in Ankole and Teso sub-regions, respectively.

3. Materials

The main materials in this survey were as follows; informed consent forms, questionnaires, glucometers and strips (On call Plus), portable digital body weighing scale (TilyExpress), and height meter (Seca 213). They were purchased from a certified (Zee pharmaceutical) store and voice recorder (Sony ICD-PX470), in Mbarara City, Uganda.

3.1. Study design and strategy

This study was both qualitative and quantitative. Qualitative design employed a cross-sectional community (household level) survey the frequently eaten vegetables, their preparation methods and consumption patterns were documented, while the quantitative aspect involved measuring the FBG levels.

3.2. Sampling strategy

Multistage sampling strategy was used. The two study sub-regions were purposively selected basing on their known remarkable differences in prevalence of hyperglycemia. The study districts and sub-counties were sampled by stratification.

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The households were, then, sampled using "spinning the bottle" method and a person responsible for meal preparation from each selected household was interviewed. Cochran's (1972) formula for finite population was used to calculate the sample size, that is,

$$n = \frac{t^2 pq}{d^2}$$

Where t is the value for selected alpha level of 0.025 in each tail = 1.96 (from the Z table), (p) is the estimated proportion of the population which has the attributes in question (vegetable consumption and prevalence of hyperglycemia) = 0.5 (since it's unknown), (q = [1-p]) is the estimate of variance = 0.5 and d is the desired level of precision (acceptable margin of error) = 0.05. By adding 10% non-response rate, the final sample size was calculated at 422 individuals. A random sample of 422 households in the target population was deemed enough to give the confidence levels of 95%.

3.3. Data collection methods

3.3.1. Community survey

An interviewer-administered questionnaire for face-to-face interviews and focus group discussions (FGDs) was used to obtain information on demographics of the respondents, frequently eaten vegetables, methods of preparation, and consumption pattern. Furthermore, the FBG levels were taken from household members (only those 18 years and above) of the sampled households using a glucometer. Ten women (responsible for meal preparation) were engaged in FGDs to assess the community-level information. A pre-test with 25 participants (from each study sub-region) was conducted to assess language clarity, ability to include information required, acceptability in terms of length, and the privacy of the participants. This also provided the Cronbach alpha of 92% which ascertained the internal validity of the questionnaire whereas the test-retest ascertained the reliability of 94%. The recorded data in the questionnaire were thereafter harmonized with the noted and recorded information from the FGDs.

Field visits were carried out to collect voucher specimens of frequently eaten vegetable species in each sub-region. Voucher specimens were identified and determined by a botanist both at Mbarara University of Science and Technology and at Makerere University Herbarium.

3.3.2. Statistical analysis

Data were entered in Microsoft Excel 2016 and exported to the Statistical Package for the Social Sciences version 20 for analysis.

Descriptive statistics were used to obtain the frequencies and percentages of demographic and socioeconomic characters of the participants, the most frequently eaten vegetables, and prevalence of hyperglycemia. Chi-square test, stepwise multi-variate logistic analysis was used to identify the variables which significantly impacted and associated with the FBG levels (hyperglycemia status).

4. Results

4.1. Demographic characteristics of participants

Results of the demographic characteristics of participants in this study are summarized in Table 1. Out of the 422 eligible respondents who participated in the study, all were female (persons responsible for meal preparation) and from the sampled households. Of these, 253 resided in Ankole sub-region and 169 in Teso sub-region. The highest number (62.1%) of them were adults and married (30 – 59 years) and the least (13.5%) were widowed and elderly (<60 years). Most had acquired a primary level of education (45.7%) and those with tertiary level were fewer (13%) than those without formal education (15.6%). Most respondents were farmers (71.9%) and the civil servants (8.6%) were less than the non-civil servants (19.3%). Overall, most of the participants were natives of their respective sub-regions, that is, they were born and had spent more than 30 years in residence.

4.2. Frequently eaten vegetables in Ankole and Teso sub-regions of Uganda

Figure 2 shows the frequently eaten leafy vegetables in both sub-regions.

Table 1. Demographic characteristics of participants

Characteristic	Sub-region					
	Ankole		Teso		Total	
Category	*n*	Weighted %	*n*	Weighted %	*n*	Weighted %
Respondents	253	100.0	169	100.0	422	100.0
Age						
18 – 29	65	25.7	38	22.5	103	24.4
30 – 59	151	59.7	111	65.7	262	62.1
≥60	37	14.6	20	11.8	57	13.5
Total	253	100.0	169	100.0	422	100.0
Marital status						
Single	24	9.5	13	7.7	37	8.8
Married	193	76.3	141	83.4	334	79.1
Divorced	14	5.5	5	3.0	19	4.5
Widowed	22	8.7	10	5.9	32	7.5
Education status						
Informal	44	17.4	22	13.0	66	15.6
Primary	100	39.5	93	55.0	193	45.7
Secondary	70	27.7	38	22.5	108	25.6
Tertiary	39	15.4	16	9.5	55	13
Occupation						
Civil servant	23	9.2	13	7.7	36	8.6
Farmers	166	66.1	136	80.5	302	71.9
Non civil servants	61	24.3	20	11.8	81	19.3
Years in residence						
0 – 10	74	29.6	42	25.1	116	27.8
10 – 20	47	18.8	42	25.1	89	21.3
20 – 30	46	18.4	42	25.1	88	21.1
>30	83	33.2	41	24.6	124	29.7

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In overall, *Amaranthus species* (*A. dubius, A. cruentus*) was the most frequently eaten green leafy vegetable by participants in this survey. *V. unguiculata*, *Hibiscus sabdariffa,* and *Balanites aegyptiaca* were eaten only by participants residing in Teso sub-region. On the other hand, *P. vulgaris* and *Solanum nigrum* sensu lato were eaten only by participants residing in Ankole sub-region (Figure 2).

4.3. Local names, habit, and habitat of the frequently eaten vegetables in Ankole and Teso sub-regions of Uganda.

Although most of the vegetable species are the same, their local names differed by study sub-region due to the different local languages spoken therein (Table 2). During the FGDs, participants mentioned that most of these frequently eaten vegetables were either cultivated, grew as weeds (escapes from cultivation) in their homesteads, or collected from the wild. Most of the vegetables were herbaceous and annual.

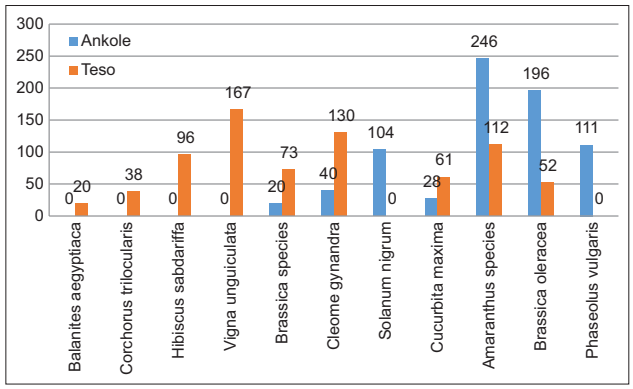

Figure 2. Frequently eaten vegetables in Ankole and Teso sub-regions of Uganda.

4.4. Vegetable collection, preparation method/preservation, amount, and frequency of consumption

All participants reported that the tender leaves and/or shoots are collected. Table 3 shows the times of vegetable collection, preparation method, amount, and frequency of consumption per week. Participants residing in Ankole sub-region collect their vegetables mostly during evening hours of the day (96.2%) whereas most residing in Teso sub-region collect in the afternoon hours (50.4%). For vegetables such as *P. vulgaris*, *V. unguiculata*, *S. nigrum,* and *H. sabdariffa*, the harder leaf parts (petiole, midrib, and harder veins) are first removed and then the remaining leaf parts left under the sun for 10 – 20 min. Depending on the tenderness of the leaves/shoots, they can either be prepared whole or chopped. The practice of drying vegetables in sunlight for preservation, in preparation for periods of scarcity such as the dry season when the climate does not favor their cultivation/growth, was mentioned by most participants from Teso sub-region only. Participants in Ankole sub-region did not preserve the vegetables due to their availability almost throughout the year. Therefore, participants from Ankole sub-region prepared their vegetables fresh, by mostly steaming on top food covered with banana leaves (98.3%) and by mixing them with stews or foods (also known as katogo) (90.3%). Almost all the participants from Teso sub-region prepared theirs fresh (during wet season) and dry (during dry season) by just boiling them alone (67.5%), and/or adding peanut/simsim paste to the vegetables (100%).

During serving of the vegetable stew, quantity was measured by number of servings (50 – 80 g of vegetable stew per serving), and the highest number of servings (five serving spoons) was eaten only by some participants from Teso sub-regions whereas those from Ankole ate at most three serving spoons. In a typical week,

Table 2. Local names, habit, and habitat of the frequently eaten vegetables in Ankole and Teso sub-regions of Uganda

Family, species, accession number	English name	Habit	Habitat	Local name and frequency in sub-region			
				Ankole (Runyankore)	Frequency %	Teso (Ateso)	Freq %
Amaranthaceae, *Amaranthus dubius* Mart. Ex Thell., 51176 and *A. cruentus**)	Amaranth	ah	w/c	Doodo, Emboga Enyabutongo	97.2	Eboga/Ekiliton	66.3
Solanaceae, *Solanum nigrum* L. sensu lato, 51174	Black nightshade	ah/ph	w/c	Enshwiga/Esiiga	41.1		0
Cleomaceae, *Cleome gynandra* L., 51178	African spider flower	ah	w/c	Esogye/Eshoje	15.8	Ecadoi/Akeo	76.9
Brassicaceae, *Brassica campestris* L. (*Acephala* group), 51168	Collard greens	ah	C	Sukumawiki/ Sukuma	7.8	Esukuma/ Sukumawiki	43.2
Brassicaceae, *Brassica oleracea* L. (*Capitata* group), 51175	Cabbage	ah	C	Cabbage	77.5	Cabbage	30.8
Fabaceae, *Vigna unguiculata* (L.) Walp., 51172	Cowpea leaves	ah	C	Omugobe	0.4	Eboo/Boyo	98.8
Malvaceae, *Hibiscus sabdariffa* L., 51177		bs	C		0	Emalakany/ Malakwang	56.8
Malvaceae, Corchorus trilocularis L. 51173	Bush okra	ah	w/c	Mutere	0	Atigo/Alilot	22.5
Fabaceae, *Phaseolus vulgaris* L., 51170	Bean leaves	ah	C	Ebijhamba	43.9		0
Zygophyllaceae, *Balanites aegyptiaca* (L.) Delile, 51169	Desert date	t	W		0	Ecomai/Ekoreete,	11.8
Cucurbitaceae, *Cucurbita maxima* Duchesne, 51171	Pumpkin leaves	ah	w/c	Ekisusha/ Ebishusha/	26.9	Asuswa/Asusa	12.4

Notes: w: Wild; c: Cultivated; w/c: Wild/cultivated; ah: Annual herb; bs: Biennual shrub; t: Tree; ph: Perennial herb; *: Not accessioned

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Table 3. Vegetable collection time, preparation methods, quantity served, and frequency of consumption

Character	Category	Sub-region and frequency (%)	
		Ankole	Teso
Collection time	Morning	56.2	43.8
	Afternoon	49.6	50.4
	Evening	96.2	3.8
Preparation method	Steaming	98.3	1.7
	Mixing with in stews or foods	90.3	9.7
	Boiling	32.4	67.6
	Frying	59.6	40.4
	Pasting	0.0	100.0
Quantity eaten	2 serving spoons	100.0	0.0
	3 serving spoons	79.4	20.6
	4 serving spoons	1.6	98.4
	5 serving spoons	0.0	100.0
Frequency of eating (weekly)	<3	88.9	11.1
	3 – 5	64.9	35.1
	More than 5	32.4	67.6

most of the participants from Ankole sub-region ate vegetables for <3 days whereas some from Teso sub-region ate them for more than 5 days.

4.5. Prevalence of hyperglycemia and the distribution of participants' characteristics

Blood glucose levels of the participants were measured during the fasting condition (i.e., FBG) and recorded as normal, pre-diabetic, and hyperglycemia, based on the WHO criteria [28]. During data analysis, the FBG levels were re-categorized into normal and hyperglycemia to better see and discuss the anti-hyperglycemia effect of vegetable consumption. Table 4 summarizes the prevalence of hyperglycemia and the distribution of participants' characteristics. The overall prevalence of hyperglycemia in the study population sample was 29.15%. However, it was higher in participants residing in Ankole sub-region (35.5%) than those in Teso sub-region (19.5%). There is a slight mean difference (5.4 – 5.9 mmol/l) of FBG levels of the participants in the two sub-regions and it is statistically insignificant.

The prevalence of hyperglycemia was highest (i.e., at 40.4%) in the elderly participants (60 years old and above), and it was lowest in youth participants (19 – 30 years old), at 20.4%. In regard to marital status, married participants had the least prevalence of hyperglycemia (at 27.2%) compared to the single, widowed, and divorced (at 35.1%, 38.7%, and 36.8%, respectively).

The participants that had received secondary level of education had the highest prevalence of hyperglycemia (at 34.3%) than those with primary, tertiary, and no formal education at all. The civil servants had the least prevalence of hyperglycemia, at 16.7% whereas the non-civil servants had the highest at 38.3%. Although data on the amount of time spent on the sub-region of residence, physical exercise and its intensity showed impact on the blood glucose levels of the participants, it was not statistically significant.

Table 4. Prevalence of hyperglycemia and distribution of participants' characteristics

Participant characteristics	Category	Hyperglycemia prevalence (frequency %)
Sub-region	Ankole	35.6
	Tes Teso	19.5
	Total	29.1
Age	19 – 30	20.4
	31 – 45	29.0
	45 – 60	31.6
	60 above	40.4
Marital status	Single	35.1
	Married	27.2
	Divorced	36.8
	Widowed	38.7
Education level	No formal education	25.8
	Primary	28.0
	Secondary	34.3
	Tertiary	27.3
Occupation	Civil servant	16.7
	Farmer	28.1
	Non-civil servants	38.3
Years lived in resident sub-region	0 – 10	27.6
	10 – 20	28.1
	20 – 30	21.6
	≥30	37.1
Physical exercise	No	37.0
	Yes	28.6
Alcohol intake	No	29.2
	Yes	29.3
Physical exercise intensity	None	34.6
	Low intensity	33.8
	Moderate intensity	20.6
	Vigorous intensity	29.6
	Total	29.1
Body mass index category	Normal weight	29.0
	Over weight	29.4

4.6. Prevalence of hyperglycemia and vegetable consumption factors

Participants who consumed vegetables for more than 5 days in a typical week had the least prevalence of hyperglycemia (at 15.5%) compared to those who consumed them for <3 days (at 33.3%) (Table 5). Participants that prepared their vegetables by adding peanut/simsim butter had the least prevalence of hyperglycemia (at 16.1%); meanwhile, those that steamed their vegetables had the highest prevalence of hyperglycemia (at 34.3%). Participants that prepared the vegetables by shallow frying them had almost similar prevalence of hyperglycemia with those that boiled them. The

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Table 5. Prevalence of hyperglycemia and vegetable consumption factors

Character	Factor	Hyperglycemia prevalence (frequency %)
Preparation method	Steaming	34.3
	Mixing	36.6
	Boiling	23.1
	Frying	27.7
	Pasting	16.1
Number of days per week of eating vegetables	<3	33.3
	3 – 5	31.9
	More than 5	15.5
Number of vegetable servings/meal	2 servings	37.2
	3 servings	33.9
	4 servings	16.1
	5 servings	16.2

prevalence of hyperglycemia with other vegetable consumption factors is shown in Table 5.

4.7. Association of hyperglycemia status with participants' characteristics

The data analyses showed that sub-region of residence, age of participant, vegetable preparation method, quantity, and frequency of the vegetable consumption were factors that independently associated with hyperglycemia status of the participants (Table 6). Participants residing in the Eastern were less likely to be hyperglycemia with an odds ratio (OR) of 0.44 (95% CI: 0.27 – 0.69). Participants of age category of 19 – 30 years were less likely to be hyperglycemia, with an OR of 4.86 (95% CI: 1.86 – 12.69) while participants that consumed <3 serving spoons of vegetables for <3 days in a typical week were more likely to be hyperglycemia with an OR of 0.31 (95% CI: 0.01 – 0.69) and 0.43 (95% CI: 0.20 – 0.90), respectively. Other independent factors are mentioned in Table 6. Other factors that we investigated for association with the hyperglycemia status, but found no association are marital status, level of education, occupation, physical exercise and its intensity, body mass index (BMI), and years lived in the residence.

A stepwise multi-variate logistic analysis was run, age and consumption of *H. sabdariffa* L. were consistently statistically significant through to step 21 of analysis with an increasing statistical significance (Table 7). The younger participants (19 – 30 years old) were less likely to be hyperglycemia compared to the elderly (>60 years old), and participants that consumed *H. sabdariffa*. were less likely to be hyperglycemia as well.

4.8. Key survey outcomes

Table 8 summarizes the key survey outcomes from the present study. The main aspects revolving around the commonly eaten vegetables in Ankole and Teso sub-regions of Uganda and the corresponding hyperglycemia prevalence in these sub-regions are highlighted, with the prevalence in Ankole sub-region being higher.

5. Discussion

The concept of this cross-sectional survey was borne from the national population-baseline survey on prevalence estimates and correlates of impaired fasting glycaemia (IFG) in Uganda [8]. This present study then aimed at finding a scientific base to the reported significant variance in prevalence of hyperglycemia in Ankole and Teso sub-regions of the country. We chose to look at the traditional diets in which vegetables are a great component of, and set out to document the consumption of the frequently eaten vegetables and prevalence of hyperglycemia in these sub-regions. Out of the 422 participants, all were female primarily because they are the persons responsible for meal preparation in households and they are also the gender that controls the power dynamics around decision-making on food procurement/collection and preparation [29]. These participants were sampled from Ankole and Teso sub-regions of Uganda and after data analysis, most were housewives of ages 30 – 59 years, they had acquired a primary level of education, subsistence farming was their occupation, and most were natives of their respective sub-region. These findings are in agreement with the 2020 National statistics on demographics, where most women got married at school going age (15 – 19 years) and ended up doing farming to fend for themselves and their families [30].

5.1. Consumption of frequently eaten vegetables in Ankole and Teso sub-regions of Uganda

Amaranth species (*A. dubius* and *A. cruentus*) were eaten in both sub-regions, although more in Akole than in Teso sub-region. *S. nigrum* was only eaten by participants in Ankole sub-region whereas *H. sabdariffa* was also eaten by only participants in Teso sub-region. These data are in agreement with the regional distribution of the African indigenous vegetable families in Uganda reported by Sseremba *et al.*, 2017 and Musinguzi *et al.*, 2011, which explains that Solanaceae family is more prevalent in Western (Ankole sub-region) than Eastern region (Teso sub-region) of Uganda [22,24]. Despite the low distribution of Amaranthaceae family in Western region, it is noteworthy that it is more eaten due to its cosmopolitan distribution, and availability since the species can be either cultivated, or grows in the wild as weeds, also called voluntary crops (EB Rubaihayo, 2002) supported by the favorable climate. Furthermore, most participants (especially residents of Teso sub-region) narrated that they preferred *Amaranthus* species because among leafy vegetables, it is the simplest to prepare, by boiling in salted water until soft; this has been so even in Northern sub-region of Uganda [31] for over four decades now.

5.2. Local names, habit, and habitat of the frequently eaten vegetables in Ankole and Teso sub-regions of Uganda

In this survey, the vegetable local/vernacular names depended on the dialect(s) spoken by the participants in the study sub-regions and since the interviewers were residents of these sub-regions, mistakes and confusion on the vegetable names were eliminated. Most of the vegetables consumed in both sub-regions

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Table 6. Association of hyperglycemia status with participants' characteristics.

Characteristics	FBG category				CI (95%)	*P*-value	OR
	Normal		Hyperglycemia				
	N	%	N	%			
Sub-region							
Ankole	163	64.4	90	35.6	0.27 – 0.69	**0.0003**	0.439
Teso	136	80.5	33	19.5			
Age							
19 – 30	82	79.6	21	20.4	1.86 – 12.69	**0.01**	4.861
31 – 45	103	71.0	42	29.0	0.880 – 4.586	0.98	2.009
45 – 60	80	68.4	37	31.6	0.77 – 3.544	0.189	1.662
60 above	34	59.6	23	40.4			1
Marital status							
Single	24	64.9	13	35.1	0.173 – 0.584	0.385	0.583
Married	243	72.8	91	27.2	0.584 – 3.475	0.437	1.424
Divorced	12	63.2	7	36.8	0.233 – 3.193	0.824	0.862
Widowed	19	61.3	12	38.7			1
Education status							
Informal	49	74.2	17	25.8	0.687 – 5.878	0.203	2.009
Primary	139	72.0	54	28.0	0.472 – 3.219	0.668	1.233
Secondary	71	65.7	37	34.3	0.350 – 2.269	0.810	0.892
Tertiary	40	72.7	15	27.3			1
Occupation							
Civil servant	30	83.3	6	16.7	2.340E008– 2.625E-007	0.000	7.839-008
Farmer	217	71.9	85	28.1	1.331E-008 – 4.695E-008	0.000	2.500E-008
Non-civil servants	50	61.7	31	38.3			1
Years in residence							
0 – 10	84	72.4	32	27.6	0.462 – 1.887	0.849	0.934
10 – 20	64	71.9	25	28.1	0.569 – 2.356	0.686	1.158
20 – 30	69	78.4	19	21.6	0.750 – 3.029	0.250	1.507
>30	78	62.9	46	37.1			1
Physical exercise							
0.00	17	63.0	10	37.0	0.303 – 1.533	0.383	0.681
1.00	282	71.4	113	28.6			
Intensity of physical exercise							
None	17	65.4	9	34.6	0.432 – 2.906	0.816	1.120
LI	45	66.2	23	33.8	0.435 – 1.529	0.525	0.815
MI	54	79.4	14	20.6	0.630 – 2.615	0.492	1.283
VI	183	70.4	77	29.6			1
BMI category							
Normal	176	71.0	72	29.0	0.663 – 1.564	1.00	1.019
Over weight	120	70.6	50	29.4			
Steaming							
No	184	74.5	63	25.5	0.998 – 2327	0.064	0.524
Yes	115	65.7	60	34.3			
Mixing in stews/foods							
No	214	74.3	74	25.7	0.074 – 2.588	**0.029**	1.667
Yes	85	63.4	49	36.6			
Boiling							
No	109	62.3	66	37.7	0.324-0.758	**0.002**	0.495
Yes	190	76.9	57	23.1			

(Contd...)

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Table 6. *(Continued)*

Characteristics	FBG category				CI (95%)	*P*-value	OR
	Normal		Hyperglycemia				
	N	%	N	%			
Frying							
No	231	70.4	97	29.6	0.54 – 1.517	0.797	0.911
Yes	68	72.3	26	27.7			
Adding peanut/simsim butter							
No	205	66.1	105	33.9	0.214 – 0.652	**0.000**	0.374
Yes	94	83.9	18	16.1			
Quantity eaten							
2 servings	76	62.8	45	37.2	0.144 – 0.685	**0.004**	0.314
3 servings	109	66.1	56	33.9	0.185 – 0.825	0.014	0.391
4 servings	52	83.9	10	16.1	0.300 – 2.096	0.640	0.793
5 servings	62	83.8	12	16.2			
Days per week							
<3	6	66.7	3	33.3	0.085 – 2.602	0.388	0.471
3 – 5	233	68.1	109	31.9	0.203 – 0.904	**0.026**	0.429
>5	60	84.5	11	15.5			1

Notes: LI: Low intensity; MI: Moderate intensity; VI: Vigorous intensity; P-value, Statistically significant participants' characteristic.

Table 7. Results from a stepwise multi-variate logistic analysis showing only significant variables

Step	Significant variable	*P*-value	95% CI
1	Age	0.01	
	19 – 30	0.00	0.60 – 0.41
	31 – 45	0.13	0.16 – 0.81
	Hibiscus sabdariffa	0.006	1.43 – 8.42
	Age	0.009	
	19 – 30	0.02	0.14 – 0.63
	Hibiscus sabdariffa	0.000	1.72 – 6.27

are annuals and were grown on small scale by intercropping them with other perennials such as coffee, cassava, and bananas. They were also grown in backyards, in kitchen gardens (for residents in town areas) for just household food security, nutrition, and health benefits in general [32-34]. Just as reported in other African countries, some participants in Teso sub-region monocropped some vegetables like *V. unguiculata* on land that is either not previously used or considered to be under-utilized, for its leaves, and seeds later harvested. Few participants planted in swampy land during dry season for household and commercial purposes. The income generated was used for the household, consequently improving on both livelihood and socioeconomic status [35,36]. In both regions, farmers considered traditional leafy vegetables low-income generating crops that did not deserve the attention like the cash crops (coffee, bananas, and cassava).

5.3. Vegetable collection, preparation method/preservation, amount, and frequency of consumption

In both sub-regions, collecting/harvesting method of leafy vegetables depended on whether they were harvested for household consumption or for commercial purposes. Tender leaves/tender stems were plucked for household consumption such that the plant would regrow the leaves and this method would continue for a minimum of 1 month to a maximum of 5 months until the plant has developed fruits. On the other hand, harvesting for commercial purposes was done by uprooting leafy vegetables, washing, and tying in bundles. This practice was also documented by Elizabeth *et al.* (2003); however, this method provides a onetime harvest of vegetables and does not allow the plant to develop seeds for both consumption (in the case of *V. unguiculata*) and sowing in the next season [37-39] affecting sustainable availability of the vegetables and germplasm.

The tender leaves/stems are briefly wilted under direct sunlight for approximately 10 – 20 min, for any insects/worms to crawl/die off. [31,40]. Residents in town councils in both study sub-regions bought vegetables from retail traders in trading centers who, in turn, bought from farmers [41].

The most common method of vegetable preparation in Ankole sub-region is steaming on top of food that is covered with banana leaves. It is because steaming method was already being used in the preparation of staple/traditional food (matooke); this saves time and fuel compared to when the vegetables are prepared separately. Another method of preparation that involves mixing vegetables in other stew or food (also called katogo) is common especially for preparation of breakfast meals [24]. To improve on the taste of stews (beans, fresh ground nut paste), vegetables are cooked together with them. In Teso sub-region, the wilted vegetable tender leaves/stems are washed and, then, boiled singly or in combination with another vegetable specie (the case of *V. unguiculata* and *Corchorus* spp.). These two species are usually cooked together so that the stiffness of *V. unguiculata* is countered by the slipperiness of *Corchorus* spp. To further tenderize the

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Table 8. Summary of the key survey outcomes

Survey outcomes	Ankole sub-region	Teso sub-region
Consumption of vegetables		
Most frequently eaten vegetables	*Amaranthus* spp., *Brassica oleracea, Phaseolus vulgaris*, and *Solanum nigrum*	*Vigna unguiculata, Cleome gynandra, Hibiscus sabdariffa*, and *Amaranthus spp*.
Collection time and vegetable state during preparation	Evening hours, prepared fresh	Afternoon hours, prepared fresh (during wet season) and dry (during dry season)
Preparation methods	Usually one vegetable specie (at a time) prepared by steaming on top of bananas, mixing with other foods (like bananas, cassava) and other stews (such as beans and fresh groundnut paste).	Usually one or two vegetable specie (s) (at a time) prepared by boiling (for some with soda ash), mixing with roasted sesame/ groundnut paste.
Quantity served and frequency eaten/ week	At most three servings, as side sauce, alongside another main source. Eaten for<3 days/week.	At most five servings as main sauce. Eaten for more than 5 days/week.
Purpose	Eaten as a side sauce, as alternative in time of need, for nutrition.	Eaten as main sauce, for food security, nutrition, and medicine.
Availability	Throughout the year	Only in wet season and preserved (by drying) for dry season.
Cultivation	Intercropped in plantations, grow voluntarily in the compounds and in the wild.	Grown in gardens, grow voluntarily in compounds and in the wild.
Prevalence of hyperglycemia		
Prevalence	35.5%	19.5%

leaves, local salt called "*Abalang*" (filtrate from ash obtained from burning of dry banana peelings, mature cotton stems, and mature amaranths plants) is added, and then, sodium chloride is added to taste. This can be eaten at this stage, or sour milk, groundnut, or sesame paste/butter are added depending on household preference to spice it [29]. *Cleome gynandra* in particular is almost always eaten pasted with groundnut paste, and if eaten when it is simply boiled, it was for relieving body ache, that is why participants in Teso sub-region called it "plant ibuprofen." Later, in 2021, Nakaziba *et al.* also listed it along with *Corchorus* spp., *Vigna unguculatta,* and *H. sabdariffa* among the medicinal vegetables in Northern region (which includes Teso sub-region), having positive effects on the various systems in the body [42]. The addition of either groundnut/sesame butter does not only make the vegetable stew tasty but also complements on its nutritional and medicinal value. Groundnut seeds are reported to contain nutrients such as carbohydrates, proteins, oil, and minerals. These nutrients are indispensable for nourishing the body. Moreover, the oil has high density lipids that are considered the "good" cholesterol because it removes the "harmful bad" type from the blood, thereby reducing its deposition, and in turn reducing body weight gain, a risk factor of hyperglycemia [43,44]. Sesame on the other hand contains a great deal of fiber content in addition to the minerals, making it ideal for improving nutritional status [45]. It is also endowed with a range of phytochemicals [46] beneficial for diabetes. The influence of these phytochemicals has been reviewed in different study deigns including clinical trials and has been shown to positively affect the glycemic makers and metabolic parameters [47,48]. A noteworthy compound in sesame is pinoresinol; it helps to control blood glucose by repressing the activity of maltase enzyme in the stomach [49].

Drying in direct sun is a traditional preservation method often applied by participants in Teso sub-region to increase the shelf life of vegetables up to dry season when they are scarce or not available at all [50]. This method of preservation works by reducing water/ moisture to a percentage low enough to prevent or delay bacterial growth and reduce the vegetable weight. However, it is not encouraged because it results in loss of vegetable nutrients such as β-carotene and vitamin C up to 58 and 84%, respectively [50]. Instead, drying in a shade with sufficient aeration was advised [51]. It is however a very rare practice in Ankole sub-region since most vegetables are intercropped in banana and coffee gardens, and during dry season, water/moisture loss is reduced by mulching of gardens with thick layers of grasses, coffee husks, and dry banana leaves and stems, and some farmers store water in built underground reservoirs with tarpaulins inside, [52,53]. In 2006, Musinguzi *et al.*, explained the further decline of preservation of vegetables in Ankole sub-region as a result of limited available knowledge on their nutritional content since more emphasis are placed on commercial, high yielding exotic plants (such as coffee, pineapples and bananas) by both the agricultural extension officers and farmers.

In this study, 86% of the respondents consumed less than the minimum recommended five servings of vegetables per day, which is in agreement with reports from the largest population-based world- wide and the nationwide cross-sectional survey to examine the prevalence of low vegetable consumption [54,55].

According to most participants in Ankole sub-region (especially in town councils), steamed vegetables are usually served as a side dish, that is, a portion (maximum of 2 serving spoonfuls) is served on the plate alongside other foods like cooking bananas (matooke), posho (corn meal which is a dish of maize flour cooked with water to porridge or dough), cassava, rice and sweet potatoes, and eaten with stew on the same or another plate [24], whereas in Teso sub-region, five or more servings of vegetables is eaten with foods such as kalo (bread made of cassava and millet flour), cassava, sweet potatoes, and posho. Clearly, consumption of vegetables is still higher in Teso sub-region than in Ankole sub-region just

like it was reported by Kabwama *et al.*, 2019 in their national survey [54]. This is due to the availability of a higher number and diversity of vegetable species, eaten interchangeably for several days of a week in households in Teso sub-region [22]. Ankole sub-region residents are mostly cattle farmers and meat consumption is rather more common [56] and generally categorizing their dietary pattern as traditional, high-fat, medium environmental impact as opposed to plant-based, and low environmental impact dietary pattern in Teso sub-region [57]. In both sub-regions, the dietary patterns are transitioning real fast, and there is need to equally fast track intervention.

5.4. Prevalence of hyperglycemia in Ankole and Teso sub-regions of Uganda

The overall prevalence of hyperglycemia was 29.1% in this study. Participants residing in Ankole sub-region had higher prevalence at 35.5% than those in Teso sub-region which was at 19.5%. Although these estimates are much higher than those from the previous national baseline survey (3.3% and 0.8%, respectively) carried out by Bahendeka *et al.*, in 2016, they agree with the trend. These differences could be due to the different definitions and measurements of hyperglycemia or the different health determinants across the population [58,59]. The elderly participants (60 and above years) had the highest prevalence compared to the youth participants (24.4%). This result concurs with that of Van Sande *et al.*, in 1997, who long reported a strong association of hyperglycemia with an advanced age [60], and similarly later by the International Diabetes Federation (IDF) in their global estimates for diabetes prevalence in 2017 [61]. The BMI of participants in our study did not show a statistical significant impact on the prevalence of hyperglycemia, whereas the nationwide baseline survey (2016) reported unclear association between the BMI and hyperglycemia status [8]. This discrepancy could be due to the fact that BMI is an indirect measure of obesity (body fat), a predisposing factor of hyperglycemia, and furthermore, BMI has a non-linear relationship with body fat [62] and hence making BMI an erroneous method of body fat measurement [63], and consequently an erroneous prediction of hyperglycemia.

Participants who were married had the least prevalence of hyperglycemia at 27.2%, this is probably because the married consumed more than five servings of vegetables daily and regularly as shown by Dias *et al.*, 2017 and Kabwama *et al.*, 2019 in their studies [54,64]. These authors explained that marriage and companionship involve social interactions which set stage for food consumption in which regular meals including vegetables are a pattern.

Respondents involved in moderate intensity of physical exercise had the least prevalence of hyperglycemia whereas those who did none at all had the highest prevalence, at 20% and 34%, respectively. This finding supports the conclusion made by Manders *et al.* (2010), who reported that low and moderate intensities of physical exercise, as opposed to high intensities, substantially reduce the prevalence of hyperglycemia. Likewise lack of regular physical exercise is a risk factor of non-communicable diseases in general due to imbalance energy homeostasis [2,65,66].

Participants who prepared vegetables by steaming showed the highest prevalence of hyperglycemia compared to those who boiled and added peanut/simsim butter. This result contradicts other study results which showed that steaming vegetables is probably the most appropriate preparation method since it retains water soluble nutrients and phytochemicals (polyphenolics) beneficial for blood glucose control [67-70]. However, these authors cautioned the duration of steaming vegetables to prevent thermal degradation of components therein, a probable explanation for their loss [71], and hence, higher prevalence of hyperglycemia even when consuming vegetables in the diet. Preparation of vegetables by boiling is preferred for vegetables having phytocompounds that are water-soluble and thermostable as long as the they are cooked in hot adequate water. In addition, the boiling method also inactivates the anti-nutritive compounds (oxalates and tannins) in them (Putriani *et al.*, 2020) [70]. Furthermore, boiling of more than one vegetable species with local salt and addition of sour milk, simsim, and groundnut paste does not only improve on the taste of the vegetable sauce but also compounds the phytochemicals responsible for reducing high blood sugar [72]; meanwhile, the tenderizing local salt is rich in plant based minerals which are beneficial for general health. Since most participants from Teso sub-region preferred this method most, it could explain the lower prevalence of hyperglycemia in the sub-region.

Stir-frying of vegetables even though used by few participants in the study, used to be a preparation method for town and city dwellers. However, due to the fast changing habits of Ugandans in general, it is becoming a traditional preparation method for some vegetables. Residents in Teso sub-region added that the adoption of frying vegetables is increasing with the decreasing availability of groundnut/simsim butter for mixing with the vegetables.

This preparation method was reported to cause the greatest loss of vegetable phytochemicals (phenols) which were repeatedly reported to be responsible for the hypoglycemic benefit [68,71].

Participants in Teso sub-region consumed more than five servings of vegetables per meal which concurs with Kabwama *et al.*, (2019) findings. This could be due to the tasty vegetable sauce resulting from addition of other ingredients (mentioned above) during preparation. In addition, only the vegetable sauce without another was often eaten in a meal for even up to 5 days/week. This frequency provides a clue on the importance of these vegetables for household food security as opposed to just for nutrition purposes. Each geographical region has a cultural identity that includes traditional staples, and our report regarding vegetables consumption is not surprising.

Most of the Ankole participants are cattle keepers therefore, they ate vegetables as a secondary/side sauce (alongside meat or fresh groundnut paste sauce), whenever the other relishes are in short supply or when famine strikes, thereby being an alternative in times of need. This could explain the higher prevalence in the sub-region since the participants consumed less than recommended amount of vegetables as stated in the American Heart Association Dietary guidelines [73] and miss out on the benefits from their

multi-dimensional health effects such as improving postprandial glucose [74], elevating parameters associated with T2DM [75,76], and benefiting health status in general [77].

As stated by a number of studies, high fasting blood sugar is a predisposing factor to diabetes mellitus and it is important to consider the other factors associated with it to prevent its development and progression to diabetes [3,16,78,79]. A singular factorial analysis was conducted and there was statistical association of hyperglycemia status with factors such as sub-region of residence, mixing, boiling, and addition of peanut/simsim butter methods of vegetable preparation [8,54,68]. Hyperglycemia status was most likely to be observed in participants residing in Ankole sub-region with OR of 0.439 (95% CI: 027 – 0.69), a prediction which has not changed for the past 3 years [8,54]. These associations are very important information for prioritization of sub-regions during implementation of prevention and/or intervention programs in the country. Preparation of more than one vegetable (for example *V. unguiculata* and *Corchorus trilocularis*) or mixing them with other stews/foods [69] compounds the variety of nutrients and phytochemicals and provides a synergistic benefit for prevention and management of hyperglycemia [72]. It also ensures adequate intake of dietary vitamins, minerals, fiber, and phytochemicals [80,81]. For example, *Amaranthus* species, in Japan, was found to contain 72.6 – 77.05 μg/g fresh weight of total phenolic index [82], *Amaranthus hybridus* in Nigeria had ascorbic acid content of 0.43 mg/g [83] and *H. sabdariffa* contained 0.18 mg/g of fresh weight [84]. Consumption of these compounds, although decrease to a certain degree after vegetable preparation, influences glycemia through different mechanisms such as limiting oxidative processes [85], modulating digestive enzymes [86], gene expressions [87], signaling pathways [88], and glucose transporters [89].

During a stepwise multi-variate logistic analysis, the association of hyperglycemia status with age of participants showed statistically significant results (Table 7), and it confirmed the reports of similar studies where the elderly compared to the youths were more likely to develop hyperglycemia [64,90]. From the same statistical analysis, consumption of *H. sabdariffa* L. showed statistically significant impact on the prevalence of hyperglycemia which is not surprising since this vegetable species has been enormously studied and reviewed worldwide for its beneficial effects on hyperglycemia, its markers, and health in general [91-96]. The lack of impact from other factors, otherwise expected, could reflect challenge in the methodology especially in the evaluation tool.

5.5. Key survey outcomes

Results show that the commonly eaten vegetables in Ankole and Teso sub-regions and how they are processed from harvesting to consumption likely plays a role in regulating occurrence of hyperglycemia, as indicated by the level of prevalence in both sub-regions, though factors such as phytochemical compounds, genetics, and social-economic status could help explain this difference further.

5.6. Study limitations

Since this study was done at one point in time, it is difficult to derive causal relationships from the data analysis, and we also carefully interpreted the associations since we do not really know if the incidence of hyperglycemia was either before or after vegetable consumption.

The prevalence depends on the incidence and length of survival after becoming hyperglycemic, so this survey is insufficient to understand the trend of hyperglycemia in each sub-region.

Information (variables) included in this analysis were self-reported and subject to recall bias.

Participants may have over-estimated their consumption of vegetables as it is a desirable behavior or under-estimated as vegetables are said to be for poor people, and hence, we may have over- or under-estimated the true consumption in both study sub-regions.

However, our survey used a standardized data collection tool. Moreover, we had a large sample size and a high response rate, and so, our findings are sub-regionally representative.

6. Conclusion

The influence of vegetable consumption is a recognized factor for not only general health but also for prevention and management of hyperglycemia. However, there are dynamics in consumption such as appropriate vegetable species, method of preparation, quantity consumed, and frequency that interplay and do weigh in on the achievement of this intended benefit. Results of this study revealed that when the appropriate vegetable species is/are prepared using appropriate method(s) and consumed at recommended amount and frequency, they do yield positive results in prevention and management of hyperglycemia. The observed remarkable difference in prevalence of hyperglycemia in Ankole and Teso sub-regions in this study is a discernible consequence of the significant difference in the vegetable consumption dynamics. Further, research on the phytochemical composition and effect in these most frequently eaten vegetable species is needed; population genetics, and other lifestyle factors in both sub-regions to get a stronger focused and clearer scientific basis for the observed difference in prevalence of hyperglycemia, ultimately guiding to the precise strategies to prevent and manage it.

Community awareness through sensitization programs on health benefits of vegetable consumption for especially women should be reinforced since women control household health behaviors by ensuring availability and preparation of vegetables in meals. This will also demystify the stigma “vegetables are for the poor” since people usually tend to act in favor of good health if they are aware, are convinced, and know how to act. “Health in all policies” [97] should be adopted in the formulation of all policies so that nutrition and health are promoted in parallel with other regional development sectors. Farmers should be supported to encourage the production of these “low income crops” (vegetables) to increase supply.

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Conflicts of Interest

The authors declare no conflicts of interest.

Availability of Data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Biocompatibility of calcitonin receptor fragment peptide-treated 3D-printed bone scaffolds: a muscle pouch implantation study

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ABSTRACT

Background and Aim: Current synthetic bone graft substitutes (BGSs) in development are limited by high resorption, poor load-bearing properties, and stress shielding. These limitations inhibit BGS from complete biointegration. In this study, we developed calcitonin receptor fragment peptide (CRFP)-treated non-biodegradable MED610 scaffold, seeded with MC3T3 stem cells, and assessed their *in vivo* biocompatibility and biointegration.
Methods: Scaffolds were fabricated with Stratasys MED610 (MED610) material, seeded with Mus musculus calvaria cells (MC3T3), and osteogenesis was induced with CRFP after the cells reached confluency and generated bone matrix. Scaffolds with and without bone matrix were implanted in male mice following a muscle pouch implantation protocol. Post-extraction, imaging, staining, and mechanical compression testing was carried after 3 weeks of scaffold implantation in the muscle to measure the ectopic bone formation and compressive strength.
Results: The implanted scaffolds showed significantly higher ($P < 0.01$) calcium deposits in comparison to the untreated scaffolds. We also found significantly higher ($P < 0.001$) mineralization on the implanted scaffolds compared to scaffolds before implantation. The mechanical properties of the scaffolds did not vary significantly.
Conclusions: MED610 scaffolds treated with CRFP *in vivo* do not cause any adverse reaction when implanted in muscle and showed significant ectopic bone formation, indicating biocompatibility and bio-integration.
Relevance for Patients: This study will aid in developing biomimetic and biocompatible artificial bones for implantation.

1. Introduction

In many orthopedic surgical procedures, metallic implants are used to fill in the defects formed due to surgery or fracture [1-3]. Metallic implants are strong and are able to withstand the load experienced by the bone. However, metallic implants lead to stress shielding and, hence, weaken the surrounding bone [4]. Moreover, metallic implants are inert to bone growth and inhibit complete implant integration [5]. The alternative for metallic implants is biological implants *vis*, autografts and allografts [6]. Autografts are osteoconductive and promote bio integration of the implant as they are extracted from the subject's body [7]. They also have a lower rate of disease transmission in comparison to allografts. However, autografts have other complications *vis*, lower availability, excessive pain, and increased hospitalization cost due to extraction surgery [8,9]. Allografts exhibit reduced operating

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cost and donor site complications, have a higher chance of antigen response and disease transmissibility [8-10]. Moreover, allografts are weaker in comparison to metallic implants leading to fractures and future revision surgeries [11,12].

Synthetic bone graft substitutes (BGSs) are being developed to overcome limitations in conventional metallic and biological implants [13]. Demineralized bone matrix (DBM), developed by removing all mineral content from bone, is the most common synthetic BGS [14,15]. However, the demineralization process makes it weak and reduces the load-bearing capacity of the implant [16-18]. Another class of implants is ceramic implants which are better load-bearing capability during implantation [19]. However, due to their high resorption rate, ceramic implants weaken with time and also form particulate matter, which may lead to an inflammatory response [20,21]. Ceramic implants are mostly used to fill in defects formed at non-load-bearing sites [22].

In recent years, three-dimensional (3D) printing technologies or additive manufacturing (AM) have facilitated the manufacturing of unique and complex shapes for a wide variety of materials. The flexibility to fabricate complex shapes and the ability to vary the design to manipulate the porosity of the BGS has led to 3D printing being used in the development of customized BGS [23-25]. These BGSs range from metallic and ceramic implants used in limb arthroplasties to polymer-based BGS, which are mostly in development stages to be introduced into mainstream applications [26-28]. The main issue with polymer-based BGS is that they are biodegradable and have a high resorption rate in the body leading to reduced strength with time and, hence, are not suitable for load-bearing sites in the body [20,29-31].

Earlier *in vitro* studies have shown that non-biodegradable materials such as ABS and Stratasys' MED610 have shown to produce long-term load-bearing implants, which are also osteoconductive and osteoinductive when pre-treated with bioactive reagents like calcitonin receptor fragment peptide (CRFP) [24,32-34]. We have earlier shown that CRFP has been found to be bioactive in differentiating stem cells into bone cells as well as enhance bone matrix production in *in vivo* studies [35,36]. In this study, we evaluate ectopic bone formation and biocompatibility of the non-biodegradable plastic MED610 scaffolds in a muscle pouch implantation model.

Ectopic bone refers to bone formation or ossification of tissue away from its typical origin *Vis*, in skin, fat, muscle, and other tissue environments [37]. Ectopic bone formation is usually used to study osteointegration in an *in vivo* setting by implanting the BGS outside its native environment. As the host's bone-forming cells are absent in this environment, ectopic bone formation is attributed to the influence of BGS [38-41]. The most common types of implantations are subcutaneous, kidney capsule, and muscle pouch implantations. Subcutaneous implantation may cause the implant to move under the skin of the rodent, causing complications, and a kidney capsule implantation requires a higher level of surgical skill to perform [37,42,43]. In this study, we adopted the muscle pouch implantation model in a mouse to assess the biocompatibility of the 3D-printed MED610 scaffold, seeded with MC3T3 stem cells, and treated with CRFP.

2. Materials and Methods

2.1. Preparation of MED610 scaffolds

The trabecular bone structure was extracted from the L5 vertebrae of a skeletally mature male mouse through a computerized tomography (CT) scanning (μCT40, Scanco Medical, Wangen-Brüttisellen, Switzerland) at an isotropic voxel resolution of 10 μm. The CT imaging (DICOM files) were processed using InVesalius software (Renato Archer Information Technology Center, Sao Paolo, Brazil) to extract a 3D model. The 3D model is then imported and processed in Geomagic DesignX software (3D systems, Rock Hill, SC, USA) to extract the trabecular shape. Using this trabecular model, we design the planar scaffold region (1 mm thickness × 3 mm length × 3 mm height). We also designed two cylindrical plates (3 mm diameter × 1 mm height) on the top and bottom of this planar region to facilitate compression studies. The scaffold's dimensions were chosen to fit in the lower extremity muscles of a mouse. The designed scaffolds were fabricated using MED610 material on a polyjet 3D printer (Objet 30 Prime; 16-μm resolution, Stratasys, Eden Prairie, MN, USA). The workflow of the design of MED610 scaffolds is presented in Figure 1.

The MED610 scaffolds were, then, washed with deionized water and sterilized in an autoclave oven at 132°C for 4 min as per the manufacturer's recommendation. The sterilized scaffolds were air-dried in a sterile cell-culture hood for 60 min. These scaffolds were, then, attached to the bottom surface of the cell culture plate using sterile grease to prevent them from floating in the cell culture media. MC3T3-E1 stem cells extracted from the C3 vertebra of a mouse were seeded with a cell density of 1 × 10^3 cells per chamber onto the scaffold surface [44]. The cell culture media (MEMα supplement with 5% fetal bovine serum and 1% penicillin/streptomycin) was changed every 3 days until 80% cell confluency was reached.

Osteogenesis was induced by adding 4 mM β-glycerol phosphate (G6P), 0.05 μg/μL ascorbic acid (AA), and 2 μM CRFP [45,46]. The cells were cultured for 3 more weeks with osteogenic reagents, with the media being changed every 3 days. In 3 weeks, the stem cells differentiate into bone cells and produce a bone matrix on the surface of the scaffolds. The scaffolds were, then, decellularized with 0.1% sodium dodecyl sulfate (SDS) solution for 5 min, washed in Dulbecco's phosphate buffer saline (DPBS) 2 times, and stored in DPBS.

2.2. Experimental design of muscle plant implantation in mice

Fourteen male C57BL/6J strain male mice of 10 – 12 week old (Charles River Laboratories, Sao Paolo, Brazil) were purchased and housed individually as males are known to fight if co-housed and tend to nibble at the healing incisions of their cage mates. The acclimatization period was 9 days at 12 h light/dark cycles, and the animals had libitum access to standard mouse chow and water. These conditions were maintained throughout the experiment. After acclimatization, mice were weighed and

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randomized into two experimental groups (n = 7): (1) Untreated scaffolds and (2) decellularized scaffolds. Decellularized scaffolds were prepared using the protocol explained in section 2.1. A set of untreated scaffolds which were exposed to the same reagents as decellularized scaffolds were also prepared, except that they do not have any stem cells seeded onto the surface.

2.3. Surgical protocol

For implantation, animals were anesthetized with isoflurane (2% induction and 1.5% maintenance) (Covetrus, Portland, ME, USA). Eye lubrication (Optixcare; Aventix, Burlington, Ontario, Canada) was applied, and the mice were prepared for the surgery by shaving the left hind limb and sterilizing the surgical site with betadine and ethanol (Sigma-Aldrich, Saint-Louis, MO, USA). A 10-mm longitudinal incision parallel to the posterior femur was created. Using a blunt dissection to prevent muscle damage, a 5-mm deep intramuscular pouch was then shaped, taking precautions not to expose the periosteum. A scaffold was, then, sterilized in 70% ethanol and implanted into the muscle pouch, and the fascia over the muscle was sutured with resorbable sutures (Ethicon, Raritan, NJ, USA) to close the muscle wound. The skin incision was closed using non-resorbable sutures (Ethicon), and topical antibiotics were applied. Buprenorphine (0.1 mg/kg) analgesia (Buprenex, Indivior, North Chesterfield, VA, USA) was administered immediately following surgery and twice daily thereafter until it was judged to be no longer necessary. The skin sutures were removed 10 – 14 days after surgery. The workflow of the surgery is illustrated in Figure 2.

The animals were monitored every day for the first 3 days and weekly thereafter. Movement around the cage and activity was observed to assess the weight-bearing on the lower extremity. The incision area was assessed for incision and quality of sutures. Grooming, vocalization, and weight loss were checked as indicators of distress. The exclusion criteria were if the animal is experiencing dehiscence, infection, pain, or distress that cannot be treated or if the animal is experiencing more than 15% weight loss.

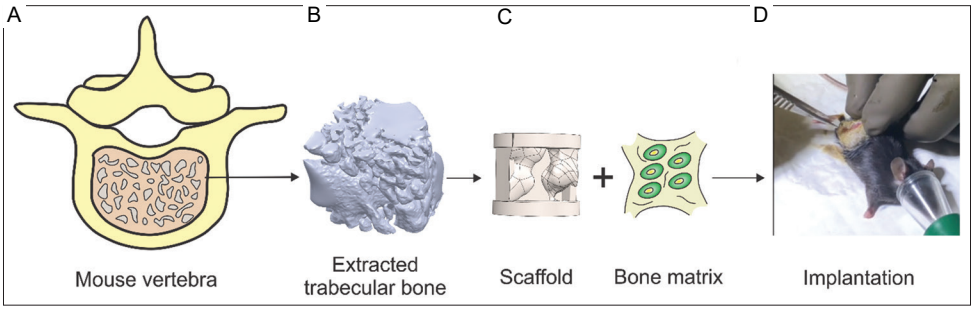

Figure 1. (A) The cross-section of a mouse vertebra with cortical bone (yellow) and trabecular bone (brown); (B) Trabecular bone extracted from μ-computed tomography scan of the vertebra; (C) MED610 scaffold designed from the extracted trabecular bone seeded with bone cells on its surface; and (D) Scaffold being implanted into the thigh muscle of a mouse.

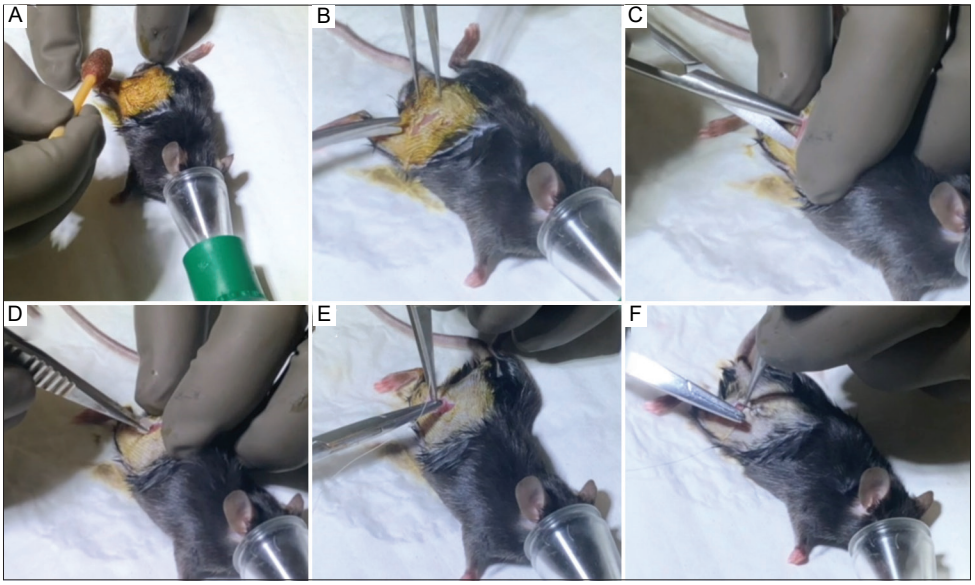

Figure 2. (A) Preparing the surgical site by scrubbing the shaved skin with Betadine: (B) Longitudinal incision made along the thigh; (C) Blunt dissection being carried out to create a muscle pouch without exposing the periosteum; (D) Implant placed in the muscle pouch; (E) muscle pouch was closed with resorbable sutures; and (F) The skin incision was sutured with non-resorbable sutures.

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2.4. Extraction and post-operative testing

After 3 weeks, the mice were euthanized with a lethal dose of isoflurane, and the scaffolds were extracted for post-operative mechanical and staining studies. The scaffolds extracted from the animals were fixed in 4% formaldehyde solution for 24 h and washed and stored in DPBS for post-operative studies. The previous studies have shown that pre-coating the BGS with CRFP leads to enhanced osteoinduction which results in the deposition of more bone matrix, contributing to improving the load-bearing capacity, that is, the compressive strength of the BGS. Therefore, we performed unconstrained mechanical compressive testing (MTEST Quattro, Admet, Norwood, MA, USA) on four sets of MED610 scaffolds (Table 1). For this purpose, the force was applied in the direction of the axis of the scaffold, as illustrated in Figure 3B. The speed of compression was set to 5 mm/min based on the ISO 604, international standards for plastics [47]. Stiffness (k), maximum compressive strength (σ_M), and compressive modulus (E_C) in the central axis were evaluated. Thereafter, Shapiro–Wilk test was carried out for the compressive test results of each type of scaffold to assess the normality of the data before carrying out the statistical analysis. A one-way analysis of variance (ANOVA) test at a significance level of 0.0083 (Bonferroni correction) was performed to compare the different strength characteristics of the various scaffolds.

Table 1. Types of MED610 scaffolds.

Type	Protocol
A	Untreated scaffolds
B	Decellularized scaffolds
C	Implanted untreated scaffolds
D	Implanted decellularized scaffolds

Post-compression tests, scanning electron microscopy (SEM) imaging (10.0 K × magnification at electron high-tension voltage of 3.0 kV) was performed to observe the surface of the different groups of scaffolds. Thereafter, staining studies with 2% Alizarin red to validate calcium deposits and Von Kossa staining to validate mineralization on the BGS surface were conducted. The Alizarin red-stained scaffolds were imaged using a confocal microscope (LSM-510; Zeiss, Oberkochen, Germany). We followed up these studies with histological studies using Nuclear Fast Red (Kernechtrot) staining for calcium and were imaged using a confocal microscope (upright DM 6000; Leica Microsystems, Wetzlar, Germany). In these staining studies, ten regions of interest (ROI) (0.7 mm × 0.7 mm) were identified for analysis in each image of the stained scaffold. For Alizarin red staining and Nuclear Fast Red staining, each ROI image was processed to isolate the red-colored pixel intensity map from the Red-Blue-Green (RGB) color image. This red color intensity map then is normalized such that "0" is the least red (100% white) and "1" is the value for the highest red value (100% red). Then, the average of this normalized intensity pixel map was calculated to represent the measure of the red intensity of the ROI. The higher red intensity measure indicates more red spots on the ROI (representing higher deposition of calcium deposits). In the case of Von Kossa staining, the RGB color image was converted to a grayscale image and normalize the grayscale image such that the "0" value refers to the brightest pixel (100% white) and "1" value refers to the darkest

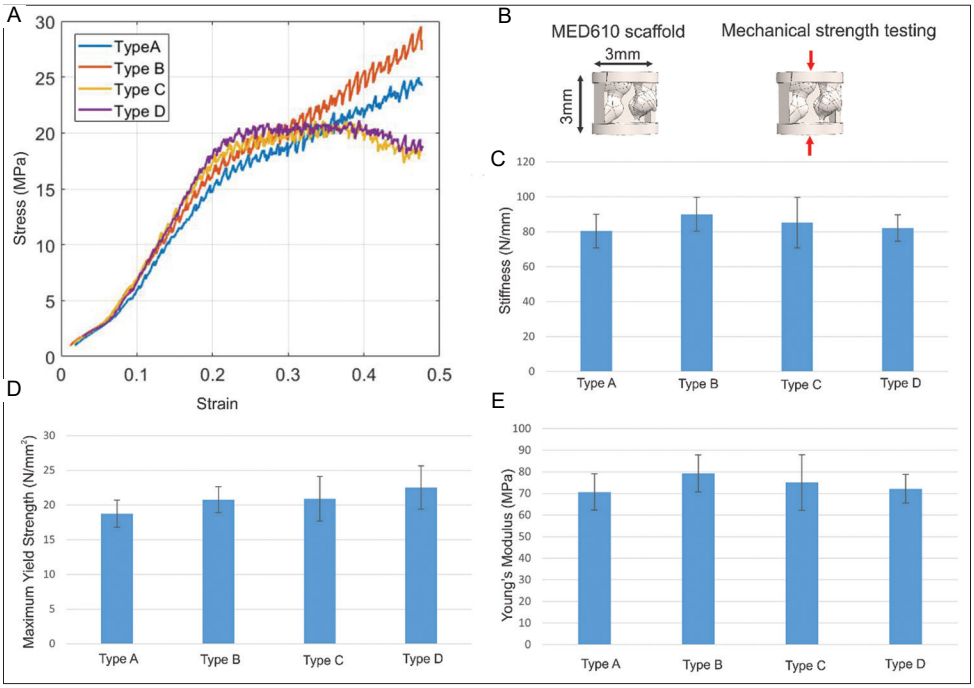

Figure 3. (A) Stress-strain curve of the four types of scaffolds and (B) the dimensions of the MED610 scaffolds and the direction of mechanical compression testing. Plots of stiffness (C), maximum compressive strength (D), and compressive modulus (E) for the four types of scaffolds (n = 7) in Table 1. Error bars represent standard deviation.

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pixel (100% black). The darker spots on the image represent the higher mineralization deposition on the ROI. One-way ANOVA analysis at a significance level of 0.0083 (Bonferroni Correction) was performed to compare unseeded scaffolds with decellularized scaffolds.

3. Results

3.1. Mechanical testing for the strength of the scaffolds

In the implantation study, the animals did not show any signs of infection or prolonged distress due to implantation throughout the duration of the study, and by the 10th day, they were able to regain complete range of motion and were able to walk/run without any signs of pain or distress. All animals maintained their weight by the end of the study and did not trigger any exclusion criteria.

In the mechanical testing of the four types of scaffolds listed in Table 1, the data followed normal distribution using Shapiro–Wilk test. Thereafter, the one-way ANOVA test showed no significant difference in stiffness (k), maximum compressive strength (σ_M), and compressive modulus (E_C). However, on plotting the stress-strain data shown in Figure 3A from the stress-strain curves, it was noted that the decellularized scaffolds with bone matrix (type B and D) show superior trends for maximum yield strength (σ_M) in comparison to untreated scaffolds without bone matrix (type A and C). These findings follow our earlier reported results [24]. The strength characteristics (k, $\sigma_{M,}$ and E_C) of all types of scaffolds are illustrated in Figure 3.

The SEM study of the scaffolds' surfaces is illustrated in Figure 4. The surface of the scaffolds shows deposition of bone matrix in the type B scaffolds, in comparison to type A scaffolds. When these scaffolds are implanted, type D scaffolds show more deposition of organic material, indicating a higher level of biointegration in comparison to type C scaffolds.

For the Alizarin red staining study for validating calcium deposits on the scaffolds' surfaces, the one-way ANOVA analysis showed a significant increase in calcium deposits from type A (untreated scaffolds) to implanted scaffolds, that is, Type C (P = 0.005) and Type D (P = 0.0027). All other comparisons between scaffolds were not statistically significant. These results are represented in Figure 5A.

For the Von Kossa staining study to validate mineralization on the surface of the scaffolds, the one-way ANOVA analysis showed that Type B and Type D (demineralized) scaffolds showed a significantly higher mineralization on the scaffold surface compared to Type A and Type C (untreated) scaffolds ($P < 0.001$ for all significant comparisons). The comparisons are illustrated in Figure 5B. The confocal microscopy results with Nuclear Fast Red staining shows a significantly higher calcium deposition in demineralized scaffolds (Types B and D) compared to untreated scaffolds (Types A and C) in the one-way ANOVA analysis ($P < 0.001$), as illustrated in Figure 6.

4. Discussion

In this study, we developed non-biodegradable BGS from Stratasys MED610 material for testing the biocompatibility of artificial bone in an *in vivo* environment. The BGS was fabricated using polyjet 3D printing to achieve a high-resolution surface that

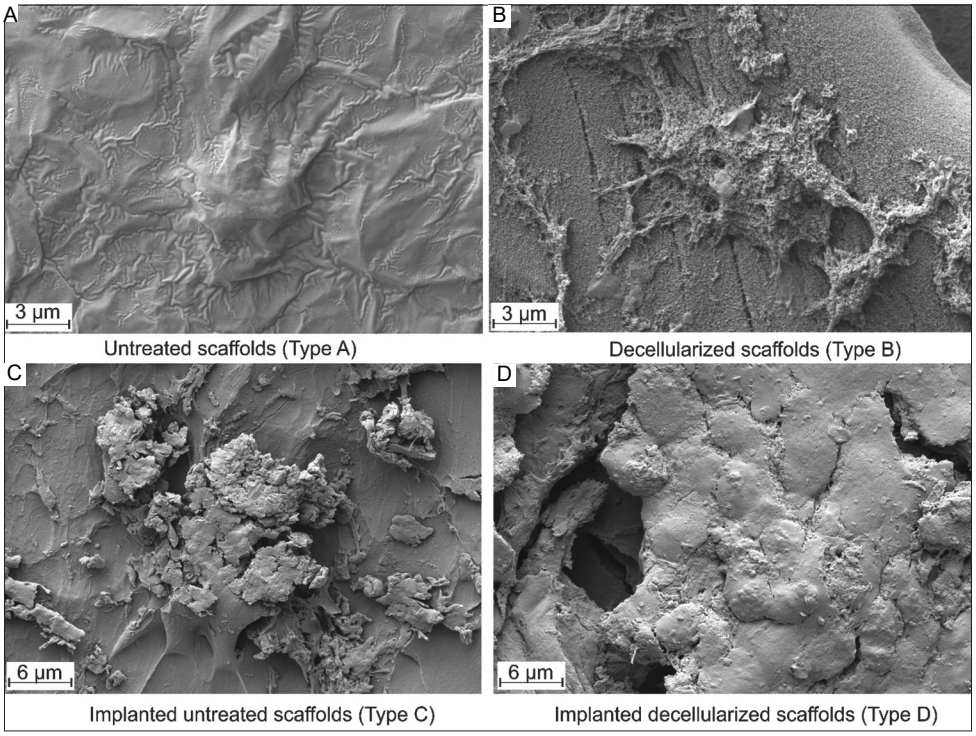

Figure 4. (A-D) Scanning electron microscopy images of different scaffolds taken at 2.0K × magnification showing the deposition of organic material on the scaffold surfaces.

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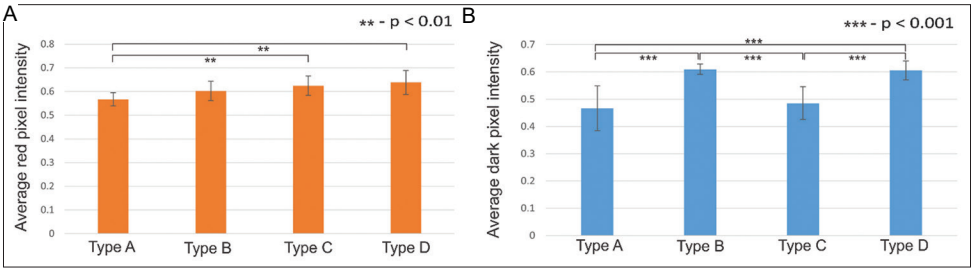

Figure 5. (A and B) Results of mean values of average red pixel intensity (orange) in Alizarin red staining for calcium deposits and mean value for average dark pixel intensity in Von Kossa staining (blue) for mineralization on scaffolds' surface (n = 10). Error bars represent the standard deviation.

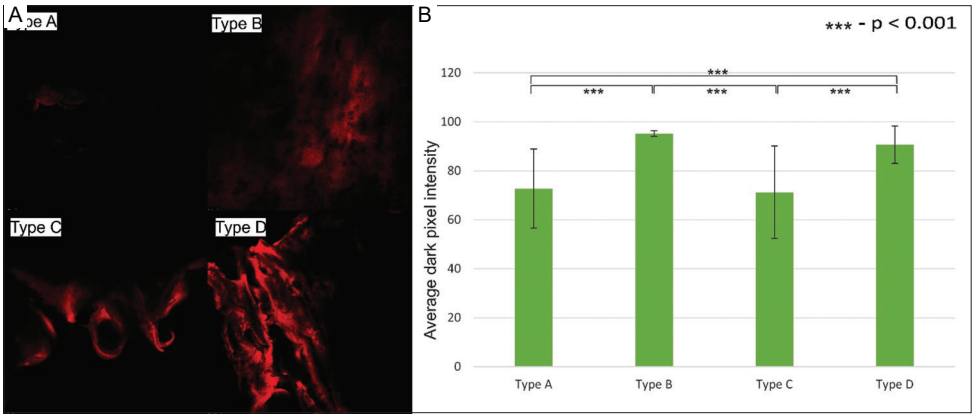

Figure 6. (A) Confocal microscopy images if the different scaffolds with nuclear fast red staining showing calcium deposition in different scaffolds. (B) Results of mean values of average red pixel intensity (green) in nuclear fast red staining for calcium deposits (n = 12). Error bars represent the standard deviation.

mimics the trabecular structure of the bone [33,48]. The previous studies suggest that most of the plastic (PLA/PLC) and ceramic (TCP) BGS being developed today show reduced strength over time and cannot consistently bare the stresses applied to the bone leading to them only being used in non-loadbearing sites [20,49]. This is due to the high resorption rate of the BGS. Our scaffolds (BGS) maintain their structural integrity and strength as they are non-biodegradable and show high potential as BGS in loadbearing areas.

In our muscle pouch implantation study, our BGS exhibited good biocompatibility, as the animals were able to accept the BGS and did not show any inflammatory response near the incision or internally [50]. By the 10th day after implantation, all animals were behaving normally without any distress and complete load bearing.

In SEM imaging study, we find that the decellularized scaffolds, when implanted, have a higher deposition of organic material on the scaffold surface in comparison to the untreated scaffolds (Figure 4). Following up with the staining studies with Alizarin red, Nuclear Fast Red, and Von Kossa staining, we find that the implanted decellularized scaffolds show a significantly higher level of calcium deposits and mineralization. We also see that there is a significantly higher level of calcium deposit on the implanted scaffolds in comparison to the untreated scaffolds. This is an indication of ectopic bone formation on the scaffold surface and bio integration on implantation.

Earlier studies reported that the strength of the BGS increases when the bone matrix is deposited on the scaffold surface [24,33]. In our mechanical strength studies, we find no significant increase in decellularized scaffolds compared to untreated scaffolds. However, the results suggest that the maximum yield strength follows similar trends to that of the *in vitro* studies in the literature, where scaffolds with bone matrix perform better than scaffolds without bone matrix [24,33,51,52].

One of the limitations of this study was the size of the scaffolds (3mm diameter × 3mm height). Due to the small size of the animals, the aim was only to observe the ectopic bone formation and biocompatibility of the MED610 material. In future, these studies will be followed up by a segmental defect model in either rats or rabbits so that the biointegration of these scaffolds with the surrounding bone can be studied and evaluate how BGS can maintain the strength at a load-bearing site.

Another limitation of this study was that we did not mimic any specific bone as done in studies by others [33,53], except taking the trabecular structure model. Despite considerable progress in the field of artificial bone development using materials like natural polymers[54-59], synthetic polymers[60-62], biocermaic and bioglass[63-66], metal[67-69], and composites[70-74], an ideal all-purpose material for scaffold-guided bone regeneration is currently not available[75]. In future biointegration studies, we plan to design the scaffolds precisely in the shape of the bone that is to be replaced to achieve complete integration structurally

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and biologically. Many iterations need to be done on the design side to achieve the balance between having the ideal porosity to facilitate the bone growth and providing the structural strength to the affected bone.

5. Conclusions

We successfully demonstrated that our MED610 3D-printed scaffolds are suitable for implantation as they are biocompatible and do not cause any adverse reaction when implanted. We also found that the CRFP-coated MED610 scaffolds generate more ectopic bone growth when implanted and contribute to bio-integration.

Acknowledgments

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Conflicts of Interst

The authors declare that there are no conflicts of interest.

Ethics Approval and Consent to Participate

No human subjects were involved in this study. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University (IACUC protocol number: 1503487).

Consent for Publication

Not applicable.

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REVIEW ARTICLE

A possible novel therapeutic targets of selinexor in acute lymphoblastic leukemia: a comprehensive review

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ABSTRACT

Background and Aim: Acute lymphoblastic leukemia (ALL) presents a formidable challenge in pediatric and adolescent healthcare due to its aggressive nature and high relapse rates. Despite therapeutic advancements, the demand for more effective treatments remains pressing. In the realm of hematologic malignancies, selective inhibitors of nuclear export (SINE) have emerged as promising agents, particularly in evading resistance observed with conventional chemotherapy in acute myeloid leukemia (AML). Selinexor, a prominent SINE compound, has exhibited promising anti-leukemic effects in murine models of AML, laying the foundation for its clinical evaluation. Furthermore, selinexor has been utilized in clinical trials both as a single-agent therapy and in combination with established regimens for a wide range of solid and liquid tumors. However, the precise impact of selinexor in the context of ALL, specifically as a single agent or in combination therapies, remains unexplored. Unraveling the mechanistic intricacies underlying selinexor's actions in ALL holds the key to optimizing its efficacy either as a monotherapy or in combination therapies. Notably, within the intricate landscape of ALL pathogenesis, critical factors including the mammalian target of rapamycin signaling cascade, aberrations in cancer glucose metabolism, occurrences of alternative splicing, perturbed expressions of dysregulated long noncoding RNAs, and impaired autophagic processes have emerged as pivotal determinants. This comprehensive review undertakes a systematic exploration of potential therapeutic targets that hold the promise of augmenting selinexor's efficacy within the unique landscape of ALL.

Relevance for Patients: This study highlights the possible therapeutic targets of selinexor in ALL. Understanding the intricate molecular mechanisms, the rational refinement of selinexor's administration, both as a single agent and as a synergistic component in combination therapies could lead to new avenues for improving the treatment outcomes in ALL patients.

1. Introduction

Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) represent two distinct forms of acute leukemia, which are fast-growing blood cancers that originate in the bone marrow and affect the white blood cells [1,2]. However, they differ in terms of the specific types of white blood cells they affect, their prevalence across various age groups, treatment strategies, and certain genetic and clinical attributes. AML primarily affects myeloid cells, responsible for producing various types of mature blood cells including red blood cells, platelets, and certain white blood cell varieties [3]. AML is linked to several genetic mutations (FLT3, NPM1, and IDH1/IDH2 mutations) that can impact treatment

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response and prognosis [3]. On the other hand, ALL predominantly target lymphoblasts, immature white blood cells belonging to the lymphoid lineage [4]. In the United States, approximately 6,540 new cases of ALL were diagnosed in the year 2023, resulting in over 1,390 deaths from the disease (American Cancer Society). ALL is characterized by specific genetic abnormalities, including chromosomal translocations such as the Philadelphia chromosome (Ph+), which is associated with a more adverse prognosis [3,4]. Among the spectrum of ALL, a distinctive subtype called B-cell precursor ALL (B-pre-ALL) emerges. Moreover, this subtype specifically targets B-cell precursors or immature B-lymphocytes, rendering it the most widespread variant of ALL, particularly prevalent among children [4,5]. In managing both AML and ALL, various therapeutic strategies are employed, including chemotherapy, immunotherapy, targeted therapy (like monoclonal antibodies), or allogeneic stem cell transplantation [6]. Due to a higher tendency of central nervous system (CNS) involvement in ALL as compared to AML, treatments with a specific focus on the CNS (such as intrathecal chemotherapy or cranial radiation) are frequently integrated into ALL treatment protocols [7]. Despite advancements in the therapeutic process, relapsed cases of ALL remain a significant challenge, exhibiting unfavorable prognoses. Thus, a critical need exists to develop effective therapies for treating relapsed ALL and to explore novel combinatorial therapeutic regimens with chemotherapy to enhance outcomes in newly diagnosed patients [8]. Elucidating the underlying molecular mechanisms that contribute to *de novo* or acquired drug resistance presents a ubiquitous obstacle in cancer therapeutics [9]. This underscores the imperative to explore novel targeted therapeutic strategies, specifically directed toward ALL [10]. Noticeably, selective inhibitors of nuclear export (SINE) are emerging as a potential therapeutic approach to overcome drug resistance in the context of AML [11].

Selinexor, an inhibitor of nuclear export, was recently demonstrated to bind reversibly and inhibit the nuclear export protein exportin-1 (XPO1), leading to the accumulation of cargo proteins inside the cell nucleus [12]. Selinexor exerts its effects on multiple myeloma by inhibiting nuclear factor kappa B (NF-kB) signaling, reactivating various tumor suppressor proteins, and reducing c-myc levels [13,14]. A recent study has indicated that selinexor treatment led to the downregulation of the mammalian (or mechanistic) target of rapamycin (mTOR) signaling pathway in sensitive and resistant AML cell lines [13]. Selinexor exhibited synergistic antimyeloma effects when combined with glucocorticoids, proteasome inhibitors (PIs), and immunomodulators in preclinical studies [14,15]. Notably, the combination of selinexor and dexamethasone (DEX) has received approval in the United States for treating patients with penta-refractory multiple myeloma [16]. Moreover, the selinexor-bortezomib-dexamethasone combination has also been approved for patients who have received ≥1 prior therapy in multiple myeloma patients [16]. The clinical trial of selinexor, either as a monotherapy or in combination, for AML patients has been shown in Table 1. However, the impact of selinexor treatment on ALL as a single agent or in combination therapies has not been explored. Gaining insights into selinexor's mechanism of action within the context of ALL is crucial for optimizing its efficacy as a standalone treatment or in synergy with combination therapies. In this review, we discuss the possible targets of selinexor in ALL, such as mTOR signaling, glucose metabolism, alternative splicing, long non-coding RNA expression, and autophagy, all of which may play critical roles in determining the pathogenesis of the disease and the effectiveness of chemotherapy. We have provided the descriptions of clinical and preclinical studies of selinexor in various cancers (Tables 1 and 2).

2. mTOR

mTOR is a conserved serine/threonine kinase that belongs to the PI3K-related kinase family and exists in two distinct signaling complexes known as mTORC1 and mTORC2 [23,24]. mTORC1 plays a significant role in mRNA translation and protein synthesis, whereas mTORC2 substantially contributes to cell survival and migration [23,24]. The mTOR pathway occupies a central position in sensing environmental cues and monitoring virtually all facets of metabolism, spanning from the cellular to the organismal level [25]. Dysregulated mTOR signaling is linked to cancer and diabetes progression, along with the aging process [26]. Given that the activation of the PI3K/Akt/mTOR network is frequently linked to a poor prognosis and chemoresistance in ALL, there remains an ongoing demand to identify novel inhibitors for the effective treatment of this disease. This is particularly relevant given the mounting evidence indicating mTOR dysregulation's association with metastatic potential, cell proliferation, and angiogenesis. [27,28]. Moreover, B-pre-ALL is characterized by constitutive activation of the PI3K/Akt/mTOR network, which is known to significantly impact cell growth and survival [29].

The application of selinexor to AML cell lines led to the reduction of mTOR activity [13]. Moreover, selinexor demonstrates synergistic effects with dexamethasone to suppress mTORC1 signaling and promote cell death in multiple myeloma [16] (Table 2). Consequently, investigating the impact of selinexor treatment on mTOR signaling in the context of ALL holds significant therapeutic importance (Figure 1A). This endeavor is pivotal for assessing the efficacy of selinexor in ALL treatments.

3. Reprogrammed Glucose Metabolism in Cancer

Aberrant glucose metabolism has emerged as a major type of metabolic reprogramming in cancer, discovered by Otto Warburg in the late 1920s [30]. The uncontrolled proliferation of cancer cells induces a heightened demand for nutrients, creating an environment of limited nutrient availability. In response to this increased nutritional stress, cancer cells undergo metabolic adaptations. Cancer cells exhibit a preference for utilizing glycolysis as their primary pathway for glucose metabolism even in oxygen-abundant conditions, rather than relying on the more efficient mitochondrial oxidative phosphorylation for ATP production [30,31]. Moreover, the cancer cells exploit elevated levels of glucose as a primary carbon source to fuel

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Table 1. Clinical trial studies of selinexor alone or in combination with chemotherapeutic drugs in AML

Drugs	Type of leukemia	Phases	Outcome	References
Selinexor	AML	Phase I	Selinexor is safe as a monotherapy in patients with relapsed or refractory AML	[17]
Selinexor + Venetoclax	AML	Phase I	This combination is a safe regimen for AML patients	NCT04898894
Selinexor + Daunorubicin + Cytarabine	AML	Phase I	This combination is a safe regimen for newly diagnosed poor-risk AML patients	[18]
Selinexor + Mitoxantrone (M) + Etoposide (E) + Cytarabine (C)	AML	Phase I	Selinexor plus MEC is a feasible treatment for patients with R/R AML	NCT02299518
Selinexor + Cytarabine + Idarubicin	AML	Phase II	Selinexor, cytarabine, and idarubicin result in a high remission rate in patients with R/R AML	[19]

AML: Acute myeloid leukemia

Table 2. Preclinical studies of selinexor alone or in combination with chemotherapeutic drugs in various cancers and their altered pathways

Drugs	*In vitro/In vivo* studies	Altered pathways	References
Selinexor	AML cell line	Downregulation of mTOR signaling; regulate p53 pathway	[13]
Selinexor + Dexamethasone	Multiple myeloma cell line and multiple myeloma mice models	Suppress mTORC1 signaling and inhibits tumor growth in both *in vitro* and *in vivo* studies	[20]
Selinexor + Azacitidine	AML cell line	Inhibit XPO1/eIF4E/c-MYC signaling	[21]
Selinexor	Gall bladder cancer cell line and mice models	Autophagy-dependent apoptosis by activating the p53/ mTOR pathway	[22]

AML: Acute myeloid leukemia; mTOR: Mammalian target of rapamycin

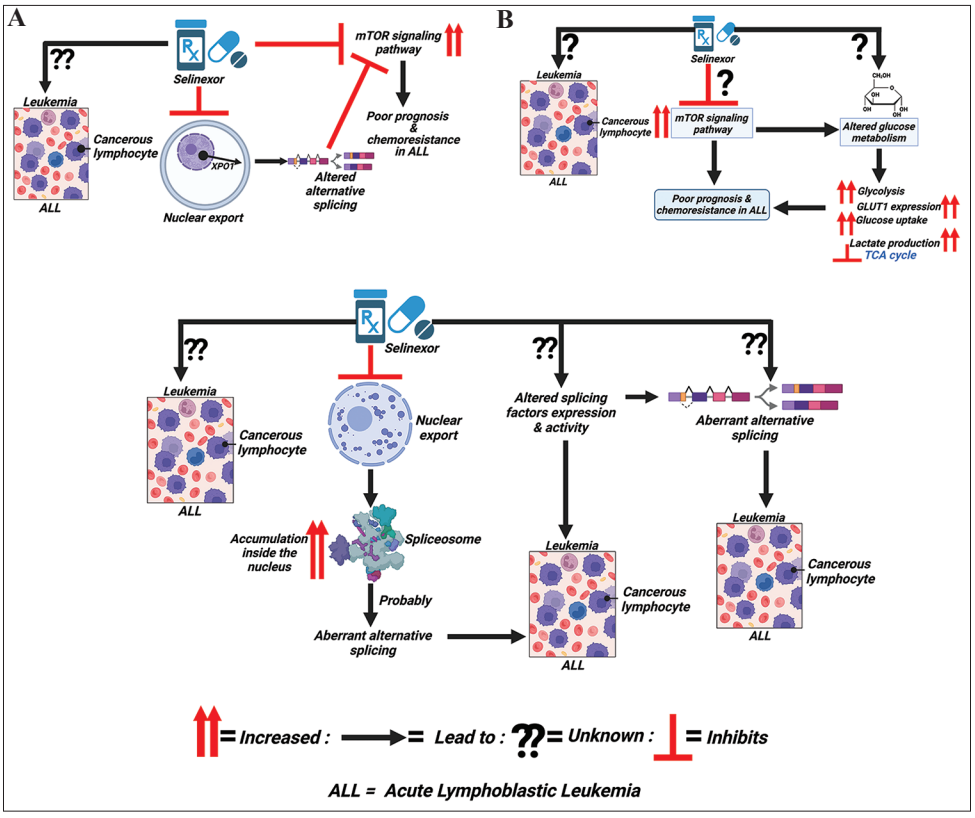

Figure 1. Mammalian target of rapamycin (mTOR) Signaling, cancer glucose metabolism, and alternative splicing as possible therapeutic targets of selinexor. **(A)** This schematic provides an overview of the potential therapeutic targets of selinexor, an inhibitor of nuclear export, in the regulation of the mTOR signaling pathway in childhood acute lymphoblastic leukemia (ALL). It also illustrates the established consequences of dysregulated mTOR signaling in ALL. **(B)** This diagram explores the potential impact of selinexor on the regulation of cancer glucose metabolism in ALL. It also highlights the regulation and consequences of altered glucose metabolism in ALL. **(C)** Alternative splicing emerges as a promising therapeutic target of selinexor in ALL. The figure portrays the various potential mechanisms by which selinexor may influence alternative splicing in ALL. The "???" in the figure represents areas that remain unexplored or unanswered.

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anabolic reactions. These reactions play pivotal roles in various aspects of cancer, including initiation, progression, metastasis, cell survival, and the development of resistance against anti-tumor therapies [32,33]. Indeed, the complete metabolic network undergoes significant reprogramming under the influence of oncogenes and tumor suppressor genes [32]. This restructuring also encompasses a redefinition of nutrient flow within metabolic networks during the process of tumor formation. In recent years, there has been a growing interest in glucose metabolism of cancer cells, which has now become an integral part of cancer biology [32]. Moreover, both mTORC1 and mTORC2 complexes play a significant role in the regulation of metabolism [34]. Gene expression profiling of pediatric patients diagnosed with ALL revealed the activation of genes that promote glycolysis, alongside the downregulation of genes associated with the tricarboxylic acid cycle [35]. Functional analysis conducted on pediatric patients with ALL demonstrated elevated expression of the glucose transport protein and glucose transporter 1 [35]. Furthermore, cell lines derived from ALL exhibited heightened lactate production and a notable susceptibility to the glycolysis inhibitor, 2-deoxy-D-glucose [35]. Mutations in genes that encode transcription factors responsible for regulating glucose metabolism, such as PAX5 and IKZF1, have been observed in more than 80% of cases of pre-B-cell ALL [36]. Notably, the combined utilization of selinexor and azacitidine exhibited synergistic effects by targeting XPO1/eIF4E/c-MYC signaling pathways in AML, offering encouraging preclinical data that suggest its potential for future clinical application [21] **(**Table 2**)**.

In preclinical models of triple-negative breast cancer, selinexor exhibits notable anti-tumor efficacy [21,37]. Selinexor treatment induces distinct alterations in AKT signaling and the expression of genes associated with metabolism in breast cancer cell lines including BT474 and MCF-7 [38]. Moreover, the combination of selinexor with tamoxifen resulted in a marked reduction in AKT signaling, and seahorse metabolic profiling revealed a significant shift in the metabolic profile of breast cancer cells. This transition shifted the cells from an energetic state to a quiescent state [38]. Notably, both the glycolytic and mitochondrial pathways were concurrently inhibited, thereby inducing autophagy [38]. In addition, selinexor induces autophagy-dependent apoptosis in gallbladder cancer by activating the p53/mTOR pathway, both *in vitro* and *in vivo* [22]. Interestingly, the inhibition of the glycolytic pathway plays a crucial role in modulating autophagy, exerting a significant impact on the survival of leukemia cells [39]. Consequently, there exists a potential for selinexor to modulate the glycolytic pathway in ALL. However, the precise effect of selinexor on cancer glucose metabolism in the context of ALL remains unknown (Figure 1B). The significance of conducting experiments aimed at evaluating the impact of selinexor treatment on the expression of PAX5 and IKZF1 cannot be overstated. These investigations will provide crucial insights into the potential effects of selinexor on these genes and their relevance in the context of ALL treatments.

4. Autophagy

Christian De Duve first coined the term “autophagy” in 1963 to describe the process of self-eating that he had discovered while studying lysosomes [40]. Since then, the role of autophagy has been explored in numerous research areas including cancer, diabetes, infectious diseases, and neurodegenerative disorders [40]. Autophagy is a multistep catabolic signaling cascade that orchestrates cytoplasmic content in a double-membrane vesicle and fuses with lysosomes, involved in the degradation of damaged organelles such as mitochondria (mitophagy), lipids, and proteins, that maintains cellular homeostasis under normal circumstances [40]. Autophagy has a multifaceted role in cancer, with well-established roles for autophagy in promoting tumor cell survival by providing recycled nutrients and modulating mitochondrial function through mitophagy, or intriguing new roles in tumor cell migration and invasion through control of focal adhesion turnover and secretion of pro-migratory cytokines/chemokines [41]. Conversely, autophagy acts as a tumor suppressor by preventing malignant transformation in mouse models defective for autophagy [42]. Therefore, autophagy has both tumor-suppressive and tumor-promoting effects in cancer depending on tumor genetics, host variables, and tumor stage [41,43]. Due to its contradictory effects, autophagy has been considered a double-edged sword in cancer, challenging researchers to further investigate how to modulate autophagy in the context of cancer therapies [43,44]. Autophagy has emerged as one of the critical molecular mechanisms involved in drug resistance. Chemotherapeutic agents are well known to induce autophagy in cancer cells [45]. The P38 stress response pathway has also been linked to therapeutic resistance and regulation of autophagy [46]. Therefore, autophagy may be exploited as a promising strategy for the therapeutic sensitization of cancer cell [43,44,47].

Selinexor treatment of sensitive AML cell lines resulted in a heightened DNA damage response [13]. Conversely, in resistant AML cell lines, the administration of selinexor led to the activation of increased stress response pathways [13]. Moreover, in the context of wild type p53 resistant cell line, selinexor treatment upregulated the autophagy pathway, while in mutant p53-resistant cells, selinexor treatment triggered an enhanced p38 stress response pathway [13]. It is worth noting that selinexor has been shown to induce autophagy-dependent apoptosis in gastric cancer [22]. Hence, based on this evidence, we propose that selinexor might have the capacity to modulate autophagy in the treatment of childhood ALL (Figure 2B).

5. Alternative Splicing

Alternative splicing is a pivotal mechanism governing the regulation of gene expression [48,49]. It entails the excision of introns from messenger RNAs, allowing exons to join together [48,49]. This process of alternative splicing is widely deregulated in various cancers, leading to the emergence of cancer-specific splicing experiences widespread dysregulation across diverse cancers, resulting in the emergence of splicing

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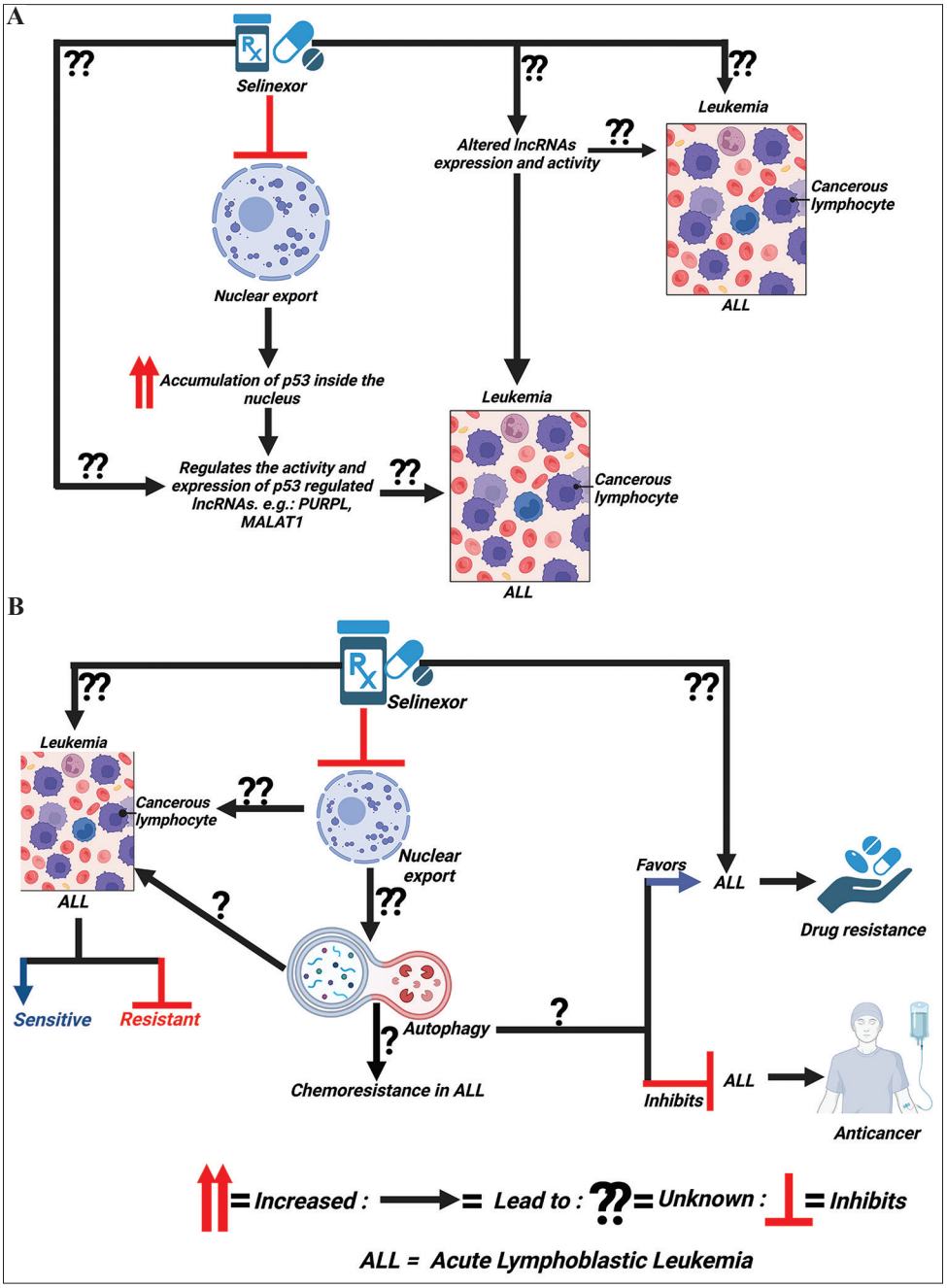

Figure 2. Long non-coding RNAs (lncRNAs) and autophagy as possible therapeutic targets of selinexor. (**A**) This schematic provides an overview of the potential therapeutic targeting of long non-coding RNAs (lncRNAs) by selinexor. It also illustrates the regulation and implications of altered lncRNA expression in acute lymphoblastic leukemia (ALL). (**B**) This diagram outlines the role of autophagy in the selinexor-mediated response in ALL. It emphasizes the significance of autophagy in ALL. The "???" in the figure symbolizes areas that remain unexplored or unanswered.

isoforms that are unique to cancer and display either absence or distinctive expression levels when contrasted with their equivalents in healthy tissue [50]. Significantly, a considerable proportion of these transcripts encompass pivotal oncogenes and tumor suppressor genes [50,51]. Among the proteins that are translocated to the nucleus in selinexor-sensitive cells, there was a notable over-presentation of KEGG terms associated with spliceosome [13]. The spliceosome holds a significant function in governing alternative splicing regulation [52]. Alternative splicing plays a pivotal role in enhancing the intricacy of proteins within the human system [34]. This intricate process is under the regulation of splicing factors [49,53], which exert control over alternative splicing. It is evident that a strong correlation exists between numerous diseases and the disruptions and errors in splicing regulation caused by these splicing factors [50,51,53]. These crucial regulatory elements, known as splicing factors, belong to

the category of trans-acting RNA binding proteins [51,53]. It is worth noting that only a limited number of RNA-binding proteins have been associated with childhood ALL. In addition, their precise contributions to childhood ALL are still emerging. The phenomenon of alternative splicing is deregulated in AML [22]. Aberrant spliced Isoforms of IKZF1 have been detected not only in leukemic cell lines but also in samples derived from patients with ALL [54]. Furthermore, the splicing patterns of IKZF1 have been associated with the development of resistance to receptor tyrosine kinase inhibitors among samples from ALL patients [55]. Likewise, a connection has been established between the splicing of isoforms in the N terminus of p53 and its involvement in ALL [56]. In AML, a particularly noteworthy aspect involves the recurrence of mutations within the machinery responsible for splicing, leading to widespread instances of irregular splicing events across the entire genome [57]. Studies looking at the role of spliceosome machinery and aberrant splicing have not been studied extensively in childhood ALL. One of the most critical challenges in contemporary cancer treatment is the emergence of resistance to therapeutic medications, ultimately culminating in the failure of treatment endeavors [10,58]. Importantly, alternative splicing holds the capacity to significantly alter the coding region of drug targets [57].

Several reports have identified significant alternative splicing events that take place in different types of cancers and contribute to resistance against cancer therapies [59]. In the context of Phase II clinical trials targeting patients with myelodysplastic syndrome (MDS), the administration of selinexor resulted in mixed outcomes, with some patients showing a positive response while others did not respond [19]. Genetic investigations have revealed a strong connection between the response to selinexor and the presence of hotspot mutations within the core RNA splicing factor SF3B1 [60]. Notably, SF3B1 mutations are frequently found in MDS and render cells more vulnerable to the impairment of normal splicing functions in remaining wild-type genes. This interplay between SF3B1 mutations and the response to selinexor is particularly significant, given SF3B1's established association with MDS and the essential role of XPO1 in the nuclear export and maturation of RNA spliceosome components [61]. After selinexor treatment, comprehensive transcriptomic sequencing of alternative splicing events in bone marrow specimens taken before and after treatment unveiled a distinct pattern [61]. Patients who achieved marrow complete remission (mCR) displayed a widespread disruption in RNA splicing, characterized by heightened intron retention (IR) in post-treatment samples compared to their pre-treatment counterparts. In contrast, those who did not achieve mCR exhibited less pronounced IR [61].

Interestingly, selinexor induced significant IR, notably in the Inhibitor of NF-kB Kinase Subunit Beta gene. This led to the inclusion of a premature stop codon, subsequently triggering nonsense-mediated decay and disrupting the NF-kB signaling pathway [61]. These observations became apparent when closely examining the most prominent instances of IR among selinexor responders [61]. Considering the above observations, it would be intriguing to explore the role of alternative splicing in the drug response of selinexor (Figure 1C). The objective would be to investigate how aberrant alternative splicing impacts the effectiveness of selinexor in the treatment of childhood ALL.

6. Long Noncoding RNAs

Long non-coding RNAs (lncRNAs: longer than 200 nucleotides) play a crucial role in regulating various aspects of gene expression. They are involved in processes such as chromatin remodeling, transcriptional control, regulation of splicing, mRNA stability, mRNA translation, miRNA processing, and protein stability [62]. Recently, a study has shed light on the involvement of lncRNAs in the etiology, progression, and treatment response of childhood ALL [63]. In addition, MALAT1, a specific long noncoding RNAs has been linked to poor prognosis in childhood AML [64]**.** Multiple studies have also implicated MALAT1 in drug resistance of various cancer types [65-70]. It would be intriguing to investigate the expression of MALAT1 in response to selinexor exposure in childhood ALL cell lines and patient samples. This raises the question of whether MALAT1 plays a role in determining the sensitivity of selinexor. Furthermore, it is worth noting that MALAT1 is a nuclear-localized lncRNA, while selinexor acts as an inhibitor of nuclear export. The impact of selinexor on MALAT1 expression, function, and regulation remains unknown.

p53, a well-studied tumor suppressor protein, has been demonstrated to govern the expression of several lncRNAs, including lncRNA-p21 [71], PANDA [72], DINO [73], and PURPL (p53 upregulated regulator of p53 levels), [74]. PURPL is an intergenic lncRNA that was identified by RNA sequencing (RNA-seq) in multiple colorectal cancer (CRC) lines [74]. The loss of PURPL has been linked to elevated basal levels of p53 and an impairment of cell growth both *in vitro* and *in vivo* [74]. Recent research has shown that PURPL production is transcriptionally regulated by the transcription factor p53, which tends to be elevated in senescent conditions [75]. Given the dependency of selinexor sensitivity on p53 levels observed in AML [13], it becomes intriguing to investigate the levels of p53-regulated lncRNAs in ALL. In addition, selinexor treatment has been shown to lead to an increased accumulation of p53 inside the nucleus [13]. However, the impact of selinexor on well-established p53-targeted lncRNAs such as lncRNA-p21, PANDA, DINO, and, PURPL, in terms of their expression, function, and regulation, remains largely unknown (Figure 2A). In addition, the role of p53-regulated noncoding RNAs in childhood ALL remains unexplored.

7. Summary and Future Prospectives

Due to the extensive disruption of nuclear transport in cancer and its pivotal involvement at the crossroads of crucial signal transduction pathways, there has been a significant focus on exploring exportins as a prime target in cancer-related research. A plethora of small molecules targeting XPO1 inhibition have been discovered. In preclinical investigations, the administration of selinexor leads to the suppression of XPO1, leading to the accumulation of its target molecules within the nucleus. The potential alternations in mTOR signaling, cancer glucose

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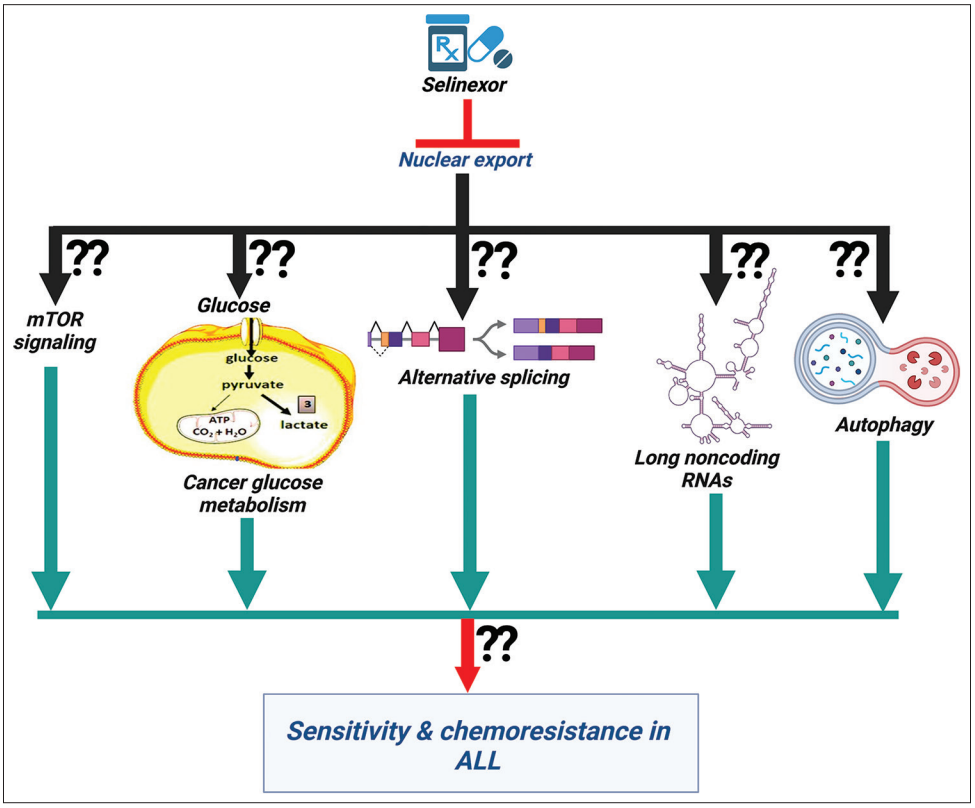

Figure 3. This diagram illustrates the multifaceted therapeutic targets of selinexor and its role in determining the effectiveness of acute lymphoblastic leukemia (ALL) treatments. Red lines represent direct effects, indicating targets directly impacted by selinexor. Black lines signify pathways that can be modulated due to selinexor's inhibition of nuclear export. Light green lines indicate the diverse responses of selinexor in ALL resulting from its interactions with different potential therapeutic targets. The "???" in the figure symbolizes areas that remain unexplored or unanswered.

metabolism, autophagy, lncRNAs expression, and alternative splicing on selinexor treatment could be employed to understand the detailed molecular mechanism of action. In instances where selinexor treatment induces heightened autophagic activity within resistant cellular lineages, the co-administration of selinexor with autophagy inhibitors holds promise for augmenting its efficacy against cell populations manifesting a resistant phenotype. Theoretically, if ALL cell lines or patient samples demonstrate certain dysregulated alternative splicing or lncRNA expression, the application of selinexor treatment in conjunction with a precise inhibitor for lncRNA expression or a splicing regulator could offer a corrective approach for specific aberrant splicing events. Further investigations are required to validate the responsiveness of selinexor in combination with mTORC1 inhibitors on both cell lines and patient samples.

Overall, we anticipate that the knowledge gained from this study can be effectively integrated into the development of innovative therapies targeting childhood ALL. These therapies hold the promise of not only prolonging the lifespan and enhancing the quality of life for ALL patients by postponing the onset of drug resistance but also serving as chemo-preventative agents to decrease the occurrence of ALL. Collectively, such focused research endeavors will significantly enhance our comprehension of the underlying factors driving childhood ALL and offer valuable pathways for its therapeutic management (Figure 3).

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Conflicts of Interest

The authors declare no competing interests.

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