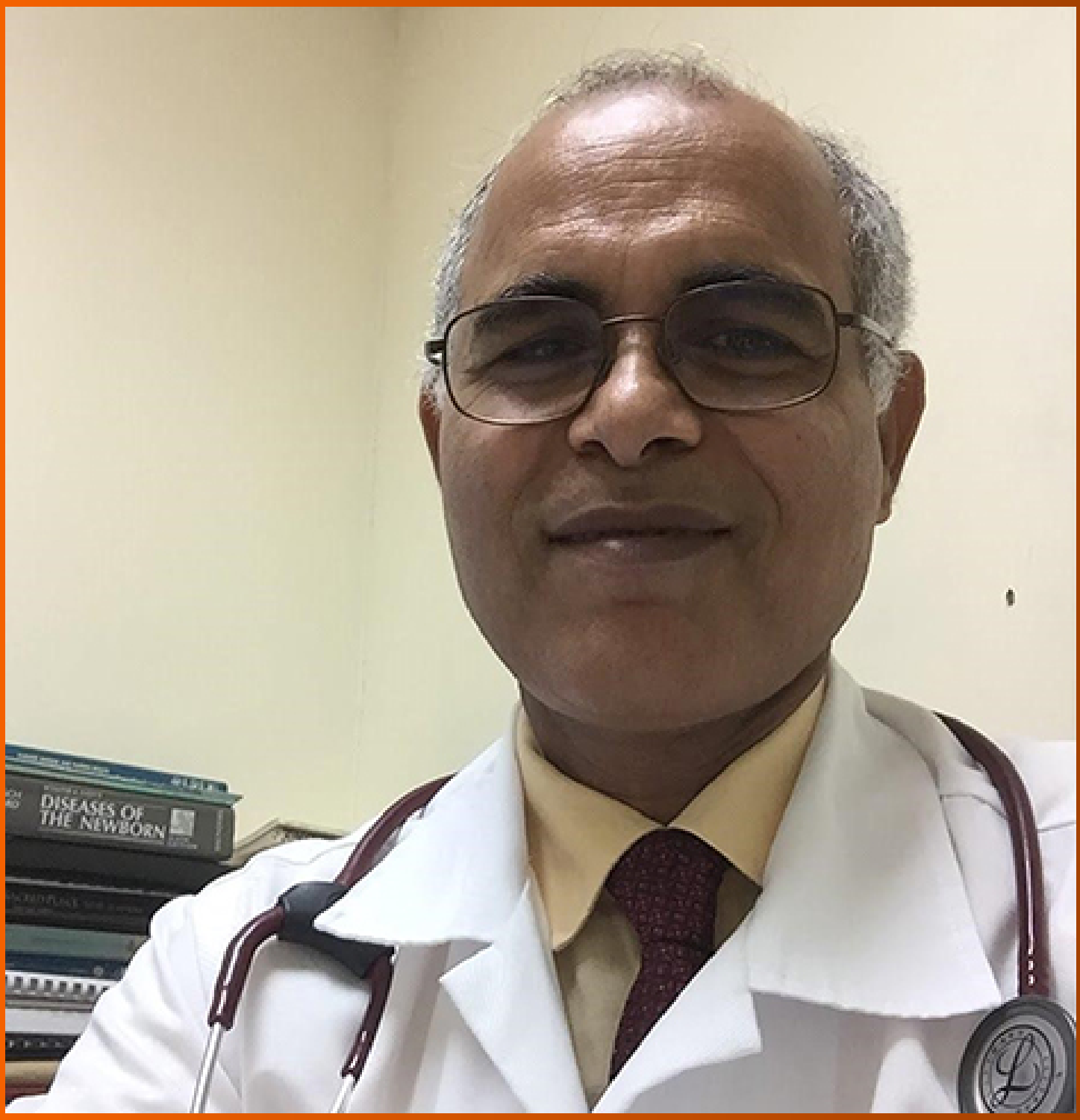
ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2023 June 20; 13(3): 29-165



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World Journal of Methodology

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The *WJM* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Quarterly

EDITORS-IN-CHIEF

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
June 20, 2023

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World J Methodol 2023 June 20; 13(3): 29-45

DOI: 10.5662/wjm.v13.i3.29

ISSN 2222-0682 (online)

REVIEW

Therapeutic potential of curcumin and its nanoformulations for treating oral cancer

Diptasree Mukherjee, Arunkumar Krishnan

Specialty type: Medical laboratory technology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Emran TB, Bangladesh; Liu J, China

Received: December 13, 2022
Peer-review started: December 13, 2022
First decision: February 8, 2023
Revised: March 14, 2023
Accepted: April 14, 2023
Article in press: April 14, 2023
Published online: June 20, 2023

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Abstract

The global incidence of oral cancer has steadily increased in recent years and is associated with high morbidity and mortality. Oral cancer is the most common cancer in the head and neck region, and is predominantly of epithelial origin (*i.e.* squamous cell carcinoma). Oral cancer treatment modalities mainly include surgery with or without radiotherapy and chemotherapy. Though proven effective, chemotherapy has significant adverse effects with possibilities of tumor resistance to anticancer drugs and recurrence. Thus, there is an imperative need to identify suitable anticancer therapies that are highly precise with minimal side effects and to make oral cancer treatment effective and safer. Among the available adjuvant therapies is curcumin, a plant polyphenol isolated from the rhizome of the turmeric plant *Curcuma longa*. Curcumin has been demonstrated to have anti-infectious, antioxidant, anti-inflammatory, and anticarcinogenic properties. Curcumin has poor bioavailability, which has been overcome by its various analogues and nanoformulations, such as nanoparticles, liposome complexes, micelles, and phospholipid complexes. Studies have shown that the anticancer effects of curcumin are mediated by its action on multiple molecular targets, including activator protein 1, protein kinase B (Akt), nuclear factor κ-light-chain-enhancer of activated B cells, mitogen-activated protein kinase, epidermal growth factor receptor (EGFR) expression, and EGFR downstream signaling pathways. These targets play important roles in oral cancer pathogenesis, thereby making curcumin a promising adjuvant treatment modality. This review aims to summarize the different novel formulations of curcumin and their role in the treatment of oral cancer.

Key Words: Oral cancer; Oral squamous cell carcinoma; Analogues; Curcumin; Adjuvant therapy; Nanocurcumin; Curcumin nanoformulations; Curcumin analogues

Core Tip: Oral cancer has a high disease burden worldwide. Oral squamous cell carcinoma is the most predominant subtype of oral cancer. The majority of oral cancers present at an advanced stage and are associated with a poor prognosis. Timely diagnosis and early treatment are critical to achieve a superior outcome. Surgery is the recommended treatment for oral cancer; other treatment modalities are radiotherapy with or without chemotherapy. Curcumin, a plant derivative, is one among the available adjuvant therapies that has been studied for its anticarcinogenic potential in the setting of various cancers. Curcumin has been proven to modulate intracellular signaling pathways that control cancer cell growth, inflammation, invasion, apoptosis, and cell death, with evidence supporting its use in cancer therapy. This review aims to summarize the molecular pathways involved in oral carcinoma pathogenesis, to explore different therapeutic interactions of curcumin, and to highlight the role of novel curcumin formulations in oral cancer treatment.

Citation: Mukherjee D, Krishnan A. Therapeutic potential of curcumin and its nanoformulations for treating oral cancer. *World J Methodol* 2023; 13(3): 29-45
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/29.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.29

INTRODUCTION

Oral cancer, a disease of predominant epithelial origin, is the most common subtype of cancer arising in the head and neck[1]. In 2020, more than 300000 new oral cancer cases were recorded globally[2]. Ninety percent of oral cancer cases are histologically diagnosed as oral squamous cell carcinomas (OSCCs)[3]. Oral cancer is widely prevalent in developing countries of South Central Asia (*e.g.*, India, Sri Lanka, Pakistan) and Melanesia, with a lesser disease burden in developed countries[4]. Oral cancer is the leading cause of death due to cancer in the Indian male population[5]. High incidence rates of oral cancer have been linked to alcohol consumption, tobacco smoking, betel nut chewing, and human papillomavirus (HPV) infection[6]. Despite diagnostic and therapeutic advances, the global 5-year survival rate remains less than 50%[7].

The primary treatment of oral cancer is based on the cancer stage. Surgery is the mainstay of multimodal therapy, which also includes radiotherapy and systemic treatment (chemotherapy and/or targeted agents)[8]. Chemotherapy has proven to increase treatment efficacy and improve overall survival, but has significant adverse effects and the potential for development of drug resistance[9]. As such, combination of these therapies with an adjuvant treatment to bolster their efficacy is urgently needed.

Among adjuvant therapies is curcumin, a phytochemical isolated from the turmeric plant *Curcuma longa*. Curcumin has been reported in a plethora of studies to have anti-infectious, antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, thrombo-suppressive, anti-arthritic, chemopreventive, and anticarcinogenic properties[10]. Studies have shown the therapeutic role of curcumin in various cancers, including oral cancer[11]. Curcumin acts on numerous molecular targets, including signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP-1), protein kinase B (PKB also known as Akt), Notch 1, nuclear factor κ-light chain enhancer of activated B cells (NF-κB), Wnt, mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), and respective downstream signaling pathways, which are known to play key roles in oral cancer pathogenesis[10,12].

The hydrophobic nature of curcumin leads to poor bioavailability, and the sensitivity of soluble curcumin in physiological pH has limited its use in clinical practice[13]. However, nanotechnology-based techniques have made possible various novel formulations of curcumin such as liposomes, nanoparticles, micelles, phospholipid complexes, and analogues, to improve its tissue-level absorption and increase its pharmacological efficacy[14]. Studies have shown favorable results with the use of nano-formulated curcumin in the setting of epithelial cancers. Thus, it follows that curcumin may have a therapeutic role in oral cancer treatment as an adjuvant[15-17]. The present review aims to summarize the key properties of curcumin and its novel formulations, along with their role in oral cancer treatment.

ORAL CANCER

Oral cancer comprises neoplasms affecting any region of the oral cavity. The oral cavity is divided into distinct anatomic subsites, including lip, oral tongue, floor of the mouth, buccal mucosa, upper and lower gingiva, retromolar trigone, and hard palate[18]. However, 90% of oral cancers are histologically diagnosed as OSCCs[3]. OSCCs commonly present as nonhealing ulcers or growths. Early in the disease, lesions can appear as flat, discolored areas (*i.e.* erythroplakia or leukoplakia)[19]. Invasion of surrounding tissues can present with neck masses, trismus, referred ear pain, or specific sensory changes[20]. In cancer of the lip, there is often an exophytic, crusted lesion invading the underlying muscle with tissue damage in the adjacent lip[21]. Oral cancers are often diagnosed late due to an asymptomatic phase with fast progression and early metastasis[22]. Furthermore, the staging of oral cancer plays a significant role in survival rate, with early-stage (I and II) and advanced-stage (III and IV) lesions having a 5-year survival rate of 80% and 50% or less, respectively[22].

There are multiple pathways involved in oral carcinogenesis leading to genetic mutation (*e.g.*, H-ras, K-ras), gene deletions (*e.g.*, loss of chromosome 9p21 or 3p), promoter methylation (*e.g.*, p16, Ras association domain family member 1), amplification of oncogenes and oncoproteins (*e.g.*, EGFR, myc, bcl-2, ras, raf, stat-3, or cyclin D1) and inactivation of tumor suppressor genes (*e.g.*, p53)[23].

PROPERTIES OF CURCUMIN

Curcumin is a yellow spice derived from the roots (rhizomes) of *Curcuma longa*, commonly known as turmeric[24]. Turmeric contains curcuminoids, comprising curcumin, demethoxy curcumin (DMC), and bis-demethoxycurcumin (BDMC)[25]. In 1910, the principal ingredient of curcumin was identified by Gupta *et al*[26] as diferuloylmethane. Curcumin is known as 1, 7-bis (4-hydroxy-3-methoxy phenyl)-1, 6-heptadiene- 3, 5-dione (1E-6E) by International Union of Pure and Applied Chemistry nomenclature. It has a molecular weight of 368.4 g/mol and a melting temperature of 183 °C[27].

Curcumin contains 2 aromatic ring systems with o-methoxy phenolic groups linked with α- and β-unsaturated β-diketone moiety (Figure 1)[28]. The absorption bands of curcumin exist in the visible spectrum (410 nm-430 nm) and the ultraviolet spectrum (250 nm–270 nm)[14]. At 488 nm, curcumin is excited by lower fluorescent yield emission in the 500 nm-530 nm range, which can be detected by flow cytometry and confocal microscopy[29]. Curcumin is insoluble in water and readily soluble in polar solvents with keto-enol tautomerism[30]. The keto-form predominates in acid or neutral solutions, with the enol-form being predominant in alkaline solutions[31].

The bioavailability of curcumin is around 1% according to various animal studies, suggesting a requirement of high doses of curcumin (3600 mg to 12000 mg) to achieve beneficial effects[32]. It is known that curcumin's solubility in water (0.0004 mg/mL at pH 7.3) is poor, giving rise to challenges with oral administration[33]. One study has shown that curcumin has no toxic effect in patients with colorectal cancer when its oral dose is at least 3600 mg[34]. Curcumin undergoes rapid metabolism in the liver and gets excreted in the feces[35]. Curcumin is transformed into dihydrocurcumin and tetrahydrocurcumin (THC) and consequently converted into glucuronide conjugates[36,37]. In intestinal mucosa, kidney, and liver, the conjugative enzyme activity for glucuronidation and sulfation of curcumin has been discovered[37,38]. Another study demonstrated that a considerable portion of orally administered curcumin was conjugated to glucuronide in the intestine; later, the conjugated compound entered the portal vein and underwent additional conjugation to form glucuronide/sulfate metabolites of curcumin in the liver[38].

Curcumin, having a vast range of effects on various human diseases, plays an anti-tumorigenic role in different cancers by affecting multiple pathways of cancer progression[10-12]. In addition, it has been shown to have different effects on normal cells *vs* cancer cells, including a higher uptake by cancer cells [39]. The anticancer effects of curcumin are predominantly mediated through its regulation of various transcription factors, growth factors, inflammatory cytokines, protein kinases, and other oncogenic molecules, as summarized in Figure 2[10,11,33]. Curcumin metabolites (THC, hexahydrocurcumin, and octahydrocurcumin) also have anticancer properties[40,41]. However, the major factor restricting the use of curcumin as a novel chemotherapeutic agent is reduced bioavailability which is attributed to its poor absorption, rapid degradation, fast metabolism, and systemic elimination. Notably, as an anti-cancer drug, curcumin should be administered in a sufficiently high concentration; however, at these concentrations, patients have shown intolerance to bulk doses of the substance[33]. Using an advanced delivery system to increase the bioavailability of curcumin with satisfactory parenteral administration is the most promising solution to these challenges.

EFFECTS OF CURCUMIN ON ORAL CANCER

Curcumin is a potent agent that inhibits cell growth and deoxyribonucleic acid (DNA) synthesis in oral cancer cells[41]. Treatment with curcumin promotes the cell cycle's G(2)/M phase arrest, accompanied

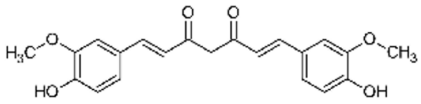

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Figure 1 Chemical structure of curcumin.

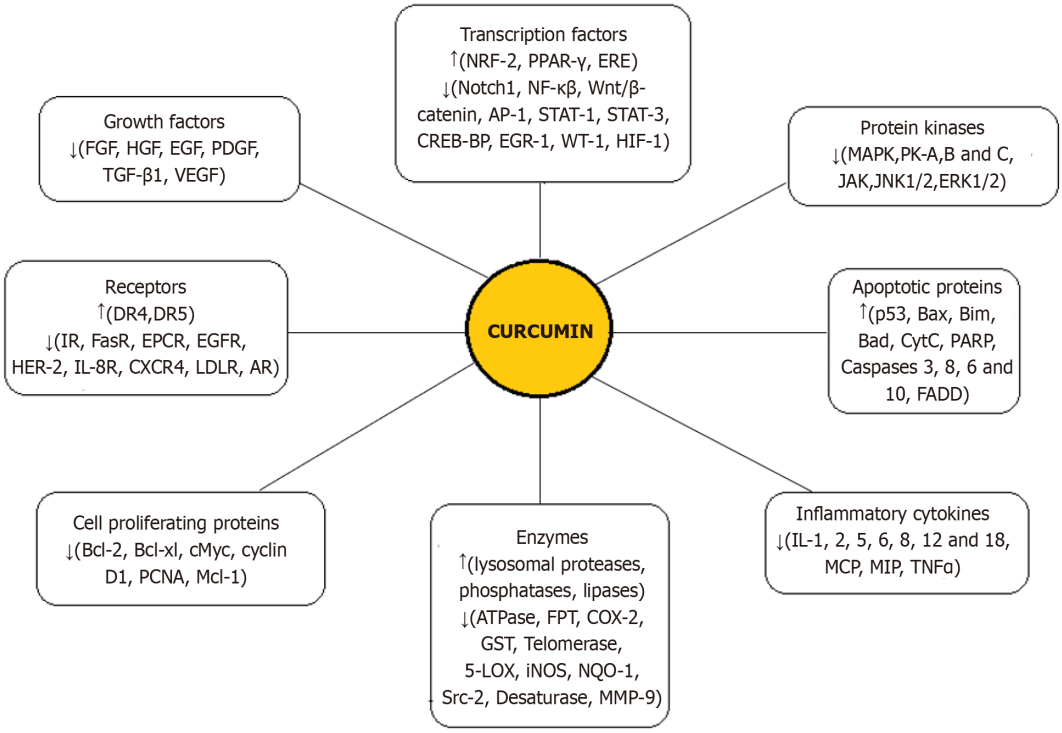

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Figure 2 Effects of curcumin on multiple molecular targets involved in carcinogenesis. ↑: Upregulated; ↓: Down regulated. AP-1: Activator protein 1; AR: Androgen receptor; CREB BP: CREB Binding protein; CXCR: Chemokine receptor; DR4, DR5: Death receptor 4, 5; EGF: Epithelial growth factor; EGFR: Epithelial growth factor; EPCR: Endothelial cell protein C receptor; ERE: Estrogen response element; FADD: FAS-associated death domain; Fas R: Fas receptor; FGF: Fibroblast growth factor; FPT: Farnesyl protein transferase; GST: Glutathione S transferase; HGF: Hepatocyte growth factor; HIF 1: Hypoxia inducible factor 1; iNOS: Inducible Nitric oxide synthase; IR: Insulin receptor; JNK: Jun N-terminal kinase; LDLR: Low density lipoprotein receptor; MAPK: Mitogen activated protein kinase; MCP: Monocyte chemo-attractant protein; MIP: Macrophage inflammatory protein; MMP: Matrix metalloproteinase; NQO-1: NAD(P)H dehydrogenase (quinone) 1; NRF 2: Nuclear factor erythroid 2-related factor 2; PARP: Poly ADP- Ribose polymerase; PCNA: Proliferating cell nuclear antigen; PDGF: Platelet derived growth factor; PK: Protein kinase; PPAR γ: Peroxisome proliferator-associated receptor γ; STAT: Signal transducer and activator of transcription; TGF β 1: Transforming growth factor β 1; TNF-α: Tumor necrosis factor α; VEGF: Vascular endothelial growth factor.

by a decrease in cyclin B/cyclin-dependent kinase 1 and cell division cycle 25C protein levels. It induces apoptosis of oral cancer cells *via* reduction of Bcl-2 levels, reduction in mitochondrial membrane potential, promotion of the active forms of caspase-3, and the release of apoptosis-inducing factor (AIF) and endonuclease G from mitochondria[42]. Curcumin and curcuminoids like DMC and BDMC have shown autophagic and apoptotic activity[43,44].

Studies have shown that curcumin significantly inhibits the carcinogen-activating enzyme cytochrome P450 family 1 subfamily A member 1, which mediates benzo(a)pyrene diol bioactivation in both OSCC cells and oral mucosa[45]. Arecoline exposure is another significant risk factor for the development of oral cancer, and treatment with curcumin markedly inhibits arecoline-induced Snail expression[46]. One study showed that administration of curcumin at 100 mg/kg for 12 wk in a rat model with 4-nitroquinolone-1-oxide (4-NQO)-induced oral cancer markedly decreased the expression of proliferating cell nuclear antigen, anti-apoptosis markers (*e.g.*, Bcl-2), suppressors of cytokine signaling 3 and 1, and STAT3. It also minimized cellular atypia and reduced expression of vimentin, E-cadherin, N-cadherin, and TWIST1, which represent epithelial-mesenchymal transition (EMT) events [47]. Combining local and systemic C3 complex (a purified mixture of curcumin, BDMC, and DMC) effectively targets cancer cell proliferation. This combination inhibits 4NQO-induced tumorigenesis *via* modulation of fibroblast growth factor-2/fibroblast growth factor receptor-2[48]. Moreover, curcumin inhibits the activation and expression of host transcription factors AP-1 and NF-κB, which bind to a cis-regulatory region of the HPV genome. This effect is concentration- and time-dependent, leading to the

suppression of HPV16/E6 transcription and the subsequent prevention of oral carcinogenesis[49]. Curcumin triggers the activation of p38, which then interacts with binding elements in insulin-like growth factor binding protein-5, leading to the activation of the transcription factor CCAAT/enhancer binding protein α (C/EBPα). This also results in the suppression of oral carcinogenesis[50].

Additionally, curcumin reduces oral cancer cell viability and invasion by downregulating Notch 1 and NF-κB[12]. It also induces G2/M phase cell cycle arrest in a dose-dependent fashion by inhibiting the phosphorylation of EGFR and its downstream signaling molecules Akt, extracellular signal-regulated kinase (ERK1/2), and STAT3[12]. Treatment of oral cancer cells with curcumin, BDMC, and DMC leads to the production of reactive oxygen species (ROS), activation of caspase-8, -9, and -3, a decrease in the levels of matrix metalloproteinases (MMP), the release of AIF, and an alteration in the expressions of EGFR, PI3K, p-AKT, NF-κB, AMP responsive protein kinase, and MAPK[44]. In an *in vivo* OSCC model, curcumin has also been observed to suppress the expression of cyclo-oxygenase-2[51].

The oncogenic microRNA miR-31 is upregulated in OSCC, and curcumin downregulates the expression of this molecule in OSCC, leading to an attenuation of AKT activation and downregulation of C/EBPβ[52]. Moreover, curcumin inhibits oral cancer cell proliferation by upregulating miR-9 expression in a dose-dependent manner and suppressing Wnt/β-catenin signaling[53]. Furthermore, curcumin can also enhance the antitumor immune response by inhibiting the expression of programmed cell death ligand 1 and pSTAT3 leading to an increase in CD8+ T-cells and a decrease in T regulatory cells and myeloid-derived suppressor cells[54]. Cancer-associated fibroblasts (CAFs) are activated fibroblasts in the tumor microenvironment that play a critical role in cancer development[55]. Curcumin can reverse the phenotype of CAFs to that of peri-tumor fibroblast-like cells by downregulating the expression of α-smooth muscle actin (a unique marker for CAFs) and inhibiting the secretion of pro-carcinogenic cytokines such as transforming growth factor-β1, MMP2, and stromal cell-derived factor-1[56]. This results in decreased cancer invasion, as evidenced by a reduced release of EMT mediators in treated CAFs and reversal of EMT in treated tumor cells[57].

Hepatocyte growth factor (HGF) signaling plays an important role in EMT induction and contributes to cancer cell invasion and metastasis[58]. Curcumin inhibits HGF-induced EMT and cell motility in oral cancer cells, acting on HGF receptor c-Met and blocking the downstream activation of the pro-survival ERK pathway[59]. It also decreases proliferation in cell lines with mesenchymal characteristics and causes cell death with a dose-dependent decrease in cell–cell adhesion[60]. Curcumin treatment has been found to suppress MMP-2, MMP-9, and MMP-10, which are linked to cancer cell migration and invasion in oral cancer[61,62].

Studies have shown that curcumin can enhance the efficacy of standard platinum-based chemotherapy for treating oral cancer, resulting in significant tumor growth suppression in cell lines and mouse xenografts[63,64]. These results highlight the potential of using subtherapeutic doses of cisplatin in combination with curcumin to effectively suppress tumor growth and minimize cisplatin's toxic side effects.

Furthermore, curcumin has a radio-sensitizer effect in OSSC and exhibits synergistic antiproliferative activity when combined with cetuximab (an anti-EGFR monoclonal antibody) in cisplatin-resistant oral cancer cells[65,66]. A study of combinations of curcumin and metformin demonstrated a reduction in tumor volume and improvement of overall survival of experimental animals, as evidenced by downregulation of cancer stem cell markers CD44 and CD133[67]. Another study showed that olaparib (a poly-ADP ribose polymerase inhibitor), when combined with curcumin *in vitro* and *in vivo* (mouse model), causes DNA damage, inhibits cell proliferation and topoisomerase activity, reduces the expression of base excision repair components, induces apoptosis, and decreases tumor volume[68].

CLINICAL TRIALS USING CURCUMIN FOR ORAL LESIONS

Multiple clinical trials are ongoing or have been completed investigating the efficacy of curcumin against human diseases including oral pathologies. Kuriakose *et al*[69] conducted a study on oral leukoplakia, a potentially malignant oral cavity lesion with no effective treatment available. In this study, subjects with oral leukoplakia underwent a randomized, double-blinded, placebo-controlled phase IIB clinical trial with curcumin. Clinical and histological response assessments showed a significantly better outcome with curcumin treatment. Notably, the therapy was well-tolerated, and a significant and long-lasting clinical response was observed after treatment with curcumin at a dose of 3.6 g administered over 6 mo[69]. Furthermore, recent studies have shown that topical curcumin effectively treats oral mucositis[70]. Currently, a phase II randomized trial (double-blind, placebo-controlled) is ongoing to assess the therapeutic effects of curcumin in patients with stage III-IV head and neck cancer and cancer-associated anorexia-cachexia[71].

NOVEL FORMULATIONS OF CURCUMIN IN ORAL CANCER TREATMENT

Curcumin analogues

Several investigations have attempted to improve curcumin's therapeutic effectiveness and pharmacokinetic profile by developing new analogues[72,73]. The synthetic curcumin analogues that have been studied include the EF series (EF24, EF31, and UBS109), the FLLL series (FLLL11, FLLL12, FLLL31, FLLL32, and FLLL62), the GO-Y series, the 4-arylidene curcumin analogues AC17, B19 [(1E,4E)-1,5-bis(2,3-dimethoxy phenyl) penta-1,4-dien-3-one], CDF (difluorinated curcumin), and 4-[3,5-bis(2-chlorobenzylidene-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid] CLEFMA, the diarylidenylpiperidones series, DM-1 (sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2- methoxy phenolate), and dimethoxycurcumin[74]. In addition, some of these new analogues have been reported to have more potent anticancer properties than curcumin, and may have more beneficial antioxidant, antimalarial, and anti-inflammatory properties than the parent compound[75-79].

A few analogues including EF24, CDF, and FLLL12, exhibit enhanced physicochemical properties such as improved solubility and bioavailability, allowing them to overcome the limitations of curcumin [80,81]. In addition, some analogues have shown to increase the efficacy of chemotherapeutic agents and to overcome issues of resistance when combined therapy is used[82,83]. These analogues have shown promising results in breast, prostate, colon, and head-neck squamous cell cancers[77,84-87]. In a study of oral cancer cells, Chuprajob *et al*[88] found that curcumin analogues with the 1,4,6-trien-3-one function are more potent than the curcuminoid types. Also, structural variations in the analogues enhanced their potency; for example, the meta-oxygen function of the aromatic ring is more potent than those in the ortho and para positions, and the free phenolic hydroxy group is more potent than in the corresponding methyl analogues[88]. Furthermore, some analogues showed fewer toxic effects than curcumin when applied to normal cells[88]. In 2017, Lin *et al*[89] found that EF24 exhibited antitumor activity on CAL-27 oral cancer cells by deactivating the MAPK/ERK signaling pathway.

Several curcumin analogues have been developed in recent years, and most of the analogues have shown mechanisms of action similar to that of curcumin. However, some have unique mechanisms that are not associated with curcumin. For instance, the B19 analogue inhibits thioredoxin reductase 1, leading to ROS-mediated endoplasmic reticulum stress, whereas the AC17 analogue blocks proteasome function by inhibiting the deubiquitinase activity of 19S regulatory particles; neither of these mechanisms are seen with curcumin[90,91]. Further studies are required to evaluate the specific benefits of these inhibition pathways in cancer treatment.

Despite their promising potential, key parameters for the clinical development of many promising analogues remain unknown, and further attention should be given to the study of their pharmacokinetics. To reduce drug-associated toxicities and improve bioavailability, targeted drug delivery through alternative formulation has been gaining attention. Some studies have shown that curcumin analogues can be conjugated to homing moieties to direct their delivery and accumulation at specific sites. Certain homing moieties have been tested, including hyaluronic acid (HA)-targeted nanomicelles, HA dendrimers, folic acid-conjugated CDF, and EF24 conjugated to coagulation factor VIIa to target tissue factor[92-95]. These potential agents have yet to undergo clinical trials and cost-effective production strategies. Further *in vivo* studies can pave the way for clinical trials and future applications.

Nanoformulations of curcumin

Nanotechnology has led the way in developing nanoscale drug delivery systems. Hydrophobic molecules such as curcumin can benefit from improved bioavailability as a result of the surface, small size, quantum size, and quantum tunnel effects of nanoparticles[96,97]. Several novel strategies have been developed to design curcumin nanoparticles as targeted drug-delivery systems, and these have been studied in various disease states, including cancer[33] (Figure 3).

The following is a summary of the different nanotechnology-based drug delivery modalities for potential use with curcumin.

Liposomes: These are spherical, closed phospholipid vesicles that incorporate drugs in the inner aqueous layer and have been widely used to enhance the bioavailability and efficacy of curcumin. In recent years, several liposomal curcumins with polymeric conjugates have been modified to achieve better clinical outcomes[98]. Nanoliposomes have shown properties such as sustained drug release, enhanced tumor targeting, minimized toxicity to healthy cells, and a lower dose[99].

Polymer micelles: These represent an excellent drug delivery system for curcumin, as they can overcome issues with poor solubility, low stability, and poor bioavailability. Encapsulating curcumin within cationic micelles like cetyltrimethylammonium bromide or dodecyl trimethyl ammonium bromide can enhance drug loading capacity, increase water solubility, reduce toxicity, and limit degradation[100]. Nanomicelle curcumin has been shown to prevent and treat oral mucositis caused by head and neck radiotherapy and chemotherapy[101,102].

Polymer nanoparticles: Polymer nanoparticles are another effective drug delivery system, owing to their high biocompatibility and ease of circulation in the bloodstream for longer periods. Synthetic

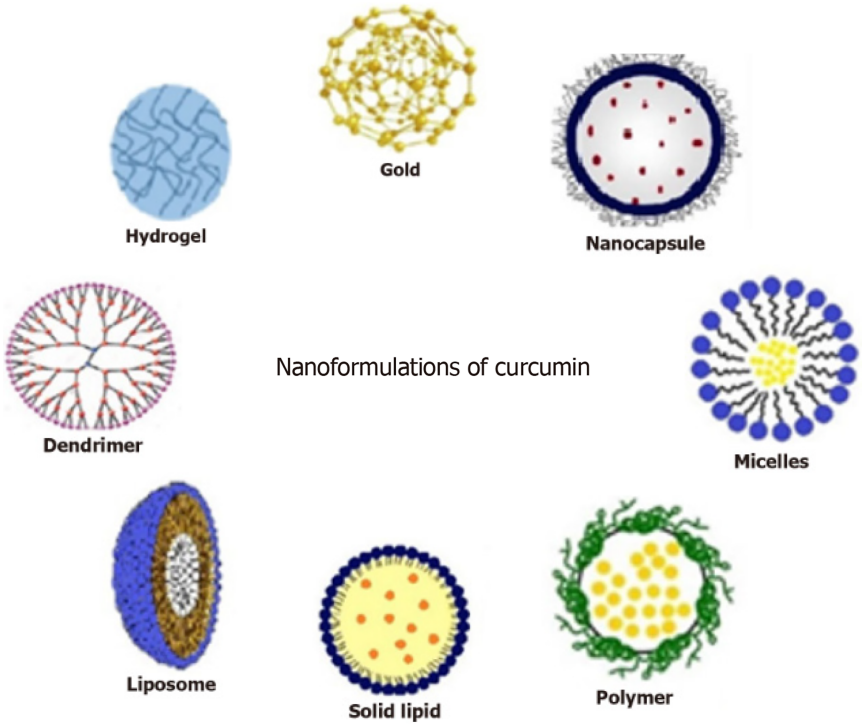

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Figure 3 Nanoformulations of curcumin used in various studies.

polymer conjugates such as chitosan, d,l-lactide-co-glycolide (PLGA), polyethylene glycol (PEG), poly (n-butyl) cyanoacrylate, silk fibroin, N-isopropyl acrylamide, and hydrophobically modified starch are commonly used. Curcumin-loaded nanoparticles using PEG-5000 as a carrier stabilizer for PLGAs have shown results in mouse models of cancer, with higher cellular uptake and induction of apoptosis both *in vitro* and *in vivo*[103].

Solid lipid nanoparticles: Solid lipid nanoparticles consist of natural lipids (*e.g.*, lecithins or triglycerides) that remain solid at 37 °C. These molecules protect labile compounds from chemical degradation and improve bioavailability. Curcumin-loaded solid lipid nanoparticles have shown enhanced cellular uptake and are promising anticancer agents against breast cancer cells *in vitro*[104].

Inclusion complexes: Inclusion complexes are formed of cyclodextrins and cyclic oligosaccharides composed of 6 to 8 glycosyl monomeric units (α-1,4 linked). They are widely used to improve stability, enhance water solubility, increase bioavailability, and reduce bitterness. β-Cyclodextrin is commonly used to form an inclusion complex with curcumin by solvent evaporation or pH shift techniques[105].

Solid dispersions: Solid dispersions have improved curcumin's physicochemical and pharmacokinetic activities. Wet-melting and subsequent freeze-drying are common strategies for preparing crystal and amorphous forms in which curcumin is dispersed in an inert carrier at a solid-state[106].

Magnetic nanoparticles: Drug-loaded magnetic nanoparticles can be directed to cancer-affected tissues under external magnetic fields to offer a targeted drug delivery option. Entrapping curcumin in an Fe3O4-curcumin conjugate with oleic acid or chitosan in the outer shell results in the formation of nano-sized, fluorescent-magnetic, water-dispersible nanoparticles with increased cellular uptake and enhanced bioavailability[107].

Microspheres and microcapsules: This approach encapsulates drugs or molecules like curcumin within polymeric particles to improve efficacy and organ-targeted bioavailability. Particles that have been used include camptothecin, rutin, zedoary oil, andrographolide, and eudragit S-100[108].

Emulsions: Emulsions refer to small-droplet dispersions comprising of oil and water mixtures that are stabilized by surfactant molecules to form interfacial films. These represent a lipid-based drug delivery system possessing numerous advantages, including thermodynamic stability, improved drug dissolution, and increased solubility[109]. High-speed and high-pressure homogenization procedures using triacylglycerol and Tween-20 as emulsifiers produce tiny microemulsion droplets. Incorporating curcuminoids into nanoemulsions has been shown to increase oral bioavailability[110].

Nanogels: Nanogels are 3-dimensional polymer networks with high drug loading capacity, high dispersion stability, targeted drug delivery efficiency, fast drug releasing properties, and increased drug delivery across cellular barriers. Moreover, they are easy to modify chemically. Curcumin has been used as a nanogel for targeted therapy[111].

Nanoparticle curcumin: Nanoparticle curcumin is a pure form of curcumin that is processed into nanoparticles of approximately 200 nm in size without carrier conjugates. *In vitro* and *in vivo* studies have shown that these nanoparticles of curcumin exhibit increased cellular uptake and enhanced anticancer effects due to their size, surface charge, and surface area[112-114].

Niosomes: Niosome nanocapsules are drug carriers composed of non-ionic surfactants that form a bilayered structure with hydrophobic and hydrophilic parts in an aqueous medium. Niosomes offer numerous advantages, including improved pharmacokinetics, drug stability, therapeutic effects, and reduced side effects of the administered drug[115].

Using nanoformulation-based combination therapy has gained popularity as a potent drug delivery system, often overcoming the limitations of conventional therapeutic agents. This delivery system has shown to improve intracellular drug concentrations and enhance the synergistic activity for cancer therapy[116-118]. Specific curcumin novel formulations have been studied for their efficacy in treating oral cancer, with encouraging findings (Table 1)[119,120].

In 2012, Lin *et al*[121] conducted a study to assess the effects of curcumin microemulsion on oral cancer cell lines. They found that exposure to curcumin-containing microemulsions for a brief period produced cytotoxic effects in the cancer cells. However, adding ultrasound enhanced these effects in OSCC-25 cells[121]. This observation is likely attributable to enhanced curcumin delivery to the cell by the fusion of microemulsion droplets with cell membranes or by overcoming transport limitations *via* ultrasound-induced mixing and/or heating. These ingestible microemulsions can be therapeutic in concentration-adjusted doses and have tissue-targeting properties when combined with ultrasound. Studies have shown that curcumin nanoparticles (Cur-NPs) possess significantly greater bioavailability and water solubility than free curcumin[122,123]. In a 2013 study by Chang *et al*[103] investigating CAL27-cisplatin-resistant human oral cancer cells (CAR cells), water-soluble PLGA Cur-NPs enhanced the drug effect. Cur-NPs increased ROS production, upregulated the expression levels of cleaved caspase-3/caspase-9, cytochrome c, apoptotic protease activating factor-1, AIF, and Bax, and downregulated the expression of Bcl-2. Cur-NPs also triggered the intrinsic apoptotic pathway by regulating the function of multiple drug resistance proteins 1 (MDR1) and the production of ROS in CAR cells[103]. Previous studies have also reported that MDR1 (a cell surface permeability glycoprotein) is a significant target of Cur-NPs[124,125]. In this study, treatment with Cur-NPs decreased MDR1 mRNA and protein levels in CAR cells, indicating the induction of CAR cell apoptosis, representing a potential treatment for cisplatin-resistant oral cancer.

Curcumin is phototoxic in the presence of oxygen[126,127]. Singh *et al*[128] demonstrated the use of organically-modified silica nanoparticles (SiNps) as a vehicle for the delivery of curcumin in human oral cancer cells. The results showed improved uptake of curcumin and phototoxicity in cancer cells. Incubation time-dependent cytotoxicity, inhibition of NF-κB activity, suppression of NF-κB-regulated proteins involved in invasion (MMP-9), angiogenesis (*via* vascular endothelial growth factor), and inflammation (tumor necrosis factor α) were observed with curcumin-SiNp. These results suggest that the curcumin–SiNp formulation has significantly improved anti-cancer effects over free curcumin in the dark and upon exposure to light[128]. These findings are likely the result of increased oxidative stress induced in the cancer cells upon visible light exposure in the presence of oxygen. The curcumin–SiNp formulation also enhances the stability of curcumin at physiological pH and increases its aqueous solubility.

In 2015, Mazzarino *et al*[129] conducted a study on the effect of mucoadhesive polycaprolactone (PCL) nanoparticles coated with chitosan and loaded with curcumin as a treatment for oral cancer. This study used the nanoprecipitation method to prepare the chitosan-coated PCL nanoparticles with curcumin loading[130]. The nanoparticles showed mucoadhesive properties, as evidenced by interaction with the glycoprotein mucin through electrostatic forces. *In vitro* studies showed that these novel curcumin nanoparticles significantly decreased the viability of SCC-9 human oral cancer cells by inducing apoptosis[129]. The study also suggested that drug retention in the mucosa after treatment with chitosan-coated curcumin-loaded nanoparticles could be helpful for local therapy in numerous diseases.

Gold nanorods (GNRs) are known for their photothermal activity and inherent tumor-targeting properties[131]. In 2018, Zhu *et al*[132] developed a novel system for combined plasmonic photothermal therapy and chemotherapy using the tumor microenvironment and near-infrared responsive gold nanorod-drug conjugates (Au NR@Curcumin). This study tested the antitumor effects of Au NR@Curcumin on human lung, liver, and oral carcinoma cells and found that it showed more potent cytotoxicity than the free drug. Additionally, oral cancer cells demonstrated cell cycle S phase arrest. The study suggested that Au NR@Curcumin could be effective at inducing instant photothermal killing of the cancer cells, even at a low irradiation power density[132]. In 2020, Ghosh *et al*[133] developed a multimodal nanoconjugate by functionalizing the GNR surface with a cytotoxic nucleoside [5-fluoro-2′-

Table 1 Oral cancer studies with novel curcumin formulations

Curcumin formulations	Study type	Results	Ref.
Liposomes	*In vitro*	Size of vesicle attributed to enhanced release of curcumin and cytotoxicity in the SCC9 cells	Gosangari *et al*[119], 2012
Cur microemulsion	*In vitro*	Damaged and ruptured OSCC 25 cells, cell death enhanced by ultrasound	Lin *et al*[121], 2012
PLGA Cur- NP	*In vitro*	Increased ROS production, upregulated caspase-3/caspase-9, cytochrome c, Apaf- 1, AIF, Bax, downregulated Bcl-2	Chang *et al*[103], 2013
Cur-SiNP	*In vitro*	Cytotoxicity by inhibition of NF-κB activity, suppression of MMP-9, angiogenesis (VEGF), and inflammation (TNF-α) in the dark as well as on exposure to light	Singh *et al*[128], 2014
Trienone analogues of curcuminoids	*In vitro*	1,4,6-trien-3-one analogue has more potent cytotoxicity than the curcuminoid type function in oral cancer cells	Chuprajob *et al*[88], 2014
Cur-loaded chitosan-coated PCL nanoparticle	*In vitro*	Mucoadhesive properties decreased SCC9 cell viability by inducing apoptosis	Mazzarino *et al*[129], 2015
Cur analogue EF24	*In vitro*	Anticancer activity on CAL-27 cancer cells *via* deactivation of the MAPK/ERK signaling pathway	Lin *et al*[89], 2017
Gold nanorod-drug conjugates (Au NR@Curcumin)	*In vitro*	Cancer cell cycle S phase arrest, the photothermal killing of the cancer cells	Zhu *et al*[132], 2018
NP Cur	*In vitro*	Chemoprotective nature of Cur towards 5-FU induced cell toxicity, antioxidant effect, altered expression of apoptotic proteins Bcl2 and Bax	Srivastava *et al*[112], 2018
	In vitro	Chemo-adjuvant property of NP Cur with Cetuximab	Mukherjee *et al*[136], 2022
	In vitro	Cytotoxicity *via* apoptosis, luminescence property of NP Cur acting as a theranostic agent	Essawy *et al*[135], 2022
PGA- Gef/Cur NP	*In vitro*	NPs internalized into SAS cells, decreased cell viability, and induced apoptotic cell death via caspase-3,9 and mitochondria-dependent pathway	Lai *et al*[134], 2019
	In vivo	Suppressed tumor size compared to the free Gef/Cur-treated group	
Mucoadhesive nanostructured Cur	*In vitro, Ex vivo*	Improved cytotoxicity, enhanced Cur release, and permeation while selectively targeting cancer cells	Ferreira *et al*[137], 2019
DNA Cur complex	*In vitro*	Enhanced cellular delivery of Cur increased cancer cell cytotoxicity in combination with FdU nucleotides	Ghosh *et al*[133], 2020
Nano micelle Cur	*In vitro*	Improved controlled-release of Cur, enhanced cellular uptake, apoptotic cell death by changing the mitochondrial membrane potential	Kumbar *et al*[120], 2022
Cur-loaded noisome	*In vitro*	Significant cytotoxicity compared to free curcumin after 24 h	Fazli *et al*[138], 2022
	In vivo	Injection use (systemic) was shown to be more effective than the use of mouthwash (topical)	

AIF: Apoptosis-inducing factor; Apaf-1: Programmed cell death ligand 1; CAR cells: Cisplatin-resistant human oral cancer cells; Cur: Curcumin; FdU: 5-fluoro-2'-deoxyuridine; 5-FU: 5-fluorouracil; MAPK: Mitogen-activated protein kinase; MDR 1: Multiple drug resistance proteins 1; MMP-9: Matrix metalloproteinase 9; Nano-CU: Nanoparticle of curcumin; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; NP: Nanoparticle; OSCC: Oral squamous cell carcinoma; PCL: Polycaprolactone; PGA- Gef/Cur NP: γ-polyglutamic acid-coated Gefitinib and curcumin-loaded nanoparticles; PLGA: D,l-lactide-co-glycolide; ROS: Reactive oxygen species; SCC: Squamous cell carcinoma; SiNp: Silica nanoparticle; TNF-α: Tumor necrosis factor α; VEGF: Vascular endothelial growth factor.

deoxyuridine (FdU)]-containing DNA hairpin followed by hydrophobic complexation of curcumin. This study showed that curcumin could be noncovalently complexed into small DNA hairpins for enhanced cellular delivery. This system caused increased cytotoxicity in SCC 131 oral cancer cells when administered in combination with FdU nucleotides, demonstrating its potential for advanced cancer therapy[133].

Several studies have investigated the potential of curcumin nanoparticles in combating oral cancer. Srivastava *et al*[112] found that Nano-CU, a curcumin nanoparticle, exhibited chemoprotective

properties against 5-fluorouracil-induced toxicity in oral cancer cells. Nano-CU was found to have an antioxidant effect, and altered the expression of apoptotic proteins Bcl-2 and Bax in treated cells[112]. Another study by Lai *et al*[134] explained the anticancer properties of gefitinib (Gef) and curcumin-loaded NPs in human oral cancer SAS cells *in vitro* and SAS cell xenografted tumors *in vivo*. The results indicated that γ-polyglutamic acid-coated (PGA)-Gef/Cur NPs could be internalized into SAS cells and significantly decrease the total cell viability. Both free Gef/Cur and γ-PGA-Gef/Cur NPs induced apoptotic cell death *via* caspase-3, caspase-9, and mitochondria-dependent pathways. *In vivo* studies showed that γ-PGA-Gef/Cur NPs significantly suppressed tumor size compared to the free Gef/Cur-treated group[134]. In 2022, Essawy *et al*[135] developed nanoparticle curcumin using a more straightforward and cost-effective solvent-antisolvent precipitation technique and studied its effect on oral cancer cells. This study found promising cytotoxic results *via* apoptosis in contrast to the necrotic effect observed using doxorubicin in the cell lines. The authors also reported the observed luminescence of the nanoparticle curcumin, qualifying it as a double theranostic agent[135]. In another study, co-treating oral cancer cells with nanoparticle curcumin (approximately 200 nm size) and cetuximab showed higher cytotoxicity than cetuximab alone[136]. The above mentioned studies highlight the potential chemo-adjuvant role of curcumin nanoparticles in combating oral cancer.

In 2019, Ferreira *et al*[137] aimed to develop nanostructured gel formulations containing curcumin for oral cancer therapy. The authors showed that the use of this novel curcumin led to rapid incorporation and localization in the hydrophobic portion of nanometer-sized polymeric micelles, resulting in increased retention after application in the oral cavity. Cytotoxicity testing showed that the formulation selectively targeted cancer cells moreso than healthy cells. Therefore, these systems may improve the physicochemical characteristics of curcumin by increasing its release and permeation and enhancing its cancer cell-targeting properties[137]. Recently in 2022, Fazli *et al*[138] found that curcumin-loaded niosomes significantly inhibited the growth and necrosis of oral cancer cells compared to free curcumin. Histopathological specimens from rats with induced oral cancer showed that niosome curcumin treatment effectively inhibited cancer growth. The authors also highlighted that the injectable curcumin-loaded niosome (*i.e.* for systemic use) was more effective than the mouthwash form of application (*i.e.* for topical use)[138]. In light of these promising findings, future studies should be designed to explore the outcomes of novel curcumin formulations in preclinical and clinical trials.

CHALLENGES AND FUTURE DIRECTIONS

Oral cancer is a highly malignant disease with a poor 5-year survival rate and limited treatment options, underscoring the importance of adjuvant therapy. Curcumin, known for its pleiotropic effects and potential therapeutic benefits, has shown promise as a treatment choice for patients with cancer. This molecule has shown improvement in the efficacy of current cancer therapeutics, including overcoming the resistance of cancer cells to chemo-radio therapy. However, several clinical and practical challenges need to be addressed before curcumin can be incorporated into regular clinical practice. The purity of the curcumin compound significantly affects its activity, and is of primary importance when used in studies or trials[139]. In addition, body tissue distribution and uptake of curcumin, which account for its biological activity, need better understanding[31]. Clinical trials with curcumin have faced various challenges, such as high metabolic instability, poor aqueous solubility, inadequate focalization, complex pharmacokinetic profile, and poor patient adherence[140].

Nanotechnology-based formulations and analogues have shown potential in overcoming the poor bioavailability issue of curcumin by improving its stability, increasing its cellular uptake, and offering controlled release. However, these formulations often lack tissue specificity. Although the various novel nano-formulations of curcumin show remarkable anti-neoplastic, theranostic, and chemo-adjuvant properties, there are technical challenges in drug development, particularly the need to regulate the size of curcumin nanoparticles for drug delivery applications. In addition, these processes are expensive and have yet to be commercialized. The effects of newer delivery systems, such as polymer nanoparticles and liposomes, on the therapeutic efficacy of curcumin need to be further investigated; while these have been shown to enhance curcumin bioavailability, the possibility of off-target toxicity has not been thoroughly studied[141]. Curcumin has shown cytotoxic and cytoprotective effects at different doses and concentrations in various cancer studies[112,135,142,143]. These findings need consideration in preclinical and clinical trials investigating newer curcumin formulations. Furthermore, the wide range of research variability in human cancer studies using these novel curcumin formulations, such as differences in study design, drug design, sample size, and route of administration, also make it difficult to conclude which formulation has the best overall pharmacokinetic properties[140].

CONCLUSION

In conclusion, encouraging findings from various studies using novel curcumin formulations indicate the need for extensive preclinical and clinical research to shed light on their pharmacokinetics, biocom-

patibility, toxicity, and dose regimens in normal and disease conditions in order to incorporate these agents in cancer treatment strategies. Systematic efforts must focus on identifying a potential curcumin formulation suitable for use in clinical trials. Collaboration between clinicians, translational scientists, medicinal chemists, and pharmacologists is necessary to advance these agents toward clinical use as oral cancer therapeutics.

FOOTNOTES

Author contributions: Mukherjee D conceptualized and wrote the original draft; Krishnan A reviewed and edited the draft; and all authors revised the manuscript for important intellectual content and approved the article's final version.

Conflict-of-interest statement: All authors report no relevant conflicts of interest for this article.

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S-Editor: Liu XF
L-Editor: Filipodia
P-Editor: Ju JL

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 46-58

DOI: 10.5662/wjm.v13.i3.46

ISSN 2222-0682 (online)

REVIEW

Evolving utility of exosomes in pancreatic cancer management

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Feliu J, Spain; Gao W, China; Zhao CF, China

Received: March 22, 2023
Peer-review started: March 22, 2023
First decision: April 17, 2023
Revised: May 2, 2023
Accepted: May 31, 2023
Article in press: May 31, 2023
Published online: June 20, 2023

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Abstract

Despite the development of newer oncological treatment, the survival of patients with pancreatic cancer (PC) remains poor. Recent studies have identified exosomes as essential mediators of intercellular communications and play a vital role in tumor initiation, metastasis and chemoresistance. Thus, the utility of liquid biopsies using exosomes in PC management can be used for early detection, diagnosis, monitoring as well as drug delivery vehicles for cancer therapy. This review summarizes the function, and clinical applications of exosomes in cancers as minimally invasive liquid biomarker in diagnostic, prognostic and therapeutic roles.

Key Words: Pancreatic cancer; Exosomes; Biomarker; Liquid biopsy; Clinical applications; Circulating biomarkers

Core Tip: The determination and identification of biomarkers using liquid biopsy can enable the early detection, monitoring, therapeutic interventions, risk of relapse, therapeutic targets and identification of resistance mechanisms in pancreatic cancer (PC). There has been a recent interest in use of exosomes as biomarker in PC management. Exosomes loaded with multiple diagnostic molecules can be isolated from different body fluids and can be used for making the exosome markers-based liquid biopsy more attractive for initial tumor detection, monitoring, and prognostic assessment of PC.

Citation: Anoop TM, Basu PK, Chandramohan K, Thomas A, Manoj S. Evolving utility of exosomes in pancreatic cancer management. *World J Methodol* 2023; 13(3): 46-58
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/46.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.46

INTRODUCTION

Pancreatic cancer (PC) is associated with poor survival outcome with a 5-year survival of 5%-10%, and a short median survival of 6-8 months after cancer diagnosis[1]. Most individuals diagnosed with advanced disease are symptomatic whereas early stages of the cancer are generally asymptomatic and often undiagnosed. Hence diagnosis is often made after dissemination. Surgery is the only curative treatment[2]. Regrettably, a large number of patients present with unresectable or metastatic disease at the time of diagnosis. Early detection of PC is essential for treatment with curative intent, typically by surgical resection in combination with neoadjuvant or adjuvant chemotherapy and chemo radiation. The majority of patients can have local recurrence or systematic metastasis even after resection. Screening methods for PC often relies on carbohydrate antigen 19-9 (CA 19-9). CA 19-9 demonstrates relatively low sensitivity and specificity in diagnosing PC[3]. Hence a diagnostic test with high sensitivity and specificity and capable of distinguishing PC at early stages from benign diseases is highly recommended. Table 1 shows common circulating biomarkers for PC[4-19]. Comparison of usefulness of various Liquid biopsies used in PC is shown in Table 2[20-30].

BIOGENESIS OF EXOSOMES

There is an emerging role of molecular profiling of liquid biopsies for cancer diagnosis and prognostication. Extracellular vesicles (EVs) derived from various body fluids and serum. There are four subclasses of EV based on different sizes like Exosomes (30-150 nm), Oncosomes (100-1000 nm), Ectoderms (100-1000 nm) and Apoptotic bodies (200-2000 nm). Exosome or Exosomes derived proteins, *etc.* are believed to serve as reliable molecular biomarkers. The circulating vesicles in the blood that originate from tumor cells contains immense proteomic and genetic information to monitor cancer progression, metastasis, and drug efficacy[31-33]. Exosomes were originally introduced during the culture of sheep reticulocytes *in vitro* by Johnstone *et al*[34]. Exosomes are EVs that are endosomal in origin with a diameter of 40-160 nm (average, 100 nm). The formation of cancer cell derived exosomes is depicted in Figure 1. Initially, exosomes are formed by inward invagination of plasma membrane to form an early endosome. These endosomes form nano-sized vesicles resulting in formation of multi vesicular body (MVB) that contain intraluminal vesicles which contain cytoplasmic components including various nucleic acids and soluble proteins[35]. These intra luminal vesicles are released to the extracellular environment by fusing the MVBs with the plasma membrane. Then with the help of exocytosis, exosomes are released in to circulation.

Exosomes contains many molecules like heat shock proteins, RNAs, DNAs, GTPase, CD63, CD81, CD9, CD82, cholesterol, sphingomyelin, and ceramides. Exosomes facilitate both the transport of essential substances like nucleic acids and proteins into various recipient cells and the communications between cells. The main sources of exosomes are plasma, serum, urine, bile, saliva and breast milk. The secreted exosomes have various cellular functions in cell-to-cell interactions and might be pivotal in the occurrence and development of tumour progression and metastasis[36]. Exosomes have definite role in inflammation, coagulation, and embryo implantation in pregnancy. However, cancer cells are capable of secreting 10 times than normal cells. Hence tumour derived exosomes can provide vast information on cancer. Furthermore, exosomes are potential surrogates of the original cells, hence they are useful for understanding cell biology.

Oncosomes are tumor derived cells and they contain different oncogenic molecules that can modify the cells to encourage cancer growth. Tumor cell-secreted exosomes are responsible for paracrine signalling during tumor progression, tumor-stromal interactions, proliferative pathway activation, and immunosuppression[37]. Tumor derived exosomes enters the cells by a variety of methods, depending on the cells that secrete them and the target cells. Metastatic breast cancer derived exosomes use transcytosis to cross the brain endothelial cells, while the "CDC42-dependent endocytic pathway" was utilized to enter astrocytes during brain metastasis[38].

EXOSOMES IN INITIATION OF PC AND METASTASIS

There are increasing evidence that exosomes are involved in the pathogenesis of development of pancreatic inflammation as well as related cancer initiation. Repeated episodes of pancreatitis are a

Table 1 Circulating biomarkers in pancreatic cancer

Biomarker	Type	Role in pancreatic cancer
CA19-9	Protein	Widely used biomarkers to aid in the diagnosis[4]
		Poor screening tool in asymptomatic patients
		Elevated in many benign gastrointestinal conditions as well as other malignancies, including pancreatitis, cirrhosis, cholangitis, and colorectal cancer[5]
		5%-10% of the caucasian population possesses a Lewis a-/b- genotype and thus does not express CA19-9
CEA	Glycoprotein	Elevated across several cancers[6]
		Non specific
		Inferior sensitivity of CEA compared to ca19-9[7]
CA125	Glycoprotein	Associated with ovarian cancer, CRC and cholangiocarcinoma[8]
		Superiority to CA19-9 in predicting resectability of PC, along with correlating with metastasis-associated disease burden
Anti-MUC1 antibody	Antibody	Anti-MUC1 antibody assays showed a sensitivity and specificity of 77% and 95%, respectively, in discriminating pancreatic cancer from pancreatitis[9]
CTCs	Tumour cells	CTCs had moderate diagnostic value in PC[10]
		Several studies have demonstrated isolation of CTCs regardless of stage among localized, locally advanced, or metastatic patients
		Conflicting evidence on CTC positivity is correlated with survivability
		In ombination with CA19-9, it was reported to have a superior sensitivity and specificity of 97.8% and 83.3% respectively, compared to when used in isolation[11]
		The presence of CTCs in 54/72 patients with confirmed PDAC (sensitivity = 75.0%, specificity = 96.4%, AUROC = 0.867, 95%CI: 0.798-0.935, and $P < 0.001$)[12]
		A cut-off of ≥ 3 CTCs in 4 mL blood could differentiate between local/regional and metastatic disease (AUROC: 0.885; 95%CI: 0.800-0.969; and $P < 0.001$)
cfDNA	DNA	Plasma ctDNA quantification of hot-spot mutations in KRAS and GNAS are useful in predicting tumor burden in patients diagnosed with PC[13]
		Digital PCR provided accurate tumor-derived mutant KRAS detection in plasma in resectable PC and improved post-resection recurrence prediction compared to CA19-9[14]
		Detection of plasma cfDNA mutations and copy number alterations may be helpful in pancreatic cancer prognosis and diagnosis
		Its sensitivity and specificity in identification of clinically relevant KRAS mutations was 87% and 99% respectively[15]
Cell-free RNA	RNA	Higher expression of lncRNA MALAT1 has been shown to correlate with poorer PDAC survival[16]
		Several microRNAs have also been associated with PDAC (*i.e.*, miR-21 and miR-155), and correlate with tumor stage or prognosis[17]
EVs	Exosomes	KRAS G12D mutations were identified in 7.4% of control patients, 67% of localized PDAC, 80% of locally advanced PDAC, and 85% of metastatic PDAC patients[18]
		GPC1 EVs could be detected in both pancreatic precursor lesions and pancreatic cancer, and could distinguish between any evidence of malignancy and healthy patients with an AUC of 1 (100% sensitivity, 100% specificity)[19]
		miRNA isolated from EVs revealed a cocktail of miRNAs (miR-1246, 4644, 3976, 4306) upregulated in 83% of pancreatic cancer derived EV
		Glypican-1 exosomes are a potential biomarker for PC

CA: Carbohydrate antigen; PDAC: Pancreatic ductal adenocarcinoma; PC: Pancreatic cancer; CEA: Carcinoembryonic antigen; CTCs: Circulating tumour cells; cfDNA: Cell-free DNA; EVs: Extracellular vesicles; AUROC: Area under the curve.

strong risk factor which can eventually increase the risk of PC. The pathogenesis and evolution of many pancreases precancerous conditions, including diabetes mellitus and pancreatitis, have been linked to crucial involvement of exosomes[39]. Exosomes can participate in promoting the transformation of various precancerous like intraductal papillary malignant neoplasm to PDAC. Exosomes are a key factor in initiating angiogenesis, cell migration, and epithelial-mesenchymal transition[40]. Cancer-associated fibroblasts, tumor-associated macrophages and pancreatic stellate cells can promote exosomes, that could promote growth, proliferation, drug resistance, epithelial mesenchymal transition, migration, invasion and metastasis of PC[41]. Interestingly, exosomes initiated from PC cells contains

Table 2 Comparison of usefulness of various liquid biopsies in pancreatic cancer

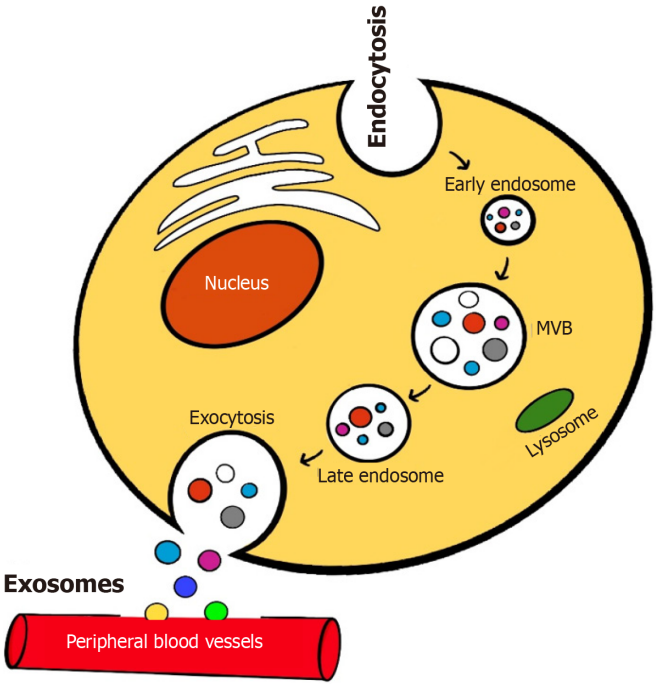
Item	CTC[20-22]	Ct DNA[21,23,24]	Exosomes[20,25-28]	CA 19-9[20,21,28-30]
Origin	Viable tumor cells	cfDNA, viable tumor cells, CTCs	DNA, proteins, lipids, RNAs metabolites, and tumor cells	Ductal cells in the pancreas, biliary system, and epithelial cells in the stomach, colon, uterus, and salivary glands
Samples used	Plasma	Frozen plasma, urine and other biofluids	Frozen plasma, urine and other biofluids	Plasma
Methods	CellSearch, MACS, Dynabeads, microfluidic, SE-iFISH, CD45/CEP8/DAPI staining-FISH, anti-EpCAM Portal-vein blood	Real-time quantitative PCR, digital PCR, droplet digital PCR, next-generation sequencing; commercial liquid biopsy platforms: GuardantTM (breast, colon, and lung cancers and multi-cancer detection) FoundationOne® (multi-cancer detection); signateraTM (colorectal cancer), Galleri (multi-cancer detection), CancerSEEK (multi-cancer detection), TempusTM (multi-cancer detection), Caris (bioinformatics testing of both circulating DNA and RNA)	Ultracentrifugation, ExoChip, precipitation, size-based isolation immunoaffinity-based isolation microfluidics-based isolation	Radio immuno assay
Mutation analysis	Yes	Yes	Yes	No
Drug delivery vehicle	No	No	Yes	No
Sensitivity	76.0%	65.0%	50.0%-85.0%	78.2%
Specificity	68.0%	75.0%	90.0%	82.8%
Usage in clinics	Diagnosis of PDAC, prognosis/prediction of PDAC	Diagnosis of PDAC; monitoring treatment efficacy; monitoring of disease progression	Diagnosis and prognosis of PDAC; prognosis/prediction of PDAC	Combining ct DNA with CA 19-9 levels could improve diagnostic sensitivity to 98%, and specificity to 97%; monitoring treatment efficacy; monitoring of disease progression

PDAC: Pancreatic ductal adenocarcinoma; CTC: Circulating tumour cells; CA: Carbohydrate antigen; ctDNA: Circulating tumor DNA.

tumor suppressor components which can inhibit the cancer cell proliferation and this could open the pandora box of potential therapeutic value of exosomes[42]. Exosomes promote cancer cell proliferation and initiate metastasis by delivering carcinogenic proteins, cytokines, adhesion molecules and miRNA. Thus, initiate proliferation of tumour by activation of different pathways like phosphoinositide 3-kinase/AKT serine/threonine kinase 1 (Akt) and mitogen-activated protein kinase pathways[43]. The features like weight loss and new-onset diabetes are characteristics of the paraneoplastic effect of PC which mostly precede the diagnosis of the PC. The biological reason of PC-associated diabetes is due to exosomal adrenomedullin, endoplasmic reticulum stress which may result in β-cell dysfunction and diabetes[44]. There is emerging evidence that suggest role of exosomemediated immunosuppression in PC. The exosomes have clear role in communications between tumor and immune cells and supposed to have a dynamic role in tumor immunity regulation. Gemcitabine chemotherapy is considered a standard treatment for PC either in combination or monotherapy, based on evidence from many studies which shown a better survival rate and more clinical benefits with median overall survival (OS) of 5 mo to 7 mo[45]. Most of patients with PC ultimately present with rapid disease progression even following chemotherapy with gemcitabine. Tumor derived exosomes can induce the progression of chemotherapy resistance in cancer cells. Chemotherapy agents could also be secreted from the extracellular matrix by exosomes is another reason for chemotherapy resistance. When exposed to gemcitabine, exosomal CAFs which are inherently insensitive to gemcitabine may also leads to chemotherapy resistance. CAF exosome secretion inhibition could decrease proliferation and drug resistance[46].

ISOLATION OF EXOSOMES

There are various methods to isolate and characterize exosomes based on their physical and chemical properties. Most popular methods are ultracentrifugation, size exclusion chromatography, magnetic activated cell sorting, membrane filtration and various commercial kits[47]. Western blotting and flow cytometry can be used to analyze and detect exosome markers. Transmission electron microscopy and



DOI: 10.5662/wjm.v13.i3.46

Figure 1 Diagrammatic representation of formation of cancer cell derived exosomes. MVB: Multi vesicular body.

nanoparticle tracking analysis are other methods to detect exosome.

Liquid biopsy to analyze exosome biomarkers could guide the diagnosis and prognosis of PC. Therefore, the identification of reliable predictive biomarkers for diagnosis and prognosis is an unmet need in PC management. The most common methods for isolation for exosome are summarized in Table 3[48-56] and quantifying methods for exosome are presented in Table 4[48-64]. Methods like Western blotting and enzyme linked immunosorbent assays needs large amounts of sample and extensive technical steps for detection. The comparison of various isolation methods used for exosomes are given in Table 5[65-76].

EXOSOMES AS DIAGNOSTIC BIOMARKER IN PC

At present scenario, early diagnosis of PC is very difficult and most are diagnosed at late stage. Mostly CT imaging are used for diagnosis and treatment. CA19-9 which is used in clinical practice has a low specificity and poor ability to distinguish benign pancreatic diseases from PC[77]. Thus, the search for novel early diagnostic markers is a concern for PC diagnosis and treatment. Exosomes are excessively produced in excess by malignant tumours. They also carry information about the tumour genetics and microenvironment, which determines its behaviour and its prognosis[78]. Circulating biomarkers are non-invasive and inexpensive for monitoring disease[79]. The circulating molecular tumor markers are circulating tumor cells, cell-free DNA, cell-free RNA, circulating tumor proteins, and exosomes. When compared to circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), exosomes are considered as the best diagnostic biomarkers in PC with a sensitivity of 93% and a specificity of 92% [80]. The ctDNA group had similar specificity 0.92 (95%CI 0.88-0.95) but lowest sensitivity. Thus, ctDNA was useful for the diagnosis of PC rather than screening. Whereas CTCs exhibits medium sensitivity and lowest specificity compared to others. The sensitivity, specificity and AUC of ctDNA were 0.6400, 0.9200, and 0.9478 respectively. Glypican 1 (GPC1) is expressed in the serum of patients with PC but not in benign pancreatic disease. Melo *et al*[19] described that Glypican-1 identifies cancer exosomes and could detects early PC. It was described that GPC1+ circulating exosomes could be used as prognostic biomarker in pre- and post-surgical patients. The GPC1+ circulating exosomes could distinguish PC precursor lesions from healthy individuals and benign pancreatic disease. The circulating GPC1+ exosomes levels were higher in PC precursor lesions than the levels in the healthy donor group and benign pancreatic diseases. There is supporting evidence that there is a potential use in early detection of pancreas cancer. Melo *et al*[19] study shows that circulating GPC1+ exosomes exhibit a sensitivity of 100% and specificity of 100%; with a positive predictive value of 100% and a negative predictive value of 100% compared to CA 19-9 which was inferior in distinguishing between pancreas cancer and healthy controls.

Table 3 Different isolation methods for exosome

Method	Sample volume	Time	Ref.
Ultracentrifugation	Low	Approximately 5 h	[48,49]
Density-gradient	Low	Approximately 5 h	[50]
Nanopillar	30 μL	Approximately 10 min	[51]
Acoustic-based	0.4-0.7 μL/min	< 30 min	[52]
Inertial lift force-based	70 μL/min	> 4 h	[53]
Surface-modified	4-16 μL/min	< 1 h	[53-55]
Nanoshearing	Not mentioned	< 3 h	[56]

Table 4 Different quantifying methods for exosome

Method	Size range	Specificity	Time	Ref.
Nanoparticle tracking analysis	10 nm-2 μm	Immunoaffinity	< 1 h	[48]
Dynamic light scattering	10 nm-8 μm	Size	< 1 h	[57]
Electron microscopy	10 nm	Size	< 1 h	[58,59]
Nanopore	> 10 nm	Size	< 1 h	[60,61]
Magnetic resonance	Wide range	Immunoaffinity	< 10 min	[62]
Electrochemical and plasmonic	Depends on binding	Immunoaffinity	< 10 min	[63,64]

The functional role of MicroRNAs has a greater opportunity in developing both prognostic and diagnostic markers. MiRNA -based biomarkers can help in the early diagnosis of disease. A recent study evaluated the expression patterns of miR-21, miR-155, miR-17-5p, and miR-196a in circulating exosomes as biomarkers for PC. The expression profile of miR-17-5p and miR-21 were increased in PC patients, The increased expression of miR-17-5p was seen in unresectable pancreatic patients[81]. miR-155 and miR-21 are over-expressed in PDAC, and can distinguish PC from benign lesions[82]. Upregulated miR-221-3p and miR-212 is associated in PDAC and they are responsible for cancer proliferation in PDAC cells. miR-128 expression is decreased in PC while non-cancerous tissue has a normal level of miR-128. Gemcitabine resistance is associated with downregulation of miRNA200b, miRNA-200c, let-7b, let-7c, let-7d, and let-7e. miR-155 and miR-1246 also have been related to gemcitabine resistance. There are miRNA that function as tumor suppressors in pancreatic ductal cancer like miR-99b, miR-100, miR-99a, miR-34a, miR-148a, miR-200a, miR-200b, and miR-200c. MicroRNAs expression profiles showed that miR-143, miR-29c, miR-148b, miR-150, and miR-96, were present in PDAC and chronic pancreatitis whereas miR-196b, miR-203, miR-196a, miR-210, miR-222, miR-210, miR216, miR-375, and miR-217, were expressed only in pancreatic carcinoma[83]. miR-190, miR-196a, miR-222, miR-15b, miR200b, miR-95, and miR-221 are elevated in pancreatic adenocarcinoma[84]. Nakamura *et al*[85], developed an exosome-based signature for non-invasive and early detection of PDAC. Previous research studies showed that serum Ephrin type-A receptor 2 in exosomes (Exo-EphA2) was expressed highly in PC cells. A study by Wei *et al*[86] the evaluated role of serum Exo-EphA2 as a potential diagnostic biomarker in PC. Serum Exo-EphA2 were higher in PC than in non-cancer pancreatic disease. Exo-EphA2 in combination with CA 19-9 was more useful to discriminate early stage of PC from non-cancer pancreatic disease. Alkaline phosphatase placental-like 2 presents in PC EVs has a potential application in liquid biopsy-based diagnostic tests. Shin *et al*[87] developed ALPPL2 direct and sandwich aptamer-linked immobilized sorbent assay for EVs, which could sensitively and specifically detect membrane protein,17 could be a potential biomarker for early diagnosis of PDAC. Recently, there are reports of exosomal migration inhibitory factor (MIF) may be an attractive sensitive biomarker for PC. MIF is an immunostimulatory cytokine associated with tumorigenesis. Costa-Silva *et al*[88] reported that the pancreatic exosomes are capable of inducing premetastatic niche formation in liver. They demonstrated that the exosome education-induced liver metastasis was abolished by silencing of exosomal MIF. The combined use of exosomal glypican-1 and MIF is a promising tool to identify very early stages of PC. The potential of MIF as a target for the treatment of PDAC should be explored in future.

Table 5 Comparison of various isolation methods for exosomes

Conventional isolation of exosomes				
Methods	**Advantages**	**Disadvantages**	**Clinical use**	**Ref.**
Ultracentrifugation	Widely used; high purity; protein and RNA components are not affected	Highly labour intensive; time-consuming; yields are typically low extensive training of personnel needed; expensive; inappropriate for the extraction of exosomes from a small amount of serum samples	Functional study of exosomes	[65, 66]
Ultrafiltration	High yield; simple; less time-consuming; do not require the use of special equipment	Low purity, clogging of pores	Study of sample concentration; used in combination with other methods	[67]
Precipitation	Widely used; economical	Co-isolation of non-EV particles	For studies with very low purity requirements that do not require omics studies	[68]
Size exclusion chromatography, OR, and gel filtration	Fast, reliable, and inexpensive; maintain the biological activity and integrity of exosomes; high purity	Nanoscale contaminants like lipoproteins; extensive laboratory equipment requirements	Suitable for exosome research in those requiring high purity, omics, and large volume samples	[69]
Immunoaffinity capture	Convenient; not affected by exosome size; no need for expensive instruments	Expensive; low capacity; low yields	Suitable for the Separation of specific exosome subgroups	[70]
Emerging isolation methods				
Stirred ultrafltration	Do not rely on equipment; less time consuming; reduces the destruction of exosomes during the process	Moderate purity of isolated exosomes; loss of exosomes during the process	Isolating exosomes from culture supernatant of bone marrow mesenchymal stem cells	[71]
ExoTIC (exosome total isolation chip)	Simple, easy-to-use, modular, and facilitates high-yield and high-purity EV isolation from biofluids	Special equipment requirements; lack of tests on clinical samples	Efficiently isolate EVs from small sample volumes; EV-based clinical testing from fingerprick quantities (10-100 μL) of blood	[72, 73]
3D ZnO Nanoarrays	Multifunction; high sensitivity; downstream analysis is possible; enhance the capture of exosomes at a high flow rate	Relatively expensive	Widely used in biosensing and analysis aspects, powerful tools for effective purification and molecular analysis of exosome	[74, 75]
Nano plasmon-enhanced scattering	Rapid, high-throughput, sensitive, and specifc method for the detection of exosomes from trace samples depending on the amount of scatter area, based on calculation of the proportion of the area that contains scattered light	High reagent cost; complex statistical tools; low capacity	Uses antibodies against the cellular markers CD81, CD63, and CD9, which are enriched on most exosome membranes	[76]

SEC: Size exclusion chromatography; EV: Extracellular vesicle.

EXOSOMES AS PROGNOSTIC BIOMARKER IN PC

The level of circulating Exo-EphA2 was higher in PC patients when compared to that of healthy controls, suggesting it could be a diagnostic and prognostic marker for PC. In a study by Wei *et al*[86] found that high expression of Exo-EphA2 in PC was associated with shorter OS. Exosomal KRAS mutations seems better than CA 19-9 Levels for the prognostic surveillance in PDAC[17]. A study by Tsuchida *et al*[89] revealed that KRAS mutation detected at baseline with a mutation frequency above 5% indicated poor clinical outcome following monitoring in the treatment course of patients with metastatic PDAC. Costa-Silva *et al*[88] found that MIF was markedly higher in exosomes from stage I PDAC patients who later developed liver metastasis. Thus, it is suggested that higher exosomal MIF may be a prognostic marker for the development of PDAC liver metastasis. Potential role of exosomal biomarkers for prognosis evaluation in PDAC was evaluated in a systematic review and meta-analysis, involving eleven studies comprising 634 patients and seen that detection of positive exosomal biomarkers increased risk of mortality and progression across disease stages. Positive exosomal biomarkers preoperatively had higher risk of mortality in resectable stages than positive exosomal biomarkers in unresectable stages[90].

The better understanding of the prognostic role of miRNAs in PC can be done by profiling miRNAs at different stages of cancer. In a study by Takahasi *et al*[91], authors suggest that plasma exosomal miR-451a may be useful to predict recurrence and prognosis in PDAC patients. The miR-451a had a significant association with tumor, stage and showed the highest upregulation in the stage II patients

who showed recurrence after surgery. In a retrospective clinical study by Namkung *et al*[92] comprising 200 pancreatic ductal adenocarcinoma tissue samples, miRNA-574-5p, miRNA-1244, miRNA-145, miRNA-328, miRNA-26b, and miRNA4321 showed association with OS and disease-free survival. Poor survival outcomes have been seen in PDAC with lower expression of *miR-183* and *miR-34a* as well as high expression of *miR-1290*, *miR-155*, *miR-203*, *miR-222*, and *miR-10b*[93]. Similarly, Microarray-based expression profiling of miRNAs derived from exosomes study revealed that miR-451a was the highest upregulated miRNA in stage II patients who developed recurrence after surgery. It was seen that survival rates of the high exosomal miR-451a patients were significantly worse than those of the low miR-451a patients[94].

TREATMENT MONITORING IN PC

Currently, one of the most common biomarkers used for so long to monitor the therapeutic responses in PC is CA 19-9. Exosomes have a significant role in monitoring response to therapy and disease progression. Melo *et al*[19] clearly demonstrated that all 190 patients with PDAC serum had higher GPC1+ exosomes than healthy individuals and was an independent prognostic marker for disease specific survival. In view of the fact that CA19-9 is not a reliable marker that correlates with clinical evolution of PC, a combination of CA19-9 together with exosome derived GPC1 could be explored for treatment monitoring and disease progression. Besides early diagnosis and prognosis, clinical utility of exosome proteins is evolving for personalized and posttreatment disease monitoring[95]. Circulating exosomal PD-L1 is an attractive option in disease monitoring. Recently, Chen *et al*[96] study explains the rationale for the application of exosome PD-L1 as a predictor for anti-PD-1 therapy.

EXOSOMES AS DRUG CARRIERS, THERAPEUTIC TARGETS AND TREATMENT

Currently, innovators are exploring the utility of exosomes for biomedical applications. Many advanced drug delivery systems that used to deliver various anticancer and antiviral agents explore the use of polymeric nanoparticles and liposomes to encapsulate drug and thus utilize for drug delivery.

Exosomes can be used as therapeutic drugs carriers because of favourable bioavailability, biocompatibility, ability to penetrate biological membranes and immunogenicity[97]. Exosomes can be used as transporters, therapeutic targets and therapeutic drugs.

Due to the favorable bioavailability and biocompatibility with the characteristics of exosomes, there appears a greater future of exosomes used either as parental exosomes or artificially modified exosomes for drug delivery vehicle. To avoid systemic toxicity, drugs can be encapsulated in exosomes and transferred to target cells[98]. Exosomes possess better biocompatibility as drug carriers. It is generally considered that injected exosomes shed from endogenous cells are tolerated with minimal immune reaction. The cargos can be delivered into the tumor microenvironment with the utility of exosomes [99]. Kamerkar *et al*[100] studied modified exosomes for cancer prevention and treatment and revealed that exosomes had a longer retention time in the circulation. Engineered exosomes specialized for malignant KRAS G12D were more successful in targeting oncogenic KRAS. Recent evidence suggests that safety and efficacy of exosomes in treating PC is not far. Exosome-based therapies for cancers have been developed due to the easy permeability of the exosome membrane, low toxic side effects and low immunogenicity. Paclitaxel -loaded exosomes have shown a great potential for delivery of chemotherapy and treatment of drug-resistant cancers[101]. Because of their rapid clearance from blood circulation after systemic administration, targeted delivery of exosomes is highly restricted. The rapid clearance after injection limits their applications for effective and durable therapeutic action. However, recent studies on modification of exosomes for targeted delivery *via* direct modification and genetic engineering to circumvent this limitation is promising. The use of MSCs-derived exosomes loaded with KRAS G12D siRNA to treat metastatic pancreas cancer (NCT03608631) is promising[102]. Mittal *et al* [103] also showed the efficacy of administration of micelles of gemcitabine and the tumor suppressor *miRNA-205* for the treatment of pancreas. Masamune *et al*[104] found that hypoxic environment in PC can release several angiogenic factors that may induce proliferation and angiogenesis. Understanding of these interactions under hypoxia is critical for angiogenic regulation in PDAC, which will also help to develop new anti-angiogenesis therapeutic strategies[105].

CONCLUSION

Even though there are several limitations in implementing exosome analysis clinically, it is a promising diagnostic and therapeutic tool for PC. The role of exosomes in cancer treatment continues to evolve.

ACKNOWLEDGEMENTS

I would like to convey my gratitude to Malavika Anoop, Standard X of ST Thomas Central School, Mukkolakkal, Thiruvananthapuram, Kerala for her invaluable assistance in completing my digital art work for this article.

FOOTNOTES

Author contributions: Anoop TM designed the study, drafted the manuscript; Basu PK participated in the design and draft of the manuscript; Chandramohan K participated in the design and draft of the manuscript; Thomas A participated in the design and draft of the manuscript; Manoj S participated in the design and draft of the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Chen YL
L-Editor: A
P-Editor: Ju JL

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 59-66

DOI: 10.5662/wjm.v13.i3.59

ISSN 2222-0682 (online)

MINIREVIEWS

Adult eosinophilic esophagitis and advances in its treatment

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Specialty type: Medical laboratory technology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Kuribayashi S, Japan; Sawada A, Japan

Received: March 1, 2023
Peer-review started: March 1, 2023
First decision: April 21, 2023
Revised: April 24, 2023
Accepted: May 15, 2023
Article in press: May 15, 2023
Published online: June 20, 2023

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Abstract

Eosinophilic esophagitis (EoE) is a chronic eosinophil inflammation that seems to be T helper type 2 antigen-driven. The disease is one of several eosinophilic gastrointestinal disorders in which there appears to be inflammation of the gastrointestinal tract without any apparent underlying causes. Differential diagnosis needs to be made with gastroesophageal reflux, which is characterized by chronic inflammation due to gastric refluxate from disorders related to motility. EoE, however, is considered a chronic allergic inflammatory disorder related to destructive tissue remodeling. There seems to be a higher prevalence of EoE in Western countries. It is typically found in atopic male individuals. Physiopathological risk factors include atopy, environmental factors, esophageal epithelial barrier dysfunctions, *etc.* EoE can cause several symptoms that include retrosternal burning sensation, dysphagia, food impaction, chronic reflux symptoms, nausea, and vomiting. Early diagnosis, which requires a biopsy to assess for esophageal inflammation, is essential for proper treatment. The aim of our brief overview is to summarize the current literature regarding the characteristics, diagnosis, complications, mechanisms of pathology, clinical features, influence of comorbidities, and treatment in patients with EoE.

Key Words: Eosinophilic esophagitis; Gastroesophageal reflux; Chronic allergic inflammatory disorder; Eosinophil inflammation; T helper 2

Core Tip: Eosinophilic esophagitis (EoE) is a chronic eosinophil inflammation. Differential diagnosis needs to be made with gastroesophageal reflux, which is characterized by chronic inflammation due to gastric refluxate from disorders related to motility. It is of clinical importance to diagnose, manage, and treat individuals with EoE. Patient outcomes, success of therapy, prevention of complications, and management of existing comorbidities depend on proper organ functioning.

Citation: Grando M, De Pauli S, Miotti G, Balbi M, Zeppieri M. Adult eosinophilic esophagitis and advances in its treatment. *World J Methodol* 2023; 13(3): 59-66
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/59.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.59

INTRODUCTION

Eosinophilic esophagitis (EoE) can be characterized as a T helper type 2 (Th2) antigen-driven disease that is due to chronic eosinophil inflammation, which causes signs and symptoms of esophageal dysfunction. The disease is part of a spectrum of eosinophilic gastrointestinal disorders that show eosinophilic inflammation of the gastrointestinal tract that can be explained by other causes. The first cases were described in the 1970s. Before 1960, interrogation of the intestinal mucosa was limited to surgical resections or postmortem analyses. An important element in the study of this disease was the advent of luminal fiberoptic endoscopy[1].

Esophageal eosinophilia was believed to be solely a manifestation of reflux esophagitis[2] and EoE was seldom diagnosed. In the mid-1990s, however, several studies described the disease as it is recognized today[3,4]. Since then, the number of publications on EoE has increased dramatically, especially considering that this condition has become more prevalent over the past 20 years as one of the main causes of upper gastrointestinal disorders. With the current diagnostic technology available, it is possible to provide a better diagnosis of gastroesophageal reflux (GERD) and EoE. GERD is characterized by chronic inflammation resulting from exposure to luminal gastric refluxate deriving from a disorder of motility. EoE, however, is defined as a chronic allergic inflammatory pathology characterized by signs, symptoms, and complications which tend to be related to destructive tissue remodeling [1].

The incidence rates of EoE continue to rise[5]. The prevalence of EoE is increasing rapidly in modern times, with rates approaching the prevalence of inflammatory bowel diseases. The prevalence of EoE follows the rising trend of allergic diseases over the last 50 years[6]. The prevalence estimates of EoE vary in different parts of the world. Studies have demonstrated a high prevalence in Western countries and a low prevalence in Eastern countries[5]. EoE occurs both in the pediatric and adult populations and tends to be more common in atopic male patients. Approximately 75% of all EoE patients tend to be male. This increased gender risk of EoE is supported by a genetic variant in the cytokine receptor-like factor 2 gene that encodes for the thymic stromal lymphopoietin receptor[6].

The aim of our minireview is to briefly assess the current literature regarding the characteristics, diagnosis, complications, mechanisms of pathology, clinical features, influence of comorbidities, and treatment in patients with EoE that have been published in the literature and considered in clinical settings since 2000. We conducted a search of the published literature from January 1, 2000 to March 1, 2023, using PubMed (https://pubmed.ncbi.nlm.nih.gov) and *Reference Citation Analysis* (*RCA*) (https://www.referencecitationanalysis.com). The main topics in this minireview can be found in Table 1.

PHYSIOPATHOLOGY

Atopy

EoE is considered an atopic disease, which is associated with food antigen-driven hypersensitivity. The majority of patients with EoE have an atopic phenotype. Individuals with EoE tend to have higher rates of other allergic conditions such as asthma, atopic dermatitis, allergic rhinitis, or increased IgE to aeroallergens and foods[6,7].

Environmental factors

Studies have shown that certain environmental factors can increase the risk of EoE, which include: Dysbiosis and dysregulation of the microbiome; residence in rural and suburban areas; cesarean birth; exposure to antibiotics; lack of breastfeeding, *etc*[8].

Table 1 Main topics of the minireview

Main topic	
Physiopathology	Atopy
	Environmental factors
	Esophageal epithelial barrier
	Th2
	Eosinophils
Clinical presentation	
Diagnosis	
Treatment	Dietary management
	Pharmacologic therapy
	Esophageal dilatation
	Biologic agents
Conclusions	

Th2: T helper type 2.

Esophageal epithelial barrier

The epithelium plays a central role in the development of EoE. In this condition, the epithelium demonstrates characteristic alteration that includes basal cell hyperplasia, dilated intra-cellular spaces, and impaired barrier function. When the epithelium barrier is broken or disrupted, it can lead to a hypersensitivity immune response to foreign antigens[6]. Several EoE patients can have an altered epithelial barrier when there are no signs of inflammation, which can predispose these individuals to allergic sensitization. Studies have reported the presence of transcriptional alterations in individuals with EoE[1]. There seems to be a downregulation of genes including filaggrin and involucrin. Other junctional proteins like claudin-1 and E-cadherin can also be decreased in EoE. Deoxyspergualin (DSG) (a transmembrane desmosomal cadherin) shows decreased activity in individuals with EoE[6]. Calpain 14 (CAPN14) has been found to be the most highly associated gene with EoE[9,10]. It is overexpressed in the esophageal epithelium in patients with EoE. Induced interleukin (IL)-13 stimulation in the esophageal epithelium can cause overexpression of CAPN14 and impaired barrier functioning. Altered expression of these genes and/or activation by type 2 cytokines such as IL-13 may predispose to barrier dysfunction.

The alteration of the barrier can also be due to peptic or other injuries. This hypothesis is derived from the clinical signs of EoE that can form after epithelial damage from trauma, acid, or infection. In these circumstances, food and other substances in direct contact with the damaged epithelium can sensitize the microenvironment of the esophageal mucosa and lead to activation of the Th2 inflammatory response. Barrier dysfunction can also occur as a self-perpetuating product of inflammation. Once the inflammation starts, the epithelium can become increasingly permissive and allow more allergenic stimulation to penetrate and develop an ongoing allergic cycle[1].

Th2

EoE is also Th2-mediated. The cascade of inflammatory response is similar to that of chronic allergic disease with an aberrant Th2 response. The lymphocytes are cells typically present in the inflammatory infiltrates found in EoE[11]. This immune response is mediated by ILs secreted by Th2, like IL-4, IL-5, and IL-13.

IL-5 is secreted by mast cells, Th2 cells, and eosinophils. This substance influences eosinophil survival and expansion and primes them to respond to activating signals in chronic allergic reactions[6]. IL-13 is one of the most important cytokines involved in EoE pathogenesis. Studies have demonstrated that IL-13 is upregulated in the esophagus of patients with EoE[12]. It can induce remodeling in the esophageal epithelial barrier; it has been shown to downregulate DSG-1, filaggrin, and involucrin[13]. IL-13 also upregulates eosinophil chemotaxis inducing the expression of chemokine ligand 26. Furthermore, it is responsible for epigenetic modification in the expression of CAPN14, an esophageal-specific protease [14]. CAPN14's substrates include signaling molecules, structural proteins, cell adhesion molecules, transcription factors, and inflammatory mediators of allergic responses, like IL-33 and STAT6[12], both of which tend to be pivotal in the development of EoE.

Eosinophils

Under normal conditions, the human esophageal epithelium has little or no eosinophilic leucocytes. The presence of intraepithelial eosinophils in the esophageal lamina propria and submucosa defines EoE [15]. Eosinophils are recruited by local chemotaxis. They have been shown to have effects on tissue damage[16]. Eosinophil also acts as antigen-presenting cells, recruiting T cells, mast cells, and basophils.

CLINICAL PRESENTATION

EoE may present with a wide variety of symptoms, which ranges from retrosternal burning sensation to dysphagia or episodes of food impaction. Some patients complain of chronic reflux symptoms, nausea, and vomiting. The clinical presentation tends to be different between adults and children[17,18]. The most typical signs and symptoms in adults include heartburn, dysphagia, chest pain, and food impaction. About 50% of adult patients initially presenting with food impaction have been shown to have a diagnosis of EoE[19]. In contrast, children present more commonly with vomiting, regurgitation, abdominal pain, and failure to thrive. Numerous patients begin to implement compensatory behaviors, such as eating food cut into small pieces, eating slowly, lubricating items with sauces, chewing carefully, diluting foods with drinking liquids, and avoiding medications and substances that induce dysphagia[8]. Several validated tools are now available to evaluate symptoms. In adults, the Eosinophilic Esophagitis Activity Index has been shown to offer good correlations between symptoms, histology, and patient-reported outcomes[20]. The most serious complication of EoE is the spontaneous rupture of the esophagus after a food impaction or episodes of vomiting (Boerhaave's syndrome). Fortunately, this complication is rare. Other associated complications that can happen in association with EoE include esophageal stricture, perforation, food impaction, and malnutrition[8].

DIAGNOSIS

There is no specific or diagnostic symptom of EoE. Diagnosis normally requires a biopsy to assess for esophageal inflammation. Endoscopic features can be misleading, thus several esophageal biopsies are needed in all individuals with risk or suspicion of EoE independent of endoscopic clinical appearance [21]. The most typical endoscopic signs in adults with EoE include mucosal rings (64%), linear furrows (80%), white plaques and/or exudates (16%), small caliber esophagus (28%), and strictures (12%)[22]. An endoscopic scoring system has recently been developed and validated to assist in the assessment and standardization of EoE signs based on the presence of rings, edema, exudates, strictures, and furrows[23].

The presence of the following criteria is required for the diagnosis based on the American College of Gastroenterology Clinical guidelines and consensus recommendations, which were reported in a review study of EoE by Gomez Torrijos *et al*[24]: (1) Clinical symptoms of esophageal dysfunction; (2) An increased number of eosinophils in the esophageal epithelium, with ≥ 15 eosinophils per high-power field, and the eosinophilia is limited to the esophagus; and (3) Exclusion of other possible causes of esophageal eosinophilia (including eosinophilic gastroenteritis, infection, hypereosinophilic syndrome, *etc*.). Characteristic histological features, which, however, are not pathognomonic, are also eosinophil aggregates or micro-abscesses and eosinophil layering along the surface of the lumen[8].

TREATMENT

The goals of the therapy of EoE are to induce histological remission, improve clinical symptoms, and prevent disease progression and complications. The management of EoE includes dietary, pharmacologic, and endoscopic interventions[8]. Commonly used treatments include an elimination diet, acid suppression with proton-pump inhibitors (PPIs), topical steroids, and, in the case of strictures, esophageal dilatation. New therapeutic options include monoclonal antibodies that are tailored to stop the underlying inflammatory processes. Increasing evidence suggests that type 2 cytokines may play key roles in EoE[25].

Dietary management

Diet therapy may prove to be very effective and can directly assist in the underlying pathological allergic mechanism. This method can also assist to identify the various food antigens that are responsible for the inflammatory response[8]. In patients with EoE, allergy tests to identify food allergens that can contribute to the pathogenesis of the disease can be useful to initiate a specific elimination diet. The main methods for testing food allergies are the skin prick test (SPT), *in vitro* specific IgE testing, and patch test (used to identify non-delayed allergic reactions). When an allergy test identifies a specific food allergy, the initial treatment is a testing-directed elimination diet. Studies in

adults and children have shown that direct diets based upon *in vitro* specific immunoglobulin E testing and SPT/patch testing can be successful to varying extents, although further studies are needed before routine use of serum-based testing can be recommended[26,27]. The patient should be referred to an allergology specialist to evaluate the prescription of epinephrine for anaphylaxis self-treatment.

Other dietary approaches are the empiric elimination diet which consists in educating the patient to avoid foods that are most likely to be allergenic (*i.e.*, SFED or the six-food elimination diet which is based on eliminating egg, cow milk, wheat, soy, fish/ shellfish, and peanuts/tree nuts). An elemental diet is an amino acid-based formula with the exclusion of all solid foods[28].

All these diets last normally for several weeks (at least eight weeks). The clinician must then check symptoms and confirm histologic improvement with esophageal biopsies. Problem foods are then slowly reintroduced one at a time, with a periodic evaluation to determine if they are tolerated.

Several prospective adult studies based on an elemental diet showed a lower histologic response in about 3 of 4 cases; however, these trials were limited by a short treatment duration of 4 wk and relatively high dropout and nonadherence due to palatability[28]. A meta-analysis, however, reported better clinical outcomes with the elemental diet when compared with specific food elimination diets [29]. Nonadherence to an elemental diet is due to limited meal variety, taste, lack of insurance coverage, and numerous endoscopies needed during food reintroduction to identify specific triggers[30].

Pharmacologic therapy

PPIs: PPIs are the first-line treatment options with dietary modification and topical glucocorticoids. *In vitro*, studies have shown that PPIs decrease cytokine secretion from the esophageal epithelium, which leads to the idea that PPIs can give an anti-inflammatory benefit[31]. The reported response rates to PPI therapy in patients with EoE can vary widely, ranging from 30%-70%[21]. There is no specific element that can predict the patient's response to these drugs. PPIs may benefit EoE patients by reducing acid production in patients with coexistent GERD. Individuals with well-established EoE can also have symptoms of GERD that are responsive to PPI treatment, where GERD can contribute to the development of EoE[8].

Topical glucocorticoids: Current steroid formulations used in the treatment of EoE are designed for airway delivery. There are various formulations, which include suspensions, puffs from inhalers, viscous slurry, or orodispersible tablets. Fluticasone given orally in the form of a spray from a metered-dose inhaler and liquid budesonide as a viscous preparation are the typical pharmacologic therapies used for EoE[32,33]. Fluticasone (440-880 μg twice daily) or budesonide (1-2 mg twice daily) for 8 wk can be used to induce remission[34]. In 2020, the European Medicines Agency approved an orodispersible budesonide tablet formulation for adults with EoE[21,22].

Long-term topical glucocorticoid therapy is indicated in EoE due to frequent recurrence with tetracycline antibiotics removal. Maintenance therapy with topical steroids and dietary restriction should be considered, especially in patients with severe symptoms (*i.e.*, dysphagia, food impaction, and weight loss), anatomical complications (high-grade esophageal strictures), and rapid relapse after initial therapy[19]. Clinicians must pay close attention to side effects, such as esophageal candidiasis.

Esophageal dilatation

This treatment is reserved for patients with EoE that do not benefit from conservative therapy or for patients with EoE that show high-grade strictures. Endoscopic dilation can provide immediate symptomatology improvement in 95% of patients with EoE that have narrow caliber esophagus or strictures [34]. Since dilation has only a mechanical effect and does not stop the inflammation of the underlying eosinophil, repeated treatments are normally required to keep symptoms under control. The association of medical therapy is recommended in these individuals[34].

Biologic agents

Current evidence suggests that type 2 cytokines play a key role in EoE[25]. Monoclonal antibodies are used in other Th2-mediated allergic diseases and have the potential of modifying the natural history of the disease. Treatments that block the underlying inflammatory processes and prevent disease progression can be useful.

IL-13 has been implicated as an important cytokine in the pathogenesis of EoE. *The New England Journal of Medicine* has recently published the results of a three-part, randomized, double-blind, placebo-controlled trial based on dupilumab, a fully human monoclonal antibody that blocks IL-13 and IL-4 signaling[35]. Eligible patients were aged 12 years or older and had a diagnosis of EoE by endoscopic biopsy despite 8 wk of high-dose PPI therapy. The two primary endpoints after 24 wk included histologic remission (≤ 6 eosinophils using a high-power field) and the decrease from baseline in the Dysphagia Symptom Questionnaire score. The study demonstrated that dupilumab at a weekly dose of 300 mg led to reductions in symptoms of EoE and enhancements in histologic outcomes amongst adolescents and adults[35]. The most frequently reported adverse event throughout the period of treatment with dupilumab was an injection-site reaction. Other common adverse reactions were respiratory tract infections, arthralgia, and herpes viral infections. Dupixent (dupilumab) was approved by the Food and Drug Administration in May of 2022 for the treatment of EoE in patients 12 years and

older weighing at least 40 kg[36].

Another biological agent that has been considered in the treatment of EoE is mepolizumab. This agent is a humanized monoclonal antibody against IL-5, a cytokine that is crucial for the recruitment of eosinophils. Studies regarding mepolizumab, however, have shown variable results[37,38]. Other biologics such as cendakimab (a monoclonal antibody inhibiting IL-13 receptor binding), lirentelimab (a monoclonal antibody to sialic acid-binding immunoglobulin-like lectin 8, a CD33 receptor present on the surface of mast cells and eosinophils), and bevacizumab (a monoclonal antibody directed against the membrane-bound IL-5 receptor α chain present on eosinophils) are in various stages of clinical trials [39]. Some of these drugs have been approved for other atopic conditions, and thus appear as potentially promising treatment options for EoE and other eosinophilic gastrointestinal diseases. Further studies are needed to determine the long-term treatment outcomes for each of these drugs[39].

CONCLUSION

EoE remains to be one of the most frequent eosinophilic gastrointestinal diseases. Standard treatments with diet modifications, PPIs, and topical corticosteroid preparations have variable rates of response. Current studies in the literature have been enriching the understanding of the mechanisms and pathogenesis involved in EoE disease, which can help find potential disease-modifying biologic therapies, such as dupilumab, as an effective treatment option. Further studies are needed to determine the long-term outcomes of these drugs. The use of these agents in EoE offers a potentially promising option for patients with severe symptoms and complications.

FOOTNOTES

Author contributions: Grando M, De Pauli S, and Zeppieri M wrote the outline and did the research and writing of the manuscript; Grando M, De Pauli S, Miotti G, Balbi M, and Zeppieri M assisted in the writing of the paper; Zeppieri M (a native English speaker, MD, PhD) was responsible for the conception and design of the study and completed the English and scientific editing; Grando M, De Pauli S, and Zeppieri M assisted in the editing and making critical revisions of the manuscript; and all authors provided the final approval of the article.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: Italy

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S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Zhang XD

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 67-78

DOI: 10.5662/wjm.v13.i3.67

ISSN 2222-0682 (online)

MINIREVIEWS

IgA nephropathy associated with Crohn's disease

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Perse M, Slovenia; Triantafillidis J, Greece

Received: March 24, 2023
Peer-review started: March 24, 2023
First decision: April 3, 2023
Revised: April 16, 2023
Accepted: May 12, 2023
Article in press: May 12, 2023
Published online: June 20, 2023

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Abstract

The relationship between IgA nephropathy (IgAN) and Crohn's disease was reported. IgAN is the most common primary glomerulonephritis and one of the leading causes of chronic kidney disease and end-stage renal failure, and up to 50% of cases progressed to end-stage renal disease within 25 years after IgAN diagnosis. However, specific and effective therapeutic strategies are still lacking. In this review, we discuss the possibility of the mechanism involved in IgAN associated with Crohn's disease based on the findings of basic and clinical studies. Although the etiology of IgAN associated with Crohn's disease is not permanent and various factors are thought to be involved, the stabilization of the disease condition of Crohn's disease is believed to help treat IgAN.

Key Words: Crohn's disease; IgA nephropathy; Immunological abnormalities; Mucosal-associated lymphoid tissue; Gut-associated lymphoid tissue; Nasopharynx-associated lymphoid tissue

Core Tip: The relationship between IgA nephropathy (IgAN) and Crohn's disease was reported. Crohn's disease (CD) immunological abnormalities may promote and activate the IgAN inflammatory process. Although the etiology of CD-IgAN is not fixed and various factors are thought to be involved, the stabilization of the disease condition of CD is believed to help treat IgAN.

Citation: Tamura H. IgA nephropathy associated with Crohn's disease. *World J Methodol* 2023; 13(3): 67-78
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/67.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.67

INTRODUCTION

Intestinal diseases include chronic nonspecific inflammatory diseases of the gastrointestinal tract. Inflammatory bowel disease (IBD) is a chronic and recurrent inflammatory disease of the gastrointestinal tract that includes crohn's disease (CD) and ulcerative colitis (UC).

In addition to intestinal inflammation, CD has numerous extraintestinal manifestations of IBD. Extraintestinal complications of renal and urological diseases in CD include internal fistula lesions such as enterovesical fistula and enterovaginal fistula, urolithiasis, and renal disease. Urolithiasis is relatively common in 19% of patients with IBD; however, comorbid renal disease is relatively rare. Interstitial nephritis and secondary renal amyloidosis caused by 5-aminosalicylic acid (5-ASA) are renal diseases that show a clear causal relationship with CD treatment or CD itself; however, glomerulonephritis has been reported in few cases, and the lack of a causal relationship is difficult to prove. Among them, the relationship between IgA nephropathy (IgAN) and CD was reported[1,2].

IgAN is the most common primary glomerulonephritis and one of the leading causes of chronic kidney disease and end-stage renal failure. Up to 50% of cases progressed to end-stage renal disease (ESRD) within 25 years after the diagnosis of IgAN. However, at present, specific and effective therapeutic strategies are still lacking[3,4]. Thus, this paper discusses the possibility of the mechanism involved in IgAN associated with CD (CD-IgAN) based on the findings of basic and clinical studies.

ABNORMAL MUCOSAL IMMUNITY AND IGAN

Because IgAN exacerbates nephropathy after upper respiratory tract infection, it has been hypothesized that mucosal immunity is involved in the pathogenesis of IgAN. IgA is an immunoglobulin that mainly acts on the mucosa, and renal glomerular IgA is a mucosal multimeric IgA1 containing a secretory component[5-8], and multimeric IgA1 is also increased in the serum[9,10]. These have also supported these hypotheses.

Conversely, which mucosal-associated lymphoid tissue (MALT) is the main responsible site for IgAN is unclear. The involvement of nasopharynx-associated lymphoid tissue (NALT) is assumed to be mainly responsible for IgAN because it worsens after upper respiratory inflammation. Gut-associated lymphoid tissue (GALT) has a huge mucosal area and is the main production site of mucosal IgA; however, its involvement in the pathology of IgAN is controversial. In addition, the exacerbation of IgAN after a mucosal infection suggests the involvement of foreign antigens; however, many points are unclear about how infection is involved.

Recently, the activation of the innate immune system triggered by infection is involved in the onset and progression of various types of nephritis, along with mechanisms such as molecular mimicry and epitope spreading[11]. However, the specific mechanism of IgAN has not been elucidated.

IgAN leads to ESRD, IgAN recurrence in approximately half of the patients who underwent kidney transplantation, and vice versa. IgAN disappears when the kidney is transplanted as a donor, suggesting that the main cause of IgAN is not kidney-specific cells but the systemic IgA immune system [5].

The facts suggesting the involvement of mucosal immunity are as described above; however, bone marrow abnormalities have also been pointed out in patients with IgAN. van den Wall Bake *et al*[12] reported that the ratio of IgA-producing plasma cells (IgA + PC) increased in the bone marrow of patients with IgAN, and IgA1 production was enhanced. These suggest that glomerular IgA1 is derived from the bone marrow[13].

Moreover, in patients with IgAN, IgA1 was significantly higher with the production of peripheral blood mononuclear cell IgA[12]. By contrast, Harper *et al*[14] reported that J-chain mRNA-positive mucosal-type IgA + PC increased in the bone marrow of patients with IgAN. Another study reported that when leukemia develops in a patient with IgAN and the patient undergoes bone marrow transplantation, not only leukemia but also IgAN improves[15]. In other words, in patients with IgAN, mucosal-type IgA + PC, which is involved in the pathology, increased in the extramucosal bone marrow, suggesting the excessive production of mucosal-type multimeric IgA1[5].

Approximately 30 years ago, van den Wall Bake *et al*[16] hypothesized the existence of "mucosa–bone marrow axis" abnormalities in IgAN[5]. Moreover, the homing mechanism of immunocompetent cells to the effective tissue was clarified. This hypothesis became more realistic because mucosa-derived effector cells were found to be translocated and stored in the lymphatic tissue.

Studies using mouse models of spontaneous IgAN have also shown that cells responsible for the abnormal IgA production related to renal onset are present in the bone marrow and spleen[17-19]. Active cell migration and immune information exchange take place between the mucosal tissue and bone marrow. However, whether the cells responsible for IgAN migrate between the MALT and bone marrow or whether only relatively localized mucosal tissue is involved is unclear.

Moreover, we discuss the possible involvement of GALT and NALT in pathologies with IgAN. Serum IgA is mainly produced in the bone marrow, and intestinal mucosa-derived IgA is thought to be scarce. Conversely, in GALT, large amounts of IgA are produced and secreted in the mucosa, which contribute

to intestinal immunity. Unlike mucosal IgA, the physiological role of serum IgA is largely unknown. Although there are IgA1 and IgA2 subclasses in humans, 80%-85% of serum IgA is IgA1[20].

As shown in the IgA1 and IgA2 ratios of IgA + PC in the mucosal execution phase, IgA2 + PC was high (30%-65%) in the intestinal mucosa, whereas it was low (7%-25%) in the peripheral lymph nodes and respiratory tract mucosa[20]. In particular, IgA2 + PC is dominant in the ileum and colon[21-24].

Human IgA2 Lacks amino acids, which are the recognition sites of hinge-specific proteases derived from intestinal bacteria, and is protease resistant, which is thought to work favorably in intestinal immunity[24]. The organogenesis mechanisms of Peyer's patch (PP) and NALT are significantly different.

In the PP, the number of $CD3^{-}CD4^{+}CD45^{+}$ inducer cells increased from the embryonic period and decreased until 3 wk after birth. In NALT, the number of inducer cells increased because of postnatal stimulation with foreign antigens and peaks at 3 wk after birth[25]. The molecular mechanisms involved in the organogenesis of the two are also different[26]. These facts suggest the difference in the basic roles and needs of both in the mucosa.

The polymeric immunoglobulin receptor (pIgR) is a protein expressed on the basolateral side of mucosal secretory epithelial cells that carry IgA and IgM produced in the mucosa to the mucosal surface.

In GALT, IgA and IgM produced from the PC of the lamina propria (LP) form dimeric IgA and pentameric IgM by the J-chain produced at the same time. pIgR easily binds to multimeric IgA and IgM containing this J-chain and efficiently transports them to the mucosal surface. The transported multimeric IgA and IgM trap antigens in the mucosa and act as a non-inflammatory mucosal immune defense mechanism[27,28].

In IBD, IgA is actively produced in the LP. Conversely, inflammatory pIgR expression and dysfunction occur, inhibiting its secretion on the mucosal surface. Therefore, mucosal IgA, which increased in the LP, was also thought to increase in the blood[29]. Mucosal IgA in the blood is increased in patients with IBD such as UC and CD[30].

The lymphotoxin-β receptor (LTβR) is essential for the formation of mucosal lymphoid tissue[31] and plays an important role in IgA production in the small intestine[32].

LIGHT is a ligand for LTβR and is expressed on activated T cells[33]. In LIGHT transgenic mice (LIGHT Tg), sustained stimulation of LIGHT *via* LTβR on T cells causes the over-induction of IgA + PC in the LP, and increased expression of MAdCAM-1 by stimulation from LTβR leads to the activation of inflammatory cells, causing severe enteritis[30].

Impaired pIgR expression and excessive IgA production caused by this intestinal inflammation induce mucosal multimeric IgA, which is not transported to the mucosa, to enter and increase their presence in the blood circulation. Because these multimeric IgAs have high affinity to the kidney, they are deposited in the kidney and cause IgAN[30].

On the contrary, the bias of Th2 cytokines may be involved in the glycosylation of IgA in the intestinal tract. Even a small amount of IgA released into the blood from the intestinal tract may cause nephropathy[34-36]. Thus, induction of glomerular deposition of intestinal IgAN is theoretically possible if conditions such as inflammation-associated pIgR dysfunction are met.

However, clinically, most patients with IgAN do not have IBD or gastrointestinal symptoms, and the so-called secondary IgAN associated with intestinal abnormalities occurs in a small proportion of patients. The involvement of IBD in IgAN is considered limited, at least in terms of IgAN in Japan.

COMPARISON OF HISTOLOGICAL FINDINGS BETWEEN CD□IGAN AND COMMON IGAN

Virchows Archiv investigated the clinical and pathological differences CD-IgAN and common IgANs (NOS-IgAN) associated with upper airway inflammation such as tonsillitis[37]. The PCs of the upper airway mucosa mainly produce IgA1[38]. However, the intestinal mucosa, especially PP, is thought to predominantly secrete IgA2 (approximately 60% in mucosal cells) rather than IgA1[39]. For IgAN with CD, intestinal IgA2 is deposited in the glomerular mesangium and may be involved in IgAN induction and progression. However, among the deposited IgA subclasses, the IgA1 subclass was predominant in both the CD-IgAN and NOS-IgAN groups, and no significant difference in the staining intensity of IgA2 was found between the two groups. Then, they examined the deposition of galactose-deficient IgA1 (Gd-IgA1) in the glomerulus of primary IgAN[40].

Gd-IgA1 is an abnormal IgA1 of the IgA1 subclass, exhibiting a structure in which the o-linked glycans in the hinge lack galactose and expose N-acetylgalactosamine (GalNAC)[41,42]. They found no difference in the extent of Gd-IgA1 deposition in the glomeruli, irrespective of CD complications, and a significant difference in Gd-IgA1 deposition was found the between CD-IgANs and NOS-IgAN. CD-IgANs were suggested to share the same pathogenesis as primary IgANs. Furthermore, negative views have emerged regarding the disease specificity of Gd-IgA1[43]. In their renal histological findings, patients with CD-IgAN were found to have significantly more severe glomerulosclerosis, arteriolar hyalinosis grade, tubular atrophy, and interstitial fibrosis than those with NOS-IgAN.

A comparison of the Oxford Classification MEST-C scores revealed that the T-scores representing tubular atrophy and interstitial fibrosis were significantly higher in CD-IgANs than in NOS-IgANs. To confirm this trend, they performed a meta-analysis comparing MEST-C scores using a large cohort of patients with IgAN reported in previous studies[44,45]. The incidence of T1 and T2 was higher in CD-IgANs than in IgANs. These histological differences were speculated to be related to the following factors: (1) CD pathophysiology (*i.e.*, diarrhea and dehydration); (2) Therapeutic agents for CD; and (3) Systemic inflammation, including the gut.

First, in the course of CD, dehydration due to diarrhea, reduced fluid intake, and surgery can reduce the circulating blood volume, which can lead to tubulointerstitial damage and glomerulosclerosis. In addition, undernutrition and hypokalemia have been reported to cause chronic tubulointerstitial injury [46]. The possible reason is that the reduced effective circulating blood volume stimulates the renin–angiotensin–aldosterone system, which in turn enhances the activation of angiotensin II, which can lead to arteriolar contraction, glomerular ischemia, and interstitial fibrosis[47,48]. In addition, evidence showed that hyperuricemia, common in CD, may exacerbate glomerulosclerosis[49].

Second, this factor is related to the effect of treatment. 5-ASA remains the main treatment for CD, and its adverse renal effects are collectively called mesalamine-associated kidney disease[50]. The mechanism by which mesalamine promotes renal injury appears to be through the salicylic acid inhibition of intrarenal prostaglandin synthesis, which is a vasoactive mediator of intrarenal blood flow and uncouples mitochondrial oxidative phosphorylation[46,51].

Several studies have also reported that mesalamine promotes histologically renal injury through interstitial nephritis[52], and similar findings have been reported in patients with CD, not on 5-ASA [53]. Therefore, distinguishing whether interstitial nephritis is due to drugs or CD is challenging.

Third, in CD pathogenesis, immune abnormalities may be involved. Macrophages and T cells produce large amounts of interleukin-23 and tumor necrosis factor (TNF)-α in immune diseases such as CD and are thought to play a central role in CD pathophysiology[54]. These cytokines also contribute to the exacerbation of IgAN tubulointerstitial lesions[54]. A study has also reported a mechanism by which dysfunctional macrophages promote intestinal fibrosis[55].

In recent years, the mechanisms and systemic responses of B cell immune abnormalities in CD have been elucidated[56-58]. Interstitial inflammation in chronic kidney disease involving IgAN was reported to involve B cell-mediated immune dysfunction[59], and immune dysfunction in CD was found to be associated with IgAN in terms of immune dysfunction, which may act as an aggravating factor for renal function. Moreover, in IgAN, complement activity was thought to promote glomerulosclerosis and interstitial fibrosis[60,61].

Previous studies have shown that in patients with CD, activated complement (predominantly C3b) stains strongly in the intestinal mucosa[62] and that resected ileocecal specimens show increased expression of complement C3 mRNA[63]. The effects of CD-associated complement activation may influence glomerular and tubulointerstitial inflammations in IgAN.

COMMON KEY GENES ASSOCIATED WITH CD AND IGAN

Several common genetic predispositions to CD and IgAN have been reported. *HLA-DR1* in IgAN[64] and *HLA-DR1 DQw5* in CD[65] are characteristic genetic predispositions. Moreover, *HLA-DR1*-positive patients may have an increased risk of IgAN and CD[66]. A genome-wide association study found that *CARD9*, *HORMAD2*, and *HLA-DQB1*susceptibility genes for IgAN were also associated with IBD. These IgAN loci encode a protein involved in maintaining the intestinal barrier and regulating mucosal immune responses to pathogens[67-70]. Yuan *et al*[71] reported that the gene for CXCL2 is critically linked to immune infiltration during CD and IgAN. CXCL2, also known as macrophage inflammatory protein-2, belongs to the CXC subfamily. It is secreted by many cell types, including monocytes, macrophages, endothelial cells, and hepatocytes, in response to infection and injury. It primarily affects the recruitment of polymorphonuclear leukocytes to sites of injury or infection, thereby modulating immune and inflammatory responses. The analysis of the relationship between CXCL2 and immune cell infiltration in CD and IgAN diseases suggested that CXCL2 is involved in immune infiltration, thereby contributing to the pathogenesis of the two diseases.

PATHOGENESIS OF IGAN ASSOCIATED WITH CROHN'S DISEASE

Recent research has revealed that the apoptosis inhibitor of macrophages (AIM) is involved in the pathogenesis of renal failure through the function of macrophages, a type of leukocytes. AIM deposited in the same sites as IgA in the glomeruli in human IgAN and a spontaneous mouse model of IgAN. As a result, AIM deficiency in the spontaneous IgAN mouse model, although IgA deposition in the glomeruli was observed, did not develop IgAN. Subsequently, when AIM was administered to mice, IgG and IgM were co-deposited in the glomeruli, proteinuria and occult blood appeared, and nephropathy was confirmed. From this, IgA deposition in the glomerulus alone does not progress to nephropathy. In

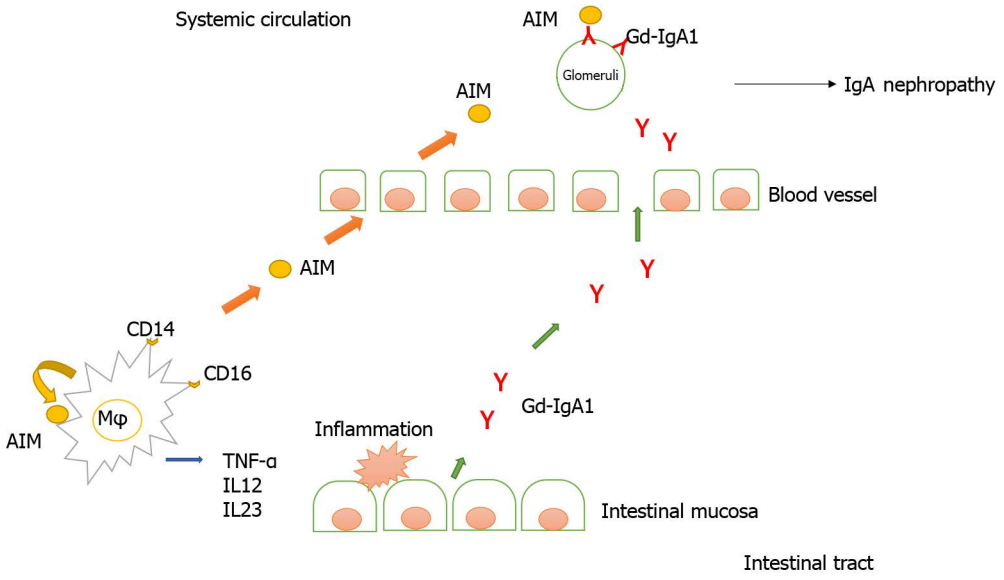

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Figure 1 Intestinal mucosal barrier disruption due to Crohn's disease enhances the production of glycosylated IgA. Apoptosis inhibitor of macrophages (AIM) produced by resident macrophages in the intestinal tissue contributes to intestinal inflammation, and active macrophage-derived AIM in the gut leads to elevated serum AIM levels. AIM is involved in the recruitment of inflammatory macrophages *via* autocrine and paracrine and induces inflammatory cytokine (TNF-α) in mesenteric adipose tissue in Crohn's disease. AIM influences the pathogenesis of Crohn's disease by inhibiting apoptosis of active intestinal macrophages and enhancing the expression of TNF-α in these mesenteric adipose tissues. Intestinal mucosal barrier disruption due to Crohn's disease enhances the production of glycosylated IgA. In IgA nephropathy, it was clarified that the deposition of IgA in the glomerulus is essential as the first step, and that AIM leads to inflammation as the second step. AIM: Apoptosis inhibitor of macrophages; CD: Crohn's disease; Mφ: Macrophages.

addition, glomerular deposition of AIM, IgG, and IgM was not induced in IgA-deficient mice. These results demonstrate that IgAN involves the deposition of IgA into the glomeruli as the first step and that co-deposition of IgG and IgM *via* AIM leads to inflammation as the second step. Furthermore, in human IgAN, AIM was co-deposited with glomerular IgA, IgG, IgM, and complement (C3). Therefore, AIM is a key molecule that initiates inflammation in IgAN[72].

Ono *et al*[73] showed that serum AIM levels were higher in patients with CD than in patients with UC, patients with intestinal BD, and healthy controls. Furthermore, AIM is expressed in CD14- and CD16-positive macrophages in the intestinal tissue.

AIM produced by resident macrophages in the intestinal tissue was believed to contribute to intestinal inflammation and that active macrophage-derived AIM in the intestine results in elevated serum AIM levels. AIM is taken up by the adipocytes *via* CD36-mediated endocytosis, which subsequently induces lipolysis[74,75]. Therefore, it may be involved in the recruitment of inflammatory macrophages and induce TNF-α in mesenteric adipose tissue in CD.

AIM may affect the pathogenesis of CD by not only inhibiting the apoptosis of active intestinal macrophages but also enhancing TNF-α expression in these mesenteric adipose tissues.

Inoue *et al*[76] reported that glycosylated IgA is produced in the gastrointestinal mucosa of CD and that glycosylated IgA correlates with CD disease activity. These results are crucial because they not only indicate that CD induced IgAN but also serve as evidence of the correlation between CD and IgAN.

We hypothesized the pathogenesis of IgAN associated with Crohn's disease (Figure 1).

IGAN: CD-ASSOCIATED OR ADALIMUMAB-INDUCED?

We searched PubMed for studies on CD with IgAN (Supplementary Figure 1). Twenty-five cases have been reported[77-92], including a self-case, with an average age of 27.5 (9–39) years, male-to-female ratio of 20:5 (male 80%), seven cases with underlying IgAN, and seven cases with underlying CD. Moreover, 17 patients had comorbidities, and one case had a simultaneous onset (Table 1).

However, certain cases had urinalysis abnormalities such as proteinuria and microscopic hematuria, which have been pointed out before the definitive diagnosis of IgAN. Because CD also progresses preclinically, identifying the time of onset of CD and IgAN is very difficult.

In the literature, the pathological findings of CD-complicated nephritis and IgAN with CD also tended to have a higher T-score, which represents tubular atrophy and interstitial fibrosis, than IgAN without CD. Five patients developed IgAN during biologic therapy, and CD and proteinuria improved after biologics were discontinued or changed.

Table 1 Reported cases of Crohn's disease with IgA nephropathy

Age (yr)	Sex	Clinical course	CD disease type	IgAN MEST-C grade	CD treatment	IgAN treatment	CD and IgAN worsened simultaneously
20	Female	CD(y)→IgAN(y)	Ileocolitis	NR	SASP	nothing	+
25	Male	CD(4y)→IgAN(12y)	Ileocolitis	NR	ED only	NR	+
18	Male	IgAN(NR)→CD(2y)	colitis	NR	5-ASA	ACE-I	NR
24	Male	CD(NR)→IgAN(3y)	NR	NR	5-ASA	NR	-
38	Male	CD(NR)→IgAN(6y)	NR	NR	PSL+5-ASA →IFX	ACE-I	+
40	Female	CD(NR)→IgAN(5y)	colitis	NR	5-ASA	ARB	+
38	Male	CD(NR)→IgAN(16y)	Ileocolitis	NR	5-ASA	NR	NR
40	Male	CD(NR)→IgAN(12y)	NR	NR	PSL+5-ASA	PSL	+
34	Female	IgAN(4y)→CD(5y)	Ileocolitis	NR	ED only	PSL	+
25	Male	CD(25y)→IgAN(33y)	NR	M1E1S1T1C1	ADA	MPT→PSL, ACE-I	-
24	Male	CD(22y)→IgAN(24y)	Ileitis	M1E0S1T1C2	IFX →UST	MPT→PSL	-
10	Male	CD(1y)→IgAN(7y)	colitis	M1E0S0T0C0	IFX	IFX→ADA, PSL	+
40	Male	CD(NR)→IgAN(39y)	NR	M1E0S1T0C1	AZA	PSL	-
31	Male	CD(16)→IgAN(18y)	Ileocolitis	M1E0S1T1C0	PSL+5-ASA	PSL	+
46	Male	IgAN(17y)→CD(40y)→IgAN(46y)	NR	M1E0S0T0C1	5-ASA, AZA, IFX	PSL	+
15	Female	CD(16)→IgAN(18y)	NR	NR	5-ASA, PSL	IFX	+
13	Male	IgAN(10y)→CD(13y)	NR	M1E0S0T0C1	PSL, SASP	PSL, CPA, ACE-I	+
9	Male	CD(9)→IgAN(11y)	Ileitis	M1E0S0T1C0	ADA	ADA →AZA; MPT, PSL, ACE-I	-
39	Female	CD(32)→IgAN(39y)	NR	M1E0S1T0C0	ADA, AZA	ARB, ADA→IFX	+
27	Male	IgAN(22y)→CD(27y)	NR	M1E0S1T2C1	5-ASA, IFX	MPT, MMF, CsA	+
18	Male	CD(9)→IgAN(11y)	NR	M1E1S1T1C2	5-ASA	PSL, CPA	+
22	Male	IgAN(8y)→CD(22y)	NR	M1E1S1T1C0	SASP, AZA	PSL, 5-ASA	+
36	Male	CD(30)→IgAN(36y)	NR	M1E0S0T2C0	5-ASA, AZA	ACE-I	-
29	Male	IgAN(24y)→CD(29y)	colitis	NR	PSL	non	+
10	Male	CD(30)→IgAN(36y)	Ileocolitis	M1E0S0T0C0	ADA, AZA, budesonide	PSL	+

CD: Crohn's disease; IgAN: IgA nephropathy; ED: Elemental diet; PSL: Prednisolone; MPT: Methylprednisolone pulse therapy; SASP: Salazosulfapyridine; 5-ASA: 5-aminosalicylic acid; CPA: Cycrophosphamid; AZA: Azathioprine; IFX: Infliximab; ADA: Adalimumab; UST: Usutekinumab; CsA: Cyclosporin; MMF: Mycophenolate mofetil; ACE-I: Angiotensin converting enzyme inhibitor; ARB: Angiotensin receptor blocker; NR: Not reported.

Simultaneous exacerbation of both diseases was observed in 17 of 23 patients, and many studies have reported that the disease activity of both diseases was related, suggesting that IgAN is an extraintestinal complication of CD. This tendency was also observed in our case (Figure 2). Considering that the disease progression of both diseases is the same, controlling the disease progression of CD is also important to prevent the progression of renal failure.

The use of immunomodulators to maintain remission in both diseases is an option. In addition, because TNF-α induces glomerular inflammation and enhances permeability[93], anti-TNF-α antibodies are theoretically expected to improve proteinuria.

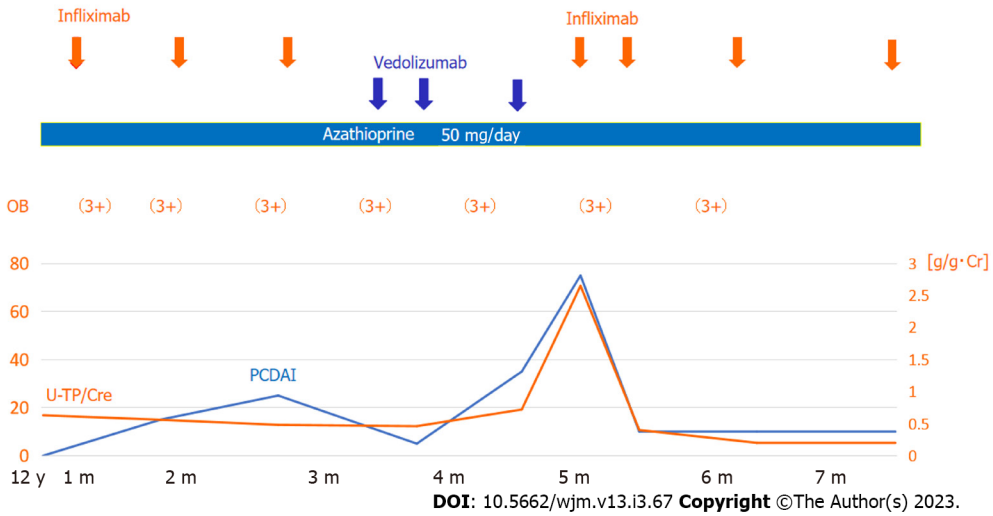

Figure 2 Clinical course after the appearance of hematuria. Hematuria and proteinuria persisted, and IgA nephropathy was suspected. Since there were some reports of IgA nephropathy caused by infliximab, we switched from infliximab to vedolizumab. After the change to vedolizumab, Crohn's disease worsened, and there was a marked increase in proteinuria. Fever and abdominal symptoms worsened, and infliximab was administered again. Thereafter, Crohn's disease improved rapidly, and decrease in the urinary protein level was noted. OB: Occult blood urine; U-TP/Cre: Urinary protein creatinine ratio; PCDAI: Pediatric Crohn's disease activity index; y: years; m: Months.

However, TNF-α inhibitors were also reported to induce systemic vasculitis and several types of glomerulonephritis, such as minimal-change disease group, membranous nephropathy, IgAN, and lupus nephritis[94-98].

Although the following hypotheses have been suggested and the following possibilities have been proposed, the mechanism of vasculitis associated with anti-TNFα therapy is unclear: (1) TNFα/anti-TNF α immune complexes may deposit in small blood vessels and induce local complementary activation [94]; (2) A cytokine imbalance with a shift from a Th1 profile to a Th2 profile could induce symptoms associated with antibody-mediated immunity[95]; and (3) In glomerulonephritis, immune complexes are thought to form by cross-reactivity of Gd-IgA1 and anti-drug antibodies to the glycan structures of TNFα inhibitors. They are deposited in the mesangium and can induce IgAN[96].

The reported detection rate of antinuclear antibodies in patients treated with anti-TNFα ranged from 29% to 76.7%. Immunological abnormalities can lead to glomerulonephritis, including membranous glomerulonephritis and lupus nephritis[97,98].

CONCLUSION

In many cases, proteinuria progressed simultaneously with CD; moreover, it is possible that IgANand Crohn's disease occur in parallel. The factors governing the simultaneous occurrence of IgAN and Crohn's disease is still unknown; however, the involvement of biologics has been pointed out. Although the etiology of CD-IgAN is not fixed and various factors are thought to be involved, the stabilization of the disease condition of CD is believed to help treat IgAN.

FOOTNOTES

Author contributions: Tamura H reviewed the patient's clinical data, wrote the initial draft of the manuscript, contributed to writing the manuscript, and revised the final version of the manuscript.

Conflict-of-interest statement: The author declare that they have no conflicts of interest.

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S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 79-97

DOI: 10.5662/wjm.v13.i3.79

ISSN 2222-0682 (online)

MINIREVIEWS

Immunotherapy for advanced gastric cancer

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Specialty type: Medical laboratory technology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D, D
Grade E (Poor): 0

P-Reviewer: Che XF, China; Liu L, China; Qin SS, China

Received: April 1, 2023
Peer-review started: April 1, 2023
First decision: May 4, 2023
Revised: May 11, 2023
Accepted: May 31, 2023
Article in press: May 31, 2023
Published online: June 20, 2023

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Abstract

Gastric cancer (GC) is believed to be the fifth most common cancer and the third most common cause of death worldwide. Treatment techniques include radiation, chemotherapy, gastrectomy, and targeted treatments are often employed. Some hopeful results from the development of GC immunotherapy have already changed treatment approaches. Along with previous combination medicines, new immunotherapies have been developed that target distinct molecules. Despite ongoing studies into the current therapeutic options and significant improvements in this field, the prognosis for the ailment is poor. Since there are few treatment options and a delay in detection, the illness actually advances, spreads, and metastasizes. The bulk of immunotherapies in use today rely on cytotoxic immune cells, monoclonal antibodies, and gene-transferred vaccines. Immune checkpoint inhibitors have become more popular. In this review, we sought to examine the viewpoint and development of several immunotherapy treatment modalities for advanced GC, as well as the clinical results thus far reported. Additionally, we outlined tumor immune escape and tumor immunosurveillance.

Key Words: Immunotherapy; Advanced gastric cancer; Personalized medicine; Biomarkers; Chemotherapy; Cancer vaccine

Core Tip: Throughout the globe, gastric cancer (GC) is ranked as the fifth most frequent cancer and the third most common cause of death. Chemotherapy, radiation, stomach resection, and targeted treatments are common treatment modalities. The development of immunotherapy for GC has already produced some encouraging outcomes and changed the treatment process. Currently, new immunotherapies that target novel molecules, as well as other combination treatments, have been developed. Immune checkpoint inhibitors are being used more and more often. In this review, we sought to examine the viewpoint, development, and reported clinical results of several immunotherapy treatment modalities for advanced GC patients.

Citation: Leowattana W, Leowattana P, Leowattana T. Immunotherapy for advanced gastric cancer. *World J Methodol* 2023; 13(3): 79-97
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/79.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.79

INTRODUCTION

The third leading cause of cancer-related mortality is gastric cancer (GC), which includes adenocarcinomas of the gastroesophageal junction (GEJ) and stomach. GC is the fifth most frequent cancer around the globe. Eastern Europe, Eastern Asia, and South America have the highest prevalence rates of GC. The majority of patients in Western nations receive their diagnoses at an advanced stage, which is defined by metastatic dissemination that is inoperable[1-3]. Long-term disease control has not yet been accomplished, despite the emergence of novel treatments. As a result, advanced GC (AGC) has a terrible prognosis, with a 5-year survival rate of only 10%. While cardiac GC is more common in North America, Australia, and the United Kingdom, non-cardiac GC is more common in Eastern Asia. The most well-known cause of GC is *Helicobacter pylori* (*H. pylori*) infection, while Epstein-Barr virus (EBV) infection has also been associated with the development of GC. Cigarette smoking, obesity, a high salt intake, a poor intake of fruits and vegetables, and a high intake of salted preserved foods are some lifestyle choices that have been linked to an increased risk of GC[4,5].

The prognosis for AGC is still bleak despite recent improvements in multimodal therapy. The GC's extremely complicated molecular basis is one of the factors contributing to its dismal prognosis. Numerous genetic and epigenetic changes, including differential gene expression, gene mutations, DNA/histone methylation, and somatic copy number changes, have been shown to contribute to the aggressive phenotype of GC. No encouraging and treatable causes of GC have yet been discovered, regardless of the fact that it is a diverse disease that is probably caused by several genetic and epigenetic abnormalities[6-8]. Although no successful therapies based on molecular characterization have been created yet, the creation of more effective treatment strategies based on novel molecular data may be feasible in the future. Nowadays, the median survival time with the best supportive care varies from 4 mo to 12 mo with standard cytotoxic treatment. Throughout the last several decades, advances in knowledge of cancer's molecular etiology and biology have resulted in the creation of innovative targeted therapy techniques that have led to higher survival rate in some contexts. These targeted therapies are also offered as small molecule inhibitors and monoclonal antibodies (mAbs), most of which are tyrosine kinase inhibitors (TKIs). Therefore, current systemic therapies for metastatic GC combine cytotoxic chemotherapy with first- and second-line therapies using targeted medicines such as trastuzumab and ramucirumab, respectively. Additionally, the establishment of immune checkpoint inhibition in the past ten years has been recognized as a significant medical and scientific advancement in the battle against malignancy; however, studies looking at the use of immunotherapy in GCs, either as a single agent or in conjunction with cytotoxic chemotherapy, have only produced limited authorization in the second-line setting, after the failure of the initial treatment, with comparably modest rates of response ranging between 5% and 30%[9-11]. The goal of this review is to quickly highlight some of the most promising immunotherapies now being researched while also summarizing the currently investigated and authorized treatments for GC.

EPIDEMIOLOGY

GC is often asymptomatic in the early stages, making it challenging to purposefully discover. Late diagnosis is mostly to account for the high mortality of GC. In order to lower GC mortality, early identification and treatment are essential[12,13]. Some East Asian nations with high relative risks have implemented their own extensive screening programs. Regardless of whether an individual has symptoms, upper gastrointestinal endoscopy is available in these nations. Endoscopic screening can lower GC mortality by 67% compared to radiography screening, according to Japanese population-

based cohort research[14]. Endoscopy was the most economical screening technique, according to data from the National Cancer Screening Program in South Korea, which may enhance survival rates[15]. In addition, the quality of endoscopic imaging has recently dramatically improved. Endoscopy using image enhancement techniques, including narrow-band imaging, can help with early GC discovery and complete endoscopic resection. Indeed, these active screening methods have resulted in earlier discovery and a higher survival rate[16,17]. The 5-year relative survival rate in Japan between 2009 and 2011 was reported at 66.6%, with more than 60% of cases of GC being discovered at stage I, according to population-based statistics collected countrywide[18]. In contrast to Asian nations, Western nations lack widespread screening programs, which causes discovery to occur later. According to the SEER-based CONCORD-2 research in the United States, only 22.1% (2001-2003) or 24.9% (2004-2009) of patients had a localized stage at diagnosis, and in comparison to Asian countries, the stated 5-year survival rate was lower (26.1% from 2001-2003 and 29.0% from 2004-2009)[19]. Western nations have a higher prevalence of GC in the proximal third. Proximal GCs are more likely to be in an advanced stage at presentation, be larger, and have a histology that is poorly differentiated. The poorer survivability in the West may be explained by this[20,21].

In particular, for intestinal-type distal carcinoma, *H. pylori* infection raises cancer risk. In comparison to Europe (47.0%) and North America (37.1%), Asia has a greater prevalence of *H. pylori* (54.7%). It is well known that the elimination of *H. pylori* causes the symptoms of atrophic gastritis to return. It is hypothesized that intestinal metaplasia in chronic gastritis caused by *H. pylori* is less likely to improve with *H. pylori* eradication than atrophic gastritis alone. The comparative risk of getting GC following the removal of *H. pylori* was 0.65, according to a meta-analysis. While extensive intestinal metaplasia occurs, there is little data to suggest that treating the *H. pylori* infection lowers the risk of GC[22]. Yan *et al*[23] recently completed a randomized, placebo-controlled study to assess the long-term impact of *H. pylori* eradication medication on the incidence and death of GC in a high-risk group. A total of 1630 asymptomatic *H. pylori*-infected people were randomly allocated to undergo conventional triple treatment for *H. pylori* eradication (n = 817) or a placebo (n = 813), and were then followed up for 26.5 years. There were 35 people in the placebo group (4.31%) and 21 people (2.57%) in the treatment group who tested positive for GC. *H. pylori* medication patients had a lower chance of developing GC in comparison to the placebo group [hazard ratio (HR): 0.57; 95%CI: 0.33-0.98]. They concluded that eradicating *H. pylori* may provide long-term protection against GC in high-risk groups, particularly in infected individuals who did not have precancerous gastric lesions at baseline.

MOLECULAR GENETIC

GC is a molecularly diverse malignancy with several genetic mutations. Based on histological results, the Lauren classification originally divided GC into two types (intestinal and diffuse). However, it is unable to reliably predict treatment outcomes and prognosis because it fails to take into consideration the variable nature of GC[24]. The Cancer Genome Atlas (TCGA), by classifying patients into four distinct molecular subtypes based on six different molecular subtypes, has provided a detailed depiction of the genetic underpinnings of GC: (1) Tumors positive for EBV, which display recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of Janus-associated kinase 2, programmed cell death ligand-1 (PD-L1), and PD-L2; (2) microsatellite unstable tumors have high mutation rates, including changes in the genes producing proteins that can be targeted to cause cancer; (3) genomically stable tumors that are enriched for mutations in Ras homolog family member A or fusions involving RHO family GTPase-activating proteins as well as diffuse histological variation; and (4) chromosomally unstable tumors with pronounced aneuploidy and localized receptor tyrosine kinase amplification (Table 1)[25]. Moreover, because of the complexity of GC, the Asian Cancer Research Group (ACRG) subtypes were introduced to enhance classification[26-28]. Although the TCGA classification is extensive and provides clinically useful information, no classification method includes all clinically meaningful signals. This would be required to best lead a customized strategy (Figure 1).

Another group, ACRG, developed a different categorization scheme by dividing GC into four subgroups based on gene expression data: Microsatellite stable with tumor protein 53 (TP53) functional loss (MSS/TP53-), MSS/TP53+ (TP53 intact), MSS/Epithelial-mesenchymal transition (EMT) (EMT signatures), and microsatellite instability (MSI) (23%)[7]. The MSI group from TCGA exhibited similarities to the MSI subtype from ACRG. Despite the fact that the EBV-positive, genomically stable, and chromosomal instability subtypes in TCGA were somewhat enriched in the MSS/TP53+, MSS/EMT, and MSS/TP53-subtypes in ACRG, respectively, there were still a number of differences seen in other subtypes. This demonstrates the distinctiveness of these two classes from TCGA and ACRG. The ACRG also included survival data, which showed the prognostic efficacy of each subtype categorization, in contrast to the TCGA. Following MSS/TP53+, MSS/TP53-, and MSS/EMT GC, MSI GC was shown to have the highest overall survival (OS) and the lowest frequency of recurrence[29]. There has been evidence of ethnic influences on molecular features. Despite the fact that the TCGA data did not reveal any significant biological differences between East Asian and other populations, certain variations in pathway-level gene expression were detected. For instance, East Asian patients had

Table 1 The Cancer Genome Atlas has presented a thorough depiction of the molecular basis of gastric cancer[25]

Subtypes	EBV-positive	MSI	GS	CIN
Frequency, %	8.8	21.7	19.7	449.8
Demographic	Male patients (81%)	Old age (median 72 yr)	Young age (median 59 yr)	Not specific
Histology	Not specific	Not specific	Diffuse	Intestinal
Main location	Fundus and body	Fundus, body, and antrum	Mostly diffuse subtype	Majority of tumors at the GEJ
Molecular alterations	EBV-CIMP: (1) PD-L1/2, JAK2 overexpression; (2) Mutation in PIK3CA, ARID1A, and BCOR; (3) CDKN2A silencing; (4) Immune cell signaling; and (5) Rare TP53 mutations	Gastric-CIMP: (1) Hypermutation in TP53, PIK3CA, ERBB2/3, and ARID1A; (2) MLH1 silencing; (3) Mitotic pathways activation; and (4) Commune changes in the genes of *CMH1*	(1) *CDH1* and *RHOA* mutation; (2) CLDN18-ARHGAP fusion; (3) Cell adhesion; (4) Angiogenesis pathways enriched; and (5) Rare *TP53* mutations	(1) TP53 mutation; (2) RTK-RAS activation; and (3) Mutations of SMAD4 and APC
Potential therapeutic points	(1) PIK3CA; (2) JAK2; and (3) PD-L1/L2	(1) PIK3CA; (2) ERBB2/3; (3) EGFR; (4) PD-L1; and (5) MLH1 silencing	(1) RHOA; and (2) CLDN18	(1) RTKs; (2) EGFR; (3) VEGFA; (4) CCNE1; (5) CCND1; and (6) CDK6

APC: Adenomatous polyposis coli; ARHGAP: Rho GTPase activating protein; ARID1A: AT-rich interactive domain-containing protein 1A; BCOR: B-cell lymphoma 6 corepressor; CCND1: Cyclin-D1; CCNE1: Cyclin-E1; CDH1: Cadherin 1; CDK6: Cyclin-dependent kinase 6; CDKN2A: Cyclin-dependent kinase inhibitor 2A; CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; CLDN18: Claudin-18; CMH1: Hypertrophic cardiomyopathy-1; GS: Genomically stable; GC: Gastric cancer; EBV: Epstein-Barr virus; EGFR: Epithelial growth factor receptor; ERBB2/3: Erb-b2 receptor tyrosine kinase 2/3; GEJ: Gastroesophageal junction; JAK2: Janus-associated kinase 2; MLH1: MutL protein homolog 1; MSI: Microsatellite instability; PD-L1/2: Programmed death ligand-1/2; PI3K: Phosphatidylinositol-3-kinase; RAS: Rat sarcoma virus; RHOA: Ras homolog family member A; RTKs: Receptor tyrosine kinases; SMAD4: Mothers against decapentaplegic homolog 4; TP53: Tumor protein 53; VEGFA: Vascular endothelial growth factor A.

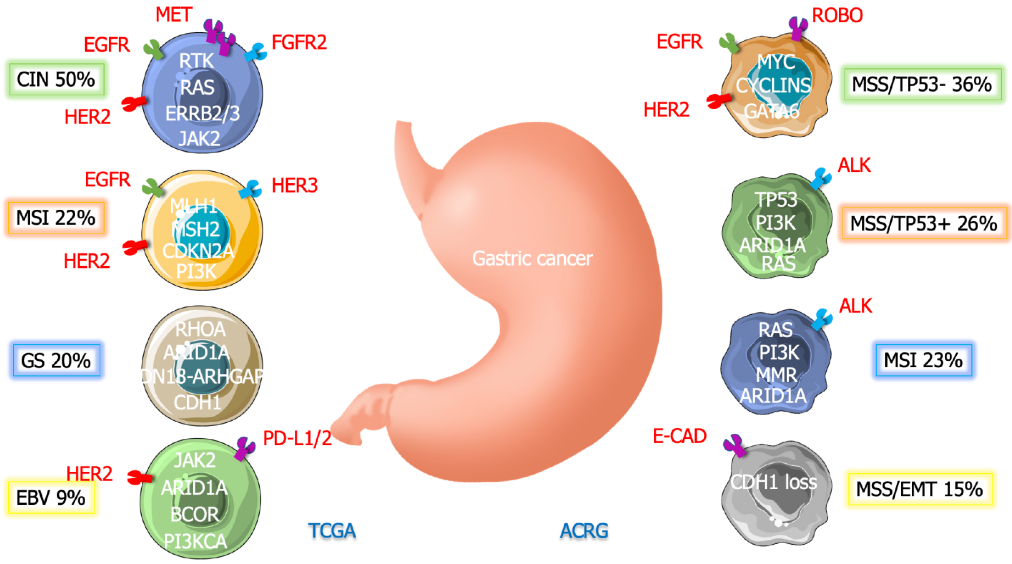

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Figure 1 The molecular findings in gastric cancer by The Cancer Genome Atlas compared with the Asian Cancer Research Group. TCGA: The Cancer Genome Atlas; ACRG: Asian Cancer Research Group; EGFR: Epithelial growth factor receptor; CIN: Chromosomal instability; HER: Human epidermal growth factor receptor; FGFR2: Fibroblast growth factor receptor 2; MSI: Microsatellite instability; GS: Genomically stable; EBV: Epstein-Barr virus; PD-L1/2: Programmed death ligand-1/2; RTKs: Receptor tyrosine kinases; RAS: Rat sarcoma virus; ERBB2/3: Erb-b2 receptor tyrosine kinase 2/3; JAK2: Janus-associated kinase 2; MLH1: MutL protein homolog 1; MSH: Melanocyte-stimulating hormones; CDKN2A: Cyclin-dependent kinase inhibitor 2A; PI3K: Phosphatidylinositol-3-kinase; ARID1A: AT-rich interactive domain-containing protein 1A; BCOR: B-cell lymphoma 6 corepressor; MSS: Microsatellite stable; TP53: tumor protein 53; MYC: Myelocytomatosis oncogene; GATA6: GATA binding protein 6; MMR: Measles-mumps-rubella; CDH1: Cadherin 1; EMT: Epithelial-mesenchymal transition; E-CAD: E-cadherin; ALK: Anaplastic lymphoma kinase.

elevated telomerase regulatory pathway expression and decreased hypoxia inducible factor-1-alpha transcription factor network expression[29]. Another study found that Asian and non-Asian GC patients had significantly different tumor immunity profiles. Non-Asian cases of GC were connected to an enrichment of T-cell gene expression patterns and a lesser expression of the immunosuppressive marker FOXP3 as compared to Asian cases of GC[30-32]. Further research with a sufficient sample size is

required to better understand how racial variations affect molecular background.

ANTITUMOR IMMUNE RESPONSES

Host immunity is now widely recognized as being important in the prevention of cancer. The findings suggest that our immune system can inhibit cancer growth through a mechanism known as immune surveillance. Dying cancer cells may generate and disseminate tumor-specific and tumor-related antigens that can be ingested and processed by tissue-resident dendritic cells (DCs). These cells then develop into antigen-presenting cells (APCs) in the presence of a favorable microenvironment, which is typically rich in activator molecules known as danger-associated molecular patterns, which are released by dying cancer cells[33,34]. Mature APCs must effectively deliver tumor antigens in the form of peptides to CD8+ T lymphocytes *via* major histocompatibility complex (MHC) class I molecules and CD4+ T lymphocytes *via* MHC class II molecules in order to trigger effective anticancer immunity. The most effective tumor antigens are non-self or altered proteins, such as those produced by somatic mutations in genes expressed by tumor cells or those encoded by viruses. Effective activation of CD8+ T cells requires both antigen presentation as a first signal and the presence of costimulatory molecules as a second signal. Once these cells are activated, they enter the tumor bed and multiply. Furthermore, T lymphocytes are able to recruit other immune cells, such as natural killer (NK) cells and M1 macrophages, that are able to further aid in the destruction of cancer cells. Additionally, T lymphocytes themselves are able to directly destroy cancer cells through the production of cytokines. This process is essential for the body to effectively fight cancer cells. The fact that not all traditional cytotoxic chemotherapeutic medicines are immunosuppressive is important in light of the practical use of immunotherapeutic methods. Recent research has revealed that a number of medications commonly used in medical practice may have the ability to kill tumor cells *via* an immunogenic cell death pathway. This pathway has the potential to activate powerful innate and adaptive anticancer immune responses. Medications found to have this effect include doxorubicin, mitoxantrone, bortezomib, oxaliplatin, and cyclophosphamide. Such findings have the potential to revolutionize cancer treatments, offering new hope for cancer patients across the world[35,36].

CANCER CELL IMMUNOESCAPE

T-cell receptor (TCR), co-stimulatory molecules, including CD28, and cytokines are all required for effective activation of cytotoxic T cells. Tumor cells can resist immunosuppression through a variety of mechanisms. The production of several co-inhibitory receptors is one of the ways to prevent T cell activation in the body. PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3) are examples of inhibitory receptors that bind to tumor cell ligands and block T cell activation. The overexpression of these receptors induces the inhibition of coinhibitory pathways, such as the B7-CD28-CTLA-4 family. This helps to ensure the physiological functioning of the immune system, which is essential in preventing tumor growth and the spread of cancer cells[37,38]. CTLA-4 is an important co-stimulatory molecule expressed on the surface of CD4+ and CD8+ T lymphocytes. It binds to molecules known as CD80 (B7-1) and CD86 (B7-2) on APCs, which are involved in the initiation and regulation of adaptive immunity. The binding of CTLA-4 to CD80 and CD86 on APCs results in the inhibition of T cell activation. Therefore, this molecule plays an important role in regulating the immune system. This inhibits TCR signaling by preventing APCs from binding to the CD28 co-stimulatory molecule. Additionally, CTLA-4 is continually produced in regulatory T cells (Treg) in order to control their immunosuppressive role. Tumor cells are thought to escape through downregulation of MHC class I expression. MHC class I molecules play a significant role in cytotoxic T cell-mediated immunity. Tumor cells have the ability to suppress immune cell activity by secreting immunosuppressive substances. These substances, such as interleukin-10, transforming growth factor-β1, galectins, tumor necrosis factor, prostaglandin E2, and vascular endothelial growth factor (VEGF), can inhibit the function of the immune cells, thus disrupting the body's natural defense system. The immunosuppressive substances also inhibit the receptors on the surface of the immune cells, making them unable to recognize and attack the tumor cells. This allows the tumor cells to grow and spread unchecked. Therefore, it is important to understand the role of these immunosuppressive substances in order to develop effective treatments for cancer[39].

By encouraging the polarization towards less cytotoxic T cell subsets and pro-inflammatory T cell subsets, the tumor microenvironment may compromise anti-tumor immunity. TH-2, TH-17, and Treg are T helper (TH) cells that are related to tumors. Tumor-associated macrophages (TAM) make up the majority of the immune cell population in the tumor microenvironment. Two separate subtypes of these macrophages, M1 and M2, exhibit anti-tumor and pro-tumorigenic actions, respectively. When a GC site was infiltrated with M2 TAM, the patient's prognosis was usually poor[40,41]. Myeloid-derived suppressor cells (MDSCs) are a diverse population of cells with the capacity to proliferate vigorously under pathological conditions like cancer. They are descended from the myeloid lineage. Both innate

and adaptive immunity against malignancies can be suppressed by these cells. Recent studies have confirmed that patients with GC are more likely to have an increased number of MDSCs. This increased presence of MDSCs in the blood samples of GC patients has been associated with poorer clinical result [42,43]. Treg cells are a significant contributor to immune suppression in the tumor microenvironment. End-stage instances of GCs were replete with Foxp3 plus CD4 plus ICOS plus effector Tregs, also known as highly suppressive Tregs[44,45].

AGC IMMUNOTHERAPY

Patients with GC exhibit "three high and three low" characteristics: High rates of incidence, metastatic condition, and mortality; low rates of early discovery, radical resection, and five-year survival. Patients might either be early-stage or advanced-stage patients. Early-stage GCs are limited to the mucosa or submucosa, regardless of the size of the lesion or the presence of lymph node metastases. AGC is defined as cancers that infiltrate into or beyond the subserosa to surrounding organs or metastasize. Middle GC is described as cancer that extends past the submucosa to penetrate the gastric muscle layer. Tumors in the advanced stage of GC include intermediate and advanced tumors. Another classification of AGC includes local unresectable GC, distant metastasis, and postoperative recurrent GC[46,47]. The therapy's efficacy and method are determined by the tumor's stage. At the moment, the primary objective of treating AGC is to ameliorate symptoms and extend patients' survival times using successive courses of chemotherapy. Systemic chemotherapy alone is becoming increasingly effective in treating AGC, yet the median survival duration with this approach is still only 4-13 mo. This demonstrates that, unfortunately, the prognosis for this disease is far from favorable. In spite of the progress made in treating AGC with chemotherapy, more research and development are needed to improve the outlook for those afflicted with it. As a result, both more effective chemotherapy medications and regimens with fewer hazardous side effects, as well as innovative therapeutic paradigms, such as targeted therapy and immunotherapy, should be investigated.

Immunotherapy is a form of cancer treatment that employs the body's own immune system to fight cancer cells. It is divided into two types: Passive and active. Passive immunotherapy is a form of therapy that utilizes antibody therapies to target cancer cells. On the other hand, active immunotherapy focuses on boosting the body's immune response against tumor cells. Examples of active immunotherapy include vaccinations and chimeric antigen receptors (CAR). CARs are designed to recognize and bind to tumor cells, triggering the body's immune system to attack the cancer cells. However, with passive immunotherapy, immune system components, such as mAbs, are generated outside of the body. Immunotherapies nowadays are frequently based on cytotoxic T cells, mAbs, and gene-transfected vaccines. Immune checkpoint inhibitors (ICIs) have become more popular since ipilimumab was originally approved in 2011 for the treatment of metastatic BRAF-negative melanoma. Following more than 1000 clinical investigations, ICIs are now recognized as a therapeutic approach in the management of both solid organ and hematologic malignancies[48-50].

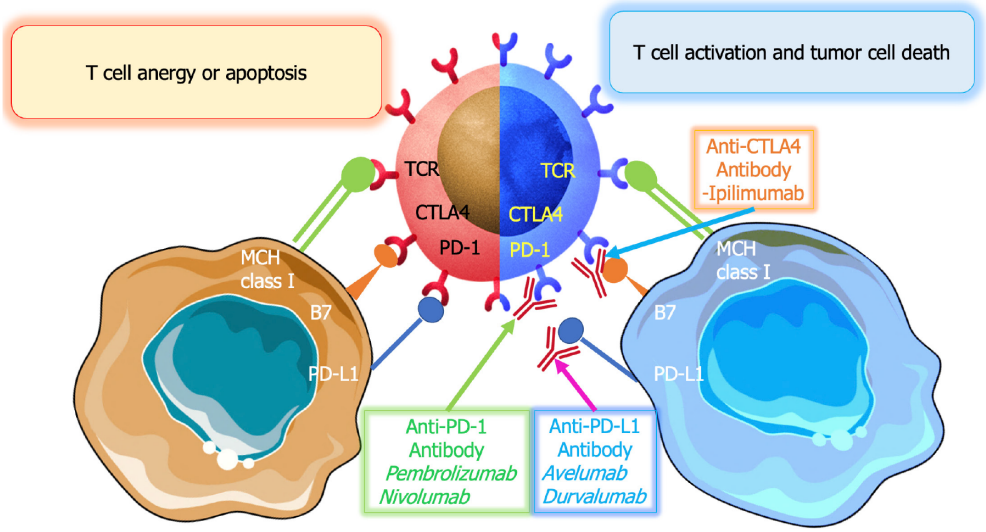
PASSIVE IMMUNOTHERAPY

ICIs

ICIs are a type of drug used in cancer immunotherapy. They work by preventing the T cells from the immune system from being suppressed by the tumor. Targeting CTLA-4, PD-1, and PD-L1, three essential ICI drug types were previously created for preclinical and clinical research. In order to lessen or decrease CD28 signaling, CTLA-4, a CD28 homolog, may bind to B7-1 and B7-2 with a greater affinity than CD28. However, PD-1 encourages tumor cell survival by inducing apoptosis in T lymphocytes that have been activated. Pharmacological blockage of its route may damage the function of immune cells such as B cells and NK cells since PD-1 is extensively expressed in many immune cells[51-53] (Figure 2).

Pembrolizumab

Pembrolizumab was the first monoclonal antibody designed to target PD-1. In 2017, the FDA authorized it for the treatment of advanced non-small cell lung cancer. In a phase I study, the anti-tumor efficacy, safety, and tolerability of pembrolizumab were assessed in 39 patients with AGC (KEYNOTE-012). According to the findings, pembrolizumab provides promising anti-tumor effectiveness with a manageable amount of toxicity in these individuals. The positive outcomes of this experiment spurred the conduct of more clinical trials testing PD-1-blocking therapy[52]. In 2018, Fuchs *et al*[53] performed the KEYNOTE-059 trial, a phase 2 global, open-label, single-arm, multicohort study that recruited 259 AGC patients from 16 countries to assess the safety and effectiveness of pembrolizumab. They discovered an 11.6% objective response rate (ORR) with a 2.3% full response rate. The median response time was 8.4 mo. ORR and median response duration were 15.5% and 16.3 mo in PD-L1-positive patients, respectively, compared to 6.4% and 6.9 mo in PD-L1-negative patients. One or more grade 3-5



DOI: 10.5662/wjm.v13.i3.79

Figure 2 Immune check point inhibitors for advanced gastric cancer treatment. CTLA4: Cytotoxic T-lymphocyte-associated protein 4; TCR: T-cell receptor; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death ligand-1; MCH: Melanin-concentrating hormone.

treatment-related adverse events (TRAEs) occurred in 46 individuals (17.8%). Two patients (0.8%) stopped due to TRAEs, and two fatalities were deemed treatment-related. They concluded that in patients with AGC or advanced gastroesophageal junction cancer (AGEJC) who had previously undergone at least two lines of treatment, Pembrolizumab monotherapy displayed promising efficacy and manageable safety. Patients with PD-L1-positive malignancies experienced long-term responses. Furthermore, Shitara *et al*[54] undertook a randomized, open-label, phase 3 research study in 30 countries to evaluate Pembrolizumab *vs* Paclitaxel in 592 patients with AGC or AGEJC who had progressed on platinum- and fluoropyrimidine-based first-line treatment. With a combined positive score (CPS) of 1 or higher, they found that 326 people passed away (151 of 196 patients in the Pembrolizumab group and 175 of 199 patients in the Paclitaxel group). With Pembrolizumab, the median OS was 9.1 mo, while with Paclitaxel, it was 8.3 mo. With Pembrolizumab, the median progression-free survival (PFS) was 1.5 mo, while with Paclitaxel, it was 4.1 mo. Furthermore, grade 3-5 TRAEs occurred in 42 (14%) of the 294 patients treated with Pembrolizumab and 96 (35%) of the 276 patients treated. They concluded that Pembrolizumab, when used as a second-line treatment for AGC or AGEJC with a PD-L1 CPS of 1 or higher, did not substantially increase OS when compared to Paclitaxel. However, Paclitaxel has a worse safety profile than Pembrolizumab (Table 2).

Bang *et al*[55] conducted the KEYNOTE-059 multicohort, phase 2, non-randomized trial in 56 patients with AGC or AGEJC in 2019. They discovered that the ORR was 60.0% in patients receiving combination treatment and 25.8% in individuals receiving Pembrolizumab monotherapy. Also, in the combination therapy cohort, 19 patients (76.0%) experienced grade 3/4 treatment-related side events; none were fatal. In the monotherapy cohort, seven patients (22.6%) experienced grade 3-5 TRAEs; one fatality was ascribed to a TRAE (pneumonitis). They concluded that in patients with previously untreated AGC or AGEJC, Pembrolizumab displayed anticancer efficacy and was well tolerated as monotherapy and in combination with chemotherapy. One year later, Kawazoe *et al*[56] performed a non-randomized, multicenter, open-label phase IIb study, KEYNOTE-659, in 54 AGC or AGEJC patients with human epidermal growth factor receptor 2-negative and PD-L1-positive to assess the safety and efficacy of Pembrolizumab combined with chemotherapy [S-1 plus oxaliplatin (SOX)] as the first-line treatment. They observed that the ORR and disease control rate (DCR) were, respectively, 72.2% and 96.3%. In terms of DOR, time to response (TTR), PFS, and OS, the median values were not attained at 1.5 mo, 9.4 mo, or not reached at all. Patients with CPSs of 1 to 10 had an ORR of 73.9%, whereas those with CPSs of more than 10 had an ORR of 70.0%. Grade 3 TRAEs occurred in 57% of participants. The most frequent grade 3 adverse events were decreased platelet count (14.8%), decreased neutrophil count (13.0%), colitis (5.6%), and adrenal insufficiency (5.6%). They came to the conclusion that SOX and Pembrolizumab demonstrated potential efficacy and a manageable safety profile for the first management of AGC or AGEJC.

Pembrolizumab was also examined in conjunction with a targeted therapy such as Lenvatinib in an open-label, single-arm, phase 2 study in 29 patients with AGC to assess the combination of Lenvatinib and Pembrolizumab. They discovered that 69% of the patients had an ORR. Hypertension, proteinuria, and a fall in platelet count were the most frequently occurring grade 3 TRAEs, occurring in 11 (38%) of the patients, five (17%) of the patients, and two (7%) of the patients, respectively. No grade 4 adverse events, no serious adverse events, and no fatalities associated with the therapy were reported. They

Table 2 Immunotherapies for advanced gastric cancer patients

Ref.	Drug(s)	Number of patients	Study phase	ORR (%)	Median OS (months)	Median PFS (months)	Results
Muro *et al* [52]	Pembrolizumab	39	1b	33	11.4	1.8	Pembrolizumab demonstrated a reasonable safety profile and potential antitumor efficacy in metastatic PD-L1-positive GC, warranting further exploration in phase 2 and 3 studies
Fuchs *et al* [53]	Pembrolizumab	259	2	15.5	5.6	2.0	Pembrolizumab is a potential new therapy option for AGC or AGEJC that has progressed following second-line treatment, demonstrating high and persistent responses. Pembrolizumab has a mechanism of action, duration of response, and toxicity profile that differs from and does not overlap with conventional treatment for gastroesophageal adenocarcinoma
Shitara *et al* [54]	Pembrolizumab *vs* Paclitaxel	395	3	-	9.1/8.3 (Pem/Pac)	1.5/4.1 (Pem/Pac)	When compared to Paclitaxel, Pembrolizumab did not significantly improve overall survival when administered as a second-line therapy for AGC or AGEJC with PD-L1 CPS of 1 or higher
Bang *et al* [55]	Pembrolizumab *vs* Pembrolizumab plus chemotherapy	56	2	25.8/60.0 (Pem/Pem+Chem)	13.8/20.7 (Pem/Pem+Chem)	3.3/6.6 (Pem/Pem+Chem)	Pembrolizumab combined chemotherapy showed acceptable tolerability and potential anticancer efficacy in AGC or AGEJC, independent of PD-L1 expression. In patients with PD-L1 CPS ≥ 1, pembrolizumab monotherapy revealed good antitumor efficacy and acceptable safety
Kawazoe *et al*[56]	Pembrolizumab plus chemotherapy	54	2b	72.2	Not reached	9.4	For the first-line treatment of AGC or AGEJC patients, chemotherapy with Pembrolizumab shown good effectiveness and a tolerable toxicity profile
Kawazoe *et al*[57]	Pembrolizumab plus Lenvatinib	29	2	69.0	Not reached	7.1	In patients with AGC, the combination of Lenvatinib and Pembrolizumab demonstrated promising anti-tumor effectiveness while maintaining a tolerable safety profile
Shitara *et al* [58]	Pembrolizumab *vs* Pembrolizumab plus chemotherapy *vs* chemotherapy	763	3	-	10.6/11.1 (Pem/chem) CPS ≥ 1; 17.0/10.8 CPS ≥ 10		In individuals with untreated AGC or AGEJC, Pembrolizumab was shown to be noninferior to chemotherapy, with less adverse effects. Pembrolizumab alone or in combination with chemotherapy did not outperform treatment in terms of OS and PFS
Kwon *et al* [60]	Pembrolizumab	18	2	55.6	-	-	A subset of MSI-H GC patients with certain immunological responses at baseline, such as stronger TMB, abundant T cell infiltration, more TCR clonal diversity, and less stem-like exhausted T cells, may not require anything more than anti-PD-1 monotherapy

Yamaguchi *et al*[63]	Pembrolizumab plus SOX *vs* Pembrolizumab plus SP	100	2b	72.2/80.4	16.9/17.1	9.4/8.3	In Japanese patients with PD-L1 positive, HER-2 negative AGC or AGEJC, the combination of Pembrolizumab plus SOX or SP as first-line treatment indicated high efficacy and reasonable tolerability
Lee *et al*[64]	Pembrolizumab plus Trastuzumab plus Capecitabine plus Cisplatin	43	1b/2	76.7	19.3	8.6	The use of a quadruplet combination as first-line therapy (Pembrolizumab plus Trastuzumab plus Capecitabine plus Cisplatin) resulted in tumor decrease in HER-2-positive AGC
Satake *et al* [65]	Pembrolizumab *vs* Pembrolizumab plus chemotherapy *vs* chemotherapy	187	3	22.6/37.7 (Pem/chem); 26.9/31.8 (PD-L1 CPS ≥ 10)	22.7/13.8 (Pem/chem); 28.5/14.8 (PD-L1 CPS ≥ 10)	4.1/6.5 (Pem/chem); 7.2/6.9 (PD-L1 CPS ≥ 10)	Pembrolizumab monotherapy was related with statistically better OS results in patients with AGC or AGEJC with PD-L1 CPS ≥ 1 and CPS ≥ 10 tumors as compared to chemotherapy alone. When compared to chemotherapy, Pembrolizumab monotherapy had a better tolerability profile
Janjigian *et al*[66]	Nivolumab plus Ipilimumab *vs* Nivolumab	160	1/2	24/12 (Niv+Ipi/Niv)	6.9/6.2 (Niv+Ipi/Niv)	1.6/1.4 (Niv+Ipi/Niv)	Nivolumab and Nivolumab in combination with Ipilimumab provide a viable treatment option for individuals with AGEJC
Kang *et al* [67]	Nivolumab *vs* placebo	493	3	11.2/0.0	5.32/4.14	1.61/1.45	Nivolumab might be a potential therapy option for people with AGC or AGEJC who have been highly pretreated
Boku *et al* [69]	Nivolumab plus SOX *vs* Nivolumab plus CapeOX	77	2	57.1/76.5	Not reached	9.7/10.6	In these individuals, Nivolumab in conjunction with SOX or CapeOX was well tolerated and showed potential efficacy
Nakajima *et al*[71]	Nivolumab plus Paclitaxel plus Ramucirumab	43	1/2	37.2	13.1	5.1	As a second-line treatment for AGC, Nivolumab in combination with Paclitaxel and Ramucirumab shown promising antitumor activity with tolerable tolerability
Janjigian *et al*[72]	Nivolumab plus chemotherapy *vs* chemotherapy	1,581	3	51/41	14.4/11.1	7.7/6.1	Nivolumab in conjunction with chemotherapy is being considered as a new standard first-line treatment for these individuals
Shah *et al* [73]	Andecaliximab plus Nivolumab *vs* Nivolumab	141	2	10/7	7.1/5.9	-	When compared to Nivolumab alone, the combination of Andecaliximab and Nivolumab exhibited a favorable safety profile but did not boost efficacy in these people
Kang *et al* [74]	Nivolumab plus oxaliplatin-based chemotherapy *vs* placebo plus oxaliplatin-based chemotherapy	724	2/3	-	17.45/17.15	10.45/8.34	In these patients, Nivolumab in conjunction with oxaliplatin-based chemotherapy improved PFS but not OS
Bang *et al* [76]	Avelumab *vs* chemotherapy	371	3	2.2/4.3	4.6/5.0	1.4/2.7	As compared to chemotherapy, treating these patients in the third-line setting with single-agent Avelumab did not improve OS or PFS. Avelumab, on the other hand, had a more manageable toxicity profile than chemotherapy
Moehler *et*	Avelumab *vs*	499	3	-	10.4/10.9	-	In patients with AGC or AGEJC

al[77]	chemotherapy	in general, or in a specified PD-L1-positive population, Avelumab maintenance therapy did not give a superior OS when compared to continuing chemotherapy

AGC: Advanced gastric cancer; AGEJC: Advanced gastroesophageal junction cancer; CapeOX: Capecitabine plus oxaliplatin C; Chem: Chemotherapy; CPS: Combined positive score; HER-2: Human epidermal growth factor receptor 2; MIS-H: High microsatellite instability; Niv: Nivolumab; Niv+Ipi: Nivolumab plus Ipilimumab; ORR: Objective response rate; OS: Overall survival; Pac: Paclitaxel; Pem: Pembrolizumab; PD-L1: Program cell death ligand 1; PFS: Progression-free survival; SOX: S-1 plus oxaliplatin; SP: S-1 and cisplatin; TCR: T-cell receptor; TMB: Tumor mutational burden.

concluded that Lenvatinib and Pembrolizumab had potential anti-tumor effectiveness with a tolerable safety profile in AGC patients[57]. A phase 3 KEYNOTE-062 randomized, controlled, and partially blinded interventional trial was carried out by Shitara *et al*[58] in 2020 in 763 patients from 29 countries who had untreated AGC or AGEJC and a PD-L1 CPS of 1 or above. Every three weeks, participants were given a random choice between receiving Pembrolizumab 200 mg, chemotherapy plus placebo, or chemotherapy combined with cisplatin 80 mg/m^2/d on day 1 plus fluorouracil 800 mg/m^2/d on days 1 through 5. After a median follow-up of 29.4 mo (median OS, 10.6 *vs* 11.1 mo), they found that Pembrolizumab was non-inferior to chemotherapy for OS in patients with a CPS of 1 or higher. Chemotherapy was not better than Pembrolizumab monotherapy in individuals with a CPS of 1 or higher. Patients with a CPS of 10 or higher experienced longer OS with pembrolizumab compared to chemotherapy (median OS, 17.4 *vs* 10.8 mo); however, the difference was not statistically significant. In terms of OS in patients with a CPS of 1 or higher (12.5 *vs* 11.1 mo), CPS of 10 or greater (12.3 *vs* 10.8 mo), or PFS in patients with a CPS of 1 or higher (6.9 *vs* 6.4 mo), Pembrolizumab in combination with chemotherapy did not outperform treatment. Pembrolizumab was found to be noninferior to chemotherapy in patients with untreated AGC or AGEJC, with fewer side effects. For the OS and PFS end points assessed, Pembrolizumab or Pembrolizumab with chemotherapy were not superior to chemotherapy. Additionally, Pembrolizumab was evaluated in PD-L1-positive (CPS ≥ 10) AGC or AGEJC patients from KEYNOTE-062 (n = 182), KEYNOTE-061 (n = 108), and KEYNOTE-059 (n = 46) to better define the specificity of CPS as a predictor of clinical outcomes. This thorough study found that pembrolizumab improved clinical outcomes in patients with CPS ≥ 10 AGC or AGEJC across many lines of treatment[59].

In 2021, Kwon *et al*[60] conducted a phase 2 study of Pembrolizumab in 18 patients with advanced high MSI (MSI-H) GC, including serial and multi-region tissue samples as well as serial peripheral blood testing, with a median follow-up of 19.5 mo. The findings showed that 6 patients (33.3%) had stable disease, 3 patients (16.7%) had a complete response (CR), 7 patients (38.9%) had a verified partial response (PR), and 3 patients (16.7%) had a CR, giving an ORR of 55.6% and a DCR of 88.9%. They proposed that a subgroup of MSI-H GC patients with a specific immunological response, as defined by a higher tumor mutational burden (TMB), abundant T cell infiltration, larger TCR clonal diversity, and fewer stem-like exhausted T cells at baseline, may not require anything more than anti-PD-1 monotherapy. Equally significant clinically was the finding of unfavorable genomic and immunologic characteristics from the outset, revealing a subset of MSI-H GC that may require additional therapy to benefit from PD-1 blocking. These findings indicated a combination therapy aimed at lowering Treg populations and/or augmenting and growing NK-cell numbers in this fraction. Synthetic model systems that mimic MSI-H biology, as well as extensive genomic and immunologic screening for therapeutic vulnerabilities, will be critical in identifying potential combinations for future testing. To stratify MSI-H cancers for therapy with either PD-1 blockade alone or cutting-edge combination approaches, the findings, however, signal that accurate pre- and post-treatment characterizations are attainable and will probably be needed.

When used as second-line therapy in the phase 3 KEYNOTE-061 study for patients with PD-L1 CPS > 1 AGC or AGEJC, Pembrolizumab did not significantly increase OS compared with Paclitaxel. Fuchs *et al*[61] conducted a trial in which they randomly assigned patients to receive Pembrolizumab 200 mg Q3W for 35 cycles or standard-dose paclitaxel and presented outcomes in the CPS 1, 5, and 10 populations after two years of follow-up. The findings revealed that 366 of 395 individuals (92.7%) with CPS ≥ 1 died. In the CPS ≥ 1 cohort, Pembrolizumab showed a tendency toward increased OS *vs* Paclitaxel; 24-mo OS rates: 19.9% *vs* 8.5%. With PD-L1 enrichment, Pembrolizumab gradually increased the OS benefit (CPS > 5: 24-mo rate, 24.2% *vs* 8.8%; CPS > 10: 24-mo rate, 32.1% *vs* 10.9%). Across treatment groups, the median PFS was similar (CPS > 1: HR, 1.25; CPS > 5: 0.98; CPS > 10: 0.79). The median DOR was 19.1 *vs* 5.2 mo, 32.7 *vs* 4.8 mo, and NR *vs* 6.9 mo; the ORR (Pembrolizumab *vs* Paclitaxel) was 16.3% *vs* 13.6% (CPS > 1), 20.0% *vs* 14.3% (CPS > 5) and 24.5% *vs* 9.1% (CPS > 10). Pembrolizumab was associated with fewer TRAEs than Paclitaxel (53% *vs* 84%). In 94 Asian patients with advanced PD-L1-positive (CPS > 1) AGC or AGEJC, 36 medical centers in China, Malaysia, South Korea, and Taiwan conducted the randomized, open-label, phase 3 study KEYNOTE-063, which compared Pembrolizumab *vs* Paclitaxel as second-line therapy. The results revealed that the median OS in Pembrolizumab plus Paclitaxel therapy was the same as 8 mo. The median PFS with Pembrolizumab

was 2 mo *vs* 4 mo with Paclitaxel. Pembrolizumab had a 13% ORR against Paclitaxel's 19%. Any-grade TRAEs occurred in 28 patients receiving Pembrolizumab (60%) and 42 patients receiving Paclitaxel (96%), respectively; grades 3-5 events occurred in 5 patients (11%) and 28 patients (64%). They stated that, due to inadequate power, decisive conclusions concerning the effectiveness of second-line Pembrolizumab in Asian patients with advanced PD-L1-positive AGC or AGEJC are restricted, however, Pembrolizumab was well tolerated in this patient population. Efficacy followed a similar pattern to that shown in the phase 3 KEYNOTE-061 experiment[62].

Yamaguchi *et al*[63] conducted an open-label phase 2b study, KEYNOTE-659, in Japan in 2022 to examine the effectiveness and safety of first-line Pembrolizumab plus SOX (cohort 1, n = 54) or S-1 and cisplatin (SP) (cohort 2, n = 46) for AGC or AGEJC. They reported that the median duration of Pembrolizumab therapy in cohorts 1 and 2 was 6.0 and 5.1 mo, respectively. SOX (cohort 1) had a median treatment length of 4.9 mo, while SP (cohort 2) had a median treatment duration of 4.4 mo. In cohort 1, 35 patients (64.8%) had their S1 dosage reduced, 47 patients (87.0%) had their oxaliplatin dose reduced, 44 patients (81.5%) had their S1 dose interrupted, and 31 patients (57.4%) had their oxaliplatin dose interrupted. In cohort 2, 33 patients (71.7%) had their S1 dosage reduced, 43 patients (93.5%) had their cisplatin dose reduced, 29 patients (63.0%) had their S1 dose interrupted, and 22 patients (47.8%) had their cisplatin dose interrupted. The ORR in cohort 1 was 72.2% (39 of 54 patients) and 80.4% (37 of 46 patients) in cohort 2. Overall, tumor reduction was observed in 52 of 54 patients (96.3%) in cohort 1 and 44 of 46 patients (95.7%) in cohort 2. DCR in cohort 1 was 96.3% (52 of 54 patients) and 97.8% (45 of 46 patients) in cohort 2. The median PFS in cohorts 1 and 2 was 9.4 mo and 8.3 mo, respectively. In cohort 1, the median OS was 16.9 mo, while in cohort 2, it was 17.1 mo. The median DOR in cohort 1 was 10.6 mo and 9.5 mo in cohort 2, whereas the median TTR in both cohorts was 1.5 mo. They proposed that the combination of Pembrolizumab with SOX or SP as first-line therapy in Japanese patients with PD-L1 positive, Human epidermal growth factor receptor (HER)-2 negative, AGC, or AGEJC demonstrated good effectiveness and tolerable safety.

Furthermore, 43 HER-2-positive AGC patients with a median follow-up of 18.2 months underwent Pembrolizumab evaluation in a single-arm, multi-institutional phase 1b/2 research to evaluate a quadruplet combination of Pembrolizumab, Trastuzumab, Capecitabine, and Cisplatin as first-line therapy. They reported an ORR of 76.7%, with 27 (62.8%) exhibiting PR and six (14.0%) patients exhibiting CR. Nine patients (20.9%) had stable disease, and the DCR was 97.7%. In 37 patients (86.0%), the total tumor burden was reduced by 30%, and in 26 (56.6%), it was reduced by 50%. The median PFS was 8.6 mo, with a 79.1% 6-mo PFS rate and a 41.9% 1-year PFS rate. The median OS was 19.3 mo, with an 80.1% 1-year OS rate. The median number of treatment cycles was 12. They concluded that utilizing a quadruplet regimen as first-line treatment resulted in tumor reduction in HER-2-positive AGC[64].

Recently, Satake *et al*[65] conducted a randomized control, phase 3 KEYNOTE-062 trial in 187 patients with AGC or AGEJC to compare the effectiveness of Pembrolizumab or Pembrolizumab with chemotherapy *vs* standard of care chemotherapy. They found that in the PD-L1 CPS ≥ 1 patients, the median OS with Pembrolizumab was 22.7 mo compared to 13.8 mo with chemotherapy. The 12-mo and 24-mo OS rates with Pembrolizumab were 69.4% and 44.8%, respectively, compared to 54.1% and 23.0% with chemotherapy. In the PD-L1 CPS ≥ 10 patients, the median OS with Pembrolizumab was 28.5 mo *vs* 14.8 mo with chemotherapy. The 12-mo and 24-mo OS rates with Pembrolizumab were 80.8% and 53.6%, respectively, compared to 68.2% and 27.3% with chemotherapy. They proposed that Pembrolizumab monotherapy was linked with numerically better OS results in patients with AGC or AGEJC with PD-L1 CPS ≥ 1 and CPS ≥ 10 tumors as compared to chemotherapy alone. When compared to chemotherapy, Pembrolizumab monotherapy had a better tolerability profile.

Nivolumab

A humanized immunoglobulin G (IgG) 4 monoclonal anti-PD-1 antibody called Nivolumab is effective against a range of tumor types. The phase 1/2 CheckMate-032 trial compared the use of Nivolumab and Ipilimumab in combination with Nivolumab monotherapy in 160 patients with AGC or AGEJC. The ORR for patients who got Nivolumab and Ipilimumab together was 24% as opposed to 12% for Nivolumab alone. Only 8% of patients in the combination arms responded to the alternate dosage (Nivolumab 3 mg/kg and Ipilimumab 1 mg/kg), suggesting that the ORR in these arms was dose-dependent. Regardless of PD-L1 expression, responses were seen. Nivolumab plus Ipilimumab treatment was linked with more severe toxicity (43%) than nivolumab alone (10%), as predicted from past combination trials[66]. Among 493 patients with unresectable AGC or AGEJC who had shown resistance to or intolerance to two or more prior chemotherapy regimens, ONO-4538-12 (ATTRACTION-2) was a multicenter, double-blind, randomized phase 3 study of Nivolumab. The ICI in GCs was the subject of the initial phase 3 placebo-controlled, randomized study. For the first time, the study demonstrated that PD-1 inhibition can improve OS in patients with severely pre-treated GC. The observed median OS with Nivolumab was 5.32 mo *vs* 4.14 mo with placebo, and the 12-month OS rate was 26.6% *vs* 10.9%[67]. The median PFS with Nivolumab was 1.61 mo *vs* 1.45 mo with placebo. The median time for a response to Nivolumab was 9.53 mo, and the ORR rate with Nivolumab was 11.2% as opposed to 0% with placebo. Nivolumab recipients had a tolerable safety profile, with 34 (10%) out of 330 patients experiencing TRAEs (grade 3 or 4), which is a rate similar to placebo recipients. However, it should be mentioned that the ATTRACTION-2 only included individuals from Asian countries,

therefore, the results might not apply to populations in Europe and North America. Emerging evidence suggests that unique gene profiles related to inflammation and immunity exist in Asian and non-Asian individuals with GC[68].

In order to assess the safety and effectiveness of Nivolumab in combination with SOX or capecitabine plus oxaliplatin (CapeOX) as first-line therapy in 77 patients with unresectable advanced or recurrent HER-2-negative AGC or AGEJC, Boku *et al*[69] conducted a randomized, phase 2 trial known as ATTRACTION-4. They discovered that Nivolumab with SOX resulted in an ORR of 57.1%, and Nivolumab plus CapeOX resulted in an ORR of 76.5%. In both groups, the median OS was not attained. The median PFS was 9.7 mo *vs* 10.6 mo. Neutropenia (14.3%) was the most common grade 3/4 TRAE in the nivolumab plus SOX group, followed by anemia (16.7%), peripheral sensory neuropathy, reduced appetite, type 1 diabetes mellitus, and nausea (11.1%) in the nivolumab with CapeOX group. They concluded that Nivolumab in combination with SOX/CapeOX was well tolerated and showed promising effectiveness in patients with unresectable advanced or recurrent HER-2-negative GC or GEJC. The ATTRACTION-2 2-year follow-up data revealed that 493 of 601 screened individuals were randomized (2:1) to receive Nivolumab (n = 330) or placebo (n = 163), and that the OS was considerably longer in the Nivolumab group compared to the placebo group (5.26 mo *vs* 4.14 mo) at the 2-year follow-up. At 1 year (27.3% *vs* 11.6%) and 2 years (10.6% *vs* 3.2%), the Nivolumab group had a greater OS rate than the placebo group. Regardless of tumor PD-L1 expression, the OS advantage was seen. The median OS for patients in the Nivolumab group who had a full or PR was 26.6 mo; the OS rates at 1 and 2 years were 87.1% and 61.3%, respectively. There were no new safety signals discovered[70]. In a phase 1/2 study, Japanese researchers investigated the safety and effectiveness of Nivolumab with Paclitaxel plus Ramucirumab in 43 patients with AGC resistant to first-line treatment. They discovered an ORR of 37.2% and a 6-mo PFS rate of 46.5%. The median OS was 13.1 mo: 13.8 mo in CPS ≥ 1 patients and 8.0 mo in CPS < 1 patients. They proposed that Nivolumab in combination with Paclitaxel and Ramucirumab showed potential anti-tumor efficacy with acceptable toxicity as a second-line therapy for AGC[71].

Janjigian *et al*[72] conducted a multicenter, randomized, open-label, phase 3 study (CheckMate 649) in 1581 patients with AGC, AGEJC, or esophageal adenocarcinoma (Nivolumab plus chemotherapy; n = 789; or chemotherapy alone; n = 792). In patients with a PD-L1 CPS greater than 5, they found that Nivolumab with chemotherapy led to substantial improvements in OS and PFS compared to chemotherapy alone. The patients with a PD-L1 CPS > 1 and all randomly assigned people showed a significant improvement in OS as well as a benefit in PFS, according to further data. Of the 782 participants in the nivolumab + chemotherapy group, 462 (59%) and the 767 patients in the chemotherapy alone group, respectively, experienced treatment-related side events. In both groups, the most prevalent any-grade treatment-related side events (25%) were nausea, diarrhea, and peripheral neuropathy. Treatment-related fatalities were determined to be 16 (2%) deaths in the nivolumab plus chemotherapy group and 4 (1%) deaths in the chemotherapy alone group. They proposed that Nivolumab in combination with chemotherapy be the new standard first-line treatment for these patients. In 2021, Shah *et al*[73] conducted a phase 2 open-label, randomized multicenter trial in 141 patients with pretreatment AGC or AGEJC to compare the effectiveness, safety, and pharmacodynamics of Andecaliximab plus Nivolumab *vs* Nivolumab alone. The ORR was 10% with Andecaliximab and Nivolumab and 7% with Nivolumab alone. The addition of Andecaliximab had no effect on response or survival. They concluded that the combination of Andecaliximab and Nivolumab had a positive safety profile but did not increase effectiveness in these individuals when compared to Nivolumab alone. Positive HER-2, greater TMB or GRB7, and lower TGF-β1 were all related to better clinical outcomes.

For the purpose of comparing the effectiveness of Nivolumab plus oxaliplatin-based chemotherapy *vs* placebo plus oxaliplatin-based chemotherapy as first-line therapy, Kang *et al*[74] conducted a randomized, multicenter, double-blind, placebo-controlled, phase 2/3 trial (ATTRACTION-4) in 724 patients with HER-2-negative, unresectable AGC or AGEJC. They discovered that the median PFS in the Nivolumab plus chemotherapy group was 10.45 mo and 8.34 mo in the placebo plus chemotherapy group. After a 26.6-mo follow-up, the median OS in the Nivolumab plus chemotherapy group was 17.45 mo and 17.15 mo in the placebo plus chemotherapy group. They hypothesized that Nivolumab in combination with oxaliplatin-based chemotherapy enhanced PFS but not OS in these patients. Shitara *et al*[75] conducted a randomized study to compare Nivolumab plus chemotherapy *vs* chemotherapy alone (n = 1581), while CheckMate-649 provided the first findings comparing Nivolumab plus Ipilimumab *vs* chemotherapy alone (n = 813). They observed that, after a 24-mo follow-up, Nivolumab with chemotherapy outperformed treatment in patients with PD-L1 CPS ≥ 5; the median OS was 14.4 mo *vs* 11.1 mo, respectively. The risk of mortality was reduced by 30%, and the proportion of patients living at 24 mo was 31% *vs* 19%, respectively. In patients with PD-L1 CPS ≥ 5 or all randomized participants, PFS and ORR were not improved by Nivolumab with Ipilimumab *vs* chemotherapy. However, in both PD-L1 CPS ≥ 5 and all randomized individuals, responses were more sustained with Nivolumab plus Ipilimumab *vs* chemotherapy (median DOR, 13.2 *vs* 6.9 mo). They proposed that the long-term clinically relevant OS and PFS benefits, enhanced and persistent responses, sustained health-related quality of life, and tolerable safety profile of Nivolumab with chemotherapy imply a favorable benefit-risk profile. These findings support the use of this regimen as a conventional first-line therapy in previously untreated AGC or AGEJC patients.

Avelumab

Bang *et al*[76] conducted a randomized, phase 3 JAVELIN Gastric 300 study in 371 patients with AGC or AGEJC in 2018 to assess the contribution of Avelumab to the physician's choice of chemotherapy as third-line treatment. The trial's primary end objectives of increasing OS (4.6 *vs* 5.0 mo) or secondary end criteria of PFS (1.4 *vs* 2.7 mo) or ORR (2.2% *vs* 4.3%) in the Avelumab *vs* chemotherapy groups, respectively, were not met. They claimed that treating these patients with single-agent Avelumab in the third-line scenario did not enhance OS or PFS when compared to chemotherapy. Avelumab, on the other hand, had a more controllable safety profile than chemotherapy. Moehler *et al*[77] conducted a worldwide, open-label, phase 3 JAVELIN Gastric 100 study in 499 AGC or AGEJC patients in 2021 to examine Avelumab maintenance treatment following first-line induction chemotherapy. They found that with Avelumab against chemotherapy, the median OS was 10.4 mo *vs* 10.9 mo, and the 24-mo OS rate was 22.1% *vs* 15.5% with no significant difference. They stated that in patients with AGC or AGEJC in general or in a predetermined PD-L1-positive population, JAVELIN Gastric 100 did not offer a superior OS with Avelumab maintenance *vs* continuing chemotherapy.

Durvalumab

Durvalumab is a human IgG1 monoclonal antibody with a high affinity for blocking PD-L1 binding to CD80 and PD-1. According to available data, 10 mg/kg of single-agent Durvalumab administered intravenously every two weeks for 12 mo showed prospective therapeutic efficacy in gastroesophageal malignancies[78,79]. Kwon *et al*[80] conducted a phase 2 open-label, single-center, non-randomized research study in 31 patients with AGC to assess the effectiveness and safety of Ceralasertib in conjunction with Durvalumab. The ORR, DCR, median PFS, and OS were reported to be 22.6%, 58.1%, 3.0 mo, and 6.7 mo, respectively. Common adverse effects were treatable by adjusting the dosage. In comparison to patients with intact ataxia telangiectasia mutated (ATM) and low sig. HRD (5.60 mo *vs* 1.65 mo), a subset of patients with ATM expression loss and/or a large proportion of mutational signature owing to homologous repair failure (high sig. HRD) had significantly higher PFS. They proposed that Ceralasertib in combination with Durvalumab had potential anticancer efficacy, with long-term responses in patients with refractory AGC.

ACTIIVE IMMUNOTHERAPY

GC vaccine

The ability of cancer vaccines to activate and boost anticancer immune responses, which are predominantly mediated by T cells that detect tumor-associated antigens, gives them therapeutic potential. The ideal vaccine should be easy to give, safe, cheap to produce, and able to induce a memory response that provides long-lasting immunity. By stimulating NK cells, B lymphocytes, and naïve and memory T cells, DCs, APCs, play a crucial role in orchestrating and coordinating antitumor immune responses[81-83]. For presentation to cytotoxic CD8+ T cells or to CD4+ helper T lymphocytes, tumor antigens are loaded by DCs as short peptides onto MHC class I or II molecules. These functional features prompted the development of several ways to use DCs in cancer immunotherapy. Despite these presumptions, the low *in vivo* viability of DC-based vaccines prevents their widespread use in clinical settings. A larger number of DCs infiltrating the tumor in GC patients was found to correspond with reduced lymph node metastases and lymphatic invasion, as well as better 5-year survival rates[84,85]. The results suggest that synthetic tumor peptides, synthetic tumor antigen mRNA, lysates, vesicles, and inactivated tumor cells can all be used as DC vaccine-loaded antigens. Potential GC vaccine antigens include the melanoma-associated antigen A3, HER-2 (p369) peptide, gastin-17 diphtheria toxoid, URLC10 or VEGFR1 epitope, and heat shock protein GP96[86]. Regrettably, there haven't been many beneficial findings using DC vaccinations in the treatment of GC. Only three patients with GC participated in the phase 1/2 clinical study, and only one of them was effective, despite showing that a Wilms tumor 1-targeted DC vaccine might be utilized to treat advanced cancer, including GC. In order to increase the effectiveness of GC vaccines, techniques to target numerous antigens have been investigated[87]. DC vaccines can be used with chemotherapy, radiation, and ICIs to increase efficacy. DC immunizations are safe for AGC patients since their toxicity and side effects include fever, flu-like symptoms, and local reactions at the injection site. DC-cytokine induced killer cell (DC-CIK) therapy is another method of using DCs to treat tumors. DC-CIK, coupled with chemotherapy, was proven in clinical trials to be effective and well tolerated in the treatment of AGC. According to a meta-analysis, patients who get DC-CIK and chemotherapy together after GC surgery have dramatically improved OS, DFS, and T cell responses. Additionally, DC-CIK combined with S-1 and cisplatin showed good PFS and OS in the treatment of AGC, and the combination therapy was safe and well tolerated in terms of toxicity[88,89]. Tumor-infiltrating DCs are linked with a better prognosis in GC; however, the tumor microenvironment contains only a few mature DCs. Therefore, DC vaccines and DC-CIK are ineffective against cancer when used as a single therapy; therefore, it is essential to determine the reasons for the ineffectiveness or combine them with other cancer therapies in order to increase the anti-tumor effects[90].

CONCLUSION

Globally, GC incidence is still high, and because early cancer screening is not extensively used and the symptoms are not frequently recognized, the majority of patients are discovered in the middle or late stages of the disease. After several years of effort, the OS has not dramatically improved the treatment of AGC. Immunotherapy, on the other hand, has given these patients hope. There are several ways to target immune cells to treat tumors, with therapy options targeting T cells having the most substantial effect and the quickest development, as well as showing strong clinical success in various solid tumors, including GC. ICIs were the most important passive immunotherapy in enhancing AGC patients' OS and PFS. These immunotherapies, however, have limitations in the treatment of GC. In certain studies, anti-PD-1/PD-L1 and anti-CTLA-4 antibodies did not improve patients' OS and PFS when compared to chemotherapy. Although encouraging results have been reported in previous clinical studies, the bulk of these populations-which do not make up the majority of AGC patients-have only benefitted from the treatment when their PD-L1 CPS, MSH-H, or TMB scores are high. Although several strategies for targeting immune cells to treat cancer have shown promise in preclinical animal models, they have not been widely used in clinical settings. Because the results of several therapy approaches' ongoing clinical trials have not yet been made public. Another explanation would be that they are only marginally effective, like with the two DC-based cancer treatment methods (vaccination and DC-CIK), which work best in combination with other therapies like chemotherapy. While others acquire primary or secondary medication resistance, the widely used ICIs only work for a portion of tumor patients. The complex microenvironment in which the tumor is located may be the cause of immunotherapy's poor effectiveness. Current immunotherapy only targets one type of cell or a specific target on a specific type of cell, whereas the immunosuppressive environment is made up of multiple cells and multiple targets. The interaction of tumor cells, immune cells, and stromal cells in the tumor microenvironment creates a massive immune suppression network that results in tumor immune escape. More than 10 distinct categories of immunosuppressive receptors expressed on T cells have been identified, and there are still other inhibitory receptors that have not yet been found. The development of therapy modalities with several targets and cells may be the following development path. On the basis of this, multi-target combination approaches for tumor therapy have been developed. Examples include the pairing of PD-1/PD-L1 inhibitors with CTLA-4 inhibitors and PD-1 inhibitors with anti-LAG-3. Moreover, therapeutic tactics targeting immune cells have shown promising outcomes when combined with other therapies, such as chemotherapy medications. As a result, anti-tumor treatment targeting immune cells has a long way to go to achieve synergy and detoxification.

FOOTNOTES

Author contributions: Leowattana W wrote the paper; Leowattana T and Leowattana P collected the data.

Conflict-of-interest statement: The authors declare no conflicts of interest related to this article.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Zhang XD

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 98-117

DOI: 10.5662/wjm.v13.i3.98

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Case Control Study

Characterization and risk factors for unexplained female infertility in Sudan: A case-control study

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Specialty type: Obstetrics and gynecology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Udo IA, Nigeria; Xu Y, China

Received: December 25, 2022
Peer-review started: December 25, 2022
First decision: February 8, 2023
Revised: February 8, 2023
Accepted: March 15, 2023
Article in press: March 15, 2023
Published online: June 20, 2023

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Abstract

BACKGROUND

Female infertility with unknown causes (unexplained) remains one of the mysteries in the reproductive health field, where the diagnostic evidence is still weak and the proposed treatments still work with unknown methods. However, several studies have proposed some possible causes and risk factors for unexplained female infertility.

AIM

To characterize and identify factors associated with unexplained infertility in Sudanese women.

METHODS

A matched (age and body mass index) case-control study was conducted from March 2021 to February 2022. The study samples were 210 women with unexplained infertility (UI) and 190 fertile women of reproductive age who were attending the maternity hospitals and fertility clinics in Khartoum, Sudan. The risk factors of unexplained infertility were identified using a structured, pre-tested questionnaire containing information on socio-demographic variables, anthropometrics, clinical diagnosis of infertility, behavioral factors, physical activity assessment, diversity, and consumption of different food groups by the

study participants.

RESULTS

The results showed a higher proportion of women diagnosed with UI were residents of rural areas than controls (21.4% *vs* 11.1%, $P < 0.05$), and previous miscarriages and/or abortions were more common in fertile women compared with infertile women (13.16% *vs* 5.71%, $P < 0.05$). Additionally, infertile women had a significantly ($P < 0.05$) higher proportion of family history of infertility (explained and unexplained) compared with controls. Finally, after controlling for the effects of potentially confounding variables using multivariable logistic regression analysis, only marital status, family history of infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, other vitamin-A-rich fruits and vegetables, and other vegetables were found to be significant ($P < 0.05$) factors associated with unexplained infertility among Sudanese women.

CONCLUSION

Married women with a family history of infertility who smoke and consume a high amount of caffeine, who live a sedentary lifestyle, and who consume more than two meals free of vitamin-A-rich fruits and/or vegetables and/or other vegetables per day are at the highest risk of developing unexplained infertility.

Key Words: Unexplained infertility; Sudanese women; Risk factors; Dietary diversity; Physical activity level

Core Tip: A matched (age and body mass index) case-control study was conducted to characterize and identify factors associated with unexplained infertility in Sudanese women. Four hundred women of reproductive age attending the maternity hospitals and fertility clinics in Khartoum, Sudan, were included in this study. The result showed that married women with a family history of infertility who smoke and consume a high amount of caffeine, who live a sedentary lifestyle, and who consume more than two meals free of vitamin-A-rich fruits and/or vegetables and/or other vegetables per day are at the highest risk of developing unexplained infertility.

Citation: Abdullah AA, Ahmed M, Oladokun A. Characterization and risk factors for unexplained female infertility in Sudan: A case-control study. *World J Methodol* 2023; 13(3): 98-117
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/98.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.98

INTRODUCTION

Unexplained infertility (UI) refers to a diagnosis (or lack of diagnosis) made in infertile couples in whom all the standard approved infertility investigations, such as tests of ovulation, tubal patency, and semen analysis, are normal[1,2]. A "diagnosis of unexplained infertility" may be very frustrating for an infertile couple and is often interpreted as if there is no explanation for the cause of infertility, so there is no effective treatment"[3]. Importantly, the prognosis of UI is worse when the duration of infertility exceeds 3 years and the female partner is > 35 years of age[4].

Furthermore, the unexplained infertility estimates vary, and the likelihood that all such tests for an infertile couple are normal (*i.e.*, that the couple has unexplained infertility) is approximately between 10% and 37% for all couples worldwide, and usually, female-related causes are responsible for 50% of it [4-9].

In Africa, the unexplained infertility prevalence is higher than that of other continents and ranges from 10%–37%[6,7]. In Sudan, a systematic review and meta-analysis found that the pooled prevalence of unexplained infertility in the Sudanese population is 17% with a confidence interval from 10% to 24% [6].

The high prevalence of unexplained infertility worldwide, especially in limited-resource countries like Sudan, implies that the current assessment of the human reproductive system is far from perfect. Nevertheless, significant improvements in diagnostic tools and assisted reproductive technologies have led to the finding of many causes of infertility that in the past had only been suspected, but, up until now, some causes of female infertility are still unknown. Subsequently, there is a need to search for more answers to the causes of female infertility to create better treatment options for patients.

Unexplained female infertility is considered one of the hot topics in reproductive medicine, and has been studied extensively; nevertheless, the studies in the area of diagnosis and treatment of unexplained female infertility didn't give concrete answers to the must-answer questions about the diagnosis and treatment of unexplained female infertility, whereby the diagnostic evidence is still weak and the proposed treatments still work with unknown methods. However, these studies have proposed some possible causes and risk factors for unexplained female infertility.

Studies conducted in low and low-middle-income countries showed that sociodemographic characteristics such as high body mass index (BMI), high waist-to-hip ratio (WHR), age older than 35 years, late marriage age, residence in rural and agricultural areas, low educational attainment, and unemployment were found to be significant risk factors for female infertility[8,10-16]. Furthermore, three systematic review and meta-analysis studies found a strong association between smoking and female infertility, whereby the pooled estimation showed that smoking women have a 1.8 times higher risk of developing infertility than women who do not smoke. Also, the same studies found a significant relationship between female infertility and alcohol consumption[10,17,18].

Additionally, other studies showed that high caffeine consumption was significantly associated with female infertility[12,19]. Meanwhile, several studies have found a negative significant association between increased physical activity level and unexplained female infertility[20], with sedentary women 3.61 times more likely to have unexplained infertility than moderately or very active women[19,21,22].

Furthermore, studies conducted in Iran and France found that women with a family history of infertility were 3.88 times more likely to develop unexplained infertility than women who did not have any close relatives who had experienced infertility previously[21,23]. This conclusion leads us to consider genetic factors as causes of unexplained infertility.

The previous history of abortion in women was one of the significant risk factors related to female infertility, whereby women with a previous history of abortion were more likely to have infertility in comparison to women without any previous history of abortion, with odds ratios of 9.33, 1.63, and 2.381, respectively, for Nigerian citizens[24], southeast Iranian citizens[23], and central Iranian citizens [25]. Some reports listed the use of contraceptives as a possible risk factor for female infertility[23,24]; in contrast, other reports found opposite results[13,26]. Therefore, there is still a need to find the association between the use of contraceptives and unexplained female infertility.

It is well known that nutritional status and the selection of food groups and supplements are crucial determinants of normal reproductive function. World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) described ten essential food groups for normal reproductive function in women of reproductive age[27,28]. Interestingly, in the available literature, there is no study about the effect of nutrition and diet diversity on unexplained female infertility, and the available evidence is only reported from cases of explained female infertility.

An 8-year prospective cohort study conducted by Chavarro *et al*[29], about the association between dietary diversity, eating habits, and the risk of female infertility, laid the basic foundation of what is now called a "fertility diet," which is a diet intended to increase the chances of women in the reproductive age of ovulation through changes in eating patterns and physical activity, whereby women with a high fertility diet score tend to have long menstrual cycles, normal reproductive hormone levels, and normal ovarian folliculogenesis. In addition, the findings of this study showed that the high fertility diet score was characterized by a lower intake of trans fat with a greater intake of monounsaturated fat; a lower intake of animal protein with a greater vegetable protein intake; a higher intake of high-fiber, low-glycemic carbohydrates; a greater preference for high-fat dairy products; a higher plant-based iron intake; and a higher frequency of multivitamin use[29]. Similarly, a nested case-control study in a Spanish cohort of 2154 female university graduates found that women with the highest adherence to the Mediterranean diet (characterized by high consumption of fruits and vegetables, fish, and poultry, low-fat products, and olive oil, which provides monounsaturated fat, and low consumption of red meat and sweets) are less likely to experience fertility difficulties[30].

Based on an extensive review of the existing literature about the risk factors of unexplained female infertility, the current study investigates sociodemographics, clinical history, dietary diversity, and consumption of different food groups as distinguishing factors associated with unexplained female infertility. More studies are needed to keep track of localized trends in infertility for better clinical management and research strategy as there is more variety and trends in unexplained infertility issues globally.

The present study highlights the characterization of unexplained infertility among Sudanese women and identifies factors associated with unexplained female infertility. The study outcome will contribute to the existing limited knowledge of unexplained female infertility and also have great significance for infertility program coordinators and policymakers as they design and implement effective strategies for preventing and managing female infertility.

MATERIALS AND METHODS

Study design and period

The objectives of this study were achieved using a prospectively matched age and BMI case-control design. The study was carried out during the period from March 2021 to February 2022.

Study population, setting, and area

Prof. El-Sir Abo Elhassan Fertility Center, Omdurman Maternity Hospital, Nile Fertility Center, Khartoum Reproductive Health Care Center, Sudan Assisted Reproductive Clinic, University of Khartoum Fertility Center, Saad Abualila Teaching Hospital, Banoun Fertility Center, and Hawwa Center for Fertility were all used to collect study samples. Those centers were purposefully selected because they are the largest centers providing assisted conception and modern antenatal care in Sudan.

Study participants' selection and eligibility criteria

The current study targeted 420 Sudanese women between the ages of 18 and 44. The case group included 210 women with unexplained infertility; the women were chosen using a systematic random sampling technique from patients who had tried but were unable to conceive for at least a year (maintain regular unprotected vaginal sexual intercourse with their partner at least twice a week for a year); cases should also have regular menstruation, a normal ovarian reserve test result, open fallopian tubes, and a normal uterus cavity size according to the hysterosalpingography[1].

In addition, their couples should have a normal spermiogram "with a concentration of at least 15 million sperm per milliliter, a motility value over 70 percent, and morphology of more than 4 percent with normal forms"[31]; the control group consisted of 210 fertile, nonpregnant women who had at least one healthy 2-year-old child (end of lactation).

Women were excluded from this study if they had any of the following illnesses: Hypertension, diabetes, endocrine disorders, autoimmune or immunocompromised conditions, a history of genetic disease, or sexually transmitted infections. In addition to that, women were also excluded if they were under the effect of anti-inflammatory medicines or if they were under the effect of hormonal contraception within the last 6 mo. Finally, any women who refused to sign the informed consent form or withdrew during the study were excluded.

Data collection procedures and instruments

To assess unexplained female infertility risk factors, a structured pre-tested questionnaire containing information on socio-demographic variables, anthropometrics, clinical diagnosis of infertility, behavioral factors, physical activity assessment, dietary diversity, and consumption of different food groups by study participants was used.

The questionnaire was prepared by reviewing several relevant published articles[19,32-34] and adopting standardized data collection tools[22,27]. Primarily, the questionnaire was prepared in the English language and translated into the country's main local language (Arabic). The consistency of the questionnaire was checked in different phases: First, by translating it back to English and reviewing it with a non-affiliated researcher with good knowledge of both languages; after that, the final form of the questionnaire was tested for consistency using internal consistency (Cronbach's alpha of 0.73); and then it was pretested by distributing it among 30 women not participating in the actual study but living in a similar setting.

After passing all quality check-ups, the questionnaire was filled out with the help of 10 research assistants who are experts in reproductive health (one in each previously mentioned healthcare facility) and who were trained on questionnaire administration skills relevant to this study by the primary investigator and charged with seeking signed informed consent from the study participants. The completeness of the data was checked each day at the end of data collection. Incomplete data was traced back and edited accordingly. The follow-up of study participants was done by tracking information (address, phone number of the participant as well as of relatives and close friends) and making periodic contact (reminders, updates) to minimize loss of follow-up, and the overall follow-up and data collection processes were coordinated and supervised by the research assistants' and principal investigator's.

Operational definition of the variables

Unexplained infertility was defined as a type of infertility that occurs when standard-approved infertility tests have not found a clear cause for the couple's inability to achieve pregnancy[35].

BMI is defined as an estimation of human body fat based on height and weight. BMI is expressed in kg/m^2, resulting from dividing body mass in kilograms by height in meters[36,37].

The WHR was defined as an estimation of fat stored around the waist and hips. The waist-hip ratio was calculated by dividing the waist measurement by the hip measurement.

Caffeine consumption: Caffeine is a central nervous system stimulant. It is used as a cognitive enhancer, increasing alertness and attentional performance. In this study, caffeine consumption was only from liquid sources of stimulants. It is used as a cognitive enhancer, increasing alertness and

attentional performance. In this study, caffeine consumption was only from liquid sources. It is used as a cognitive enhancer, increasing alertness and attentional performance. In this study, caffeine consumption was only from liquid sources (tea, coffee, sodas, and energy drinks) and was estimated based on the number of drunk cups in 24 h, whereby no consumption was considered if the woman didn't "drink any cups of caffeine," low consumption if the woman "drank a cup or two a day," moderate consumption if the woman "drunk 3 or 4 cups in 24 h," and high consumption if the woman "drunk more than 4 cups in 24 h".

Physical activity level is a way to express a person's daily physical activity as a number and is used to estimate a person's total energy expenditure. The physical activity level was estimated using a list of the physical activities a woman performs within a 24-h period and the amount of time spent on each activity. The following shows physical activity levels for several lifestyles: (Inactive = hospital patient with limited physical mobility; sedentary = office worker getting little or no exercise; moderately active = moderate physical activity at work or leisure; very active = considerable physical activity at work, *e.g.*, agricultural worker (non-mechanized) or office workers who take at least moderate exercise for two or more hours per day; and extremely active = professional athlete or sports person, *e.g.*, football player) [38]. Dietary diversity is a qualitative measure of food consumption that reflects women's access to a variety of foods, including grains, nuts, dairy products, meat, eggs, fruits, and vegetables. Dietary diversity was determined based on a 7-d recall method. For each group, no consumption was considered "no servings per week," low consumption "1 or 2 servings per week," moderate consumption "3 servings per week," and high consumption "more than 3 servings per week." Servings per week were defined considering the consumption considered adequate in the African and Arabic diets[27,39].

Data management and statistical analysis plan

Data collected from this study were sorted and recorded in Microsoft Excel 2016, cleaned, and then transferred to STATA software, version 16.0 (Stata Corp LLC, 77845 Texas, United States), and Jeffrey's Amazing Statistics Program, version 0.16.4.0 (JASP), for analysis. To characterize the study population, descriptive statistics, frequencies, and percentages for categorical data and summary statistics (mean standard deviation (SD) with a 95% confidence interval (CI) for continuous data normally distributed and median and interquartile range for continuous data not normally distributed) were used. In addition, tables and figures were used for data presentation. The normal distribution of the study variables (univariate, pairwise, and multivariate[40]) was performed using the Shapiro-Wilk test, and the data were considered normally distributed if the p-value was greater than 0.05.

The association between the categorical variables in the study population was checked using the chi-square test at the statistically significant level of $P = 0.05$. Meanwhile, this association was estimated using Cramer's V statistic effect size test, according to which it was classified, according to Kim[41], 2017, into small, medium, and large effect sizes.

A binary logistic regression analysis (bi-variable and multivariable) was carried out to identify the independent predictors of unexplained infertility. All independent variables with a P value of less than 0.05 in the bivariable logistic regression model were considered candidate variables for the multivariable model. Finally, the relationship was presented using a crude odds ratio and an adjusted odds ratio (AOR) with their corresponding 95% confidence intervals, and a P value of 0.05 or less was considered to be statistically significant.

RESULTS

Anthropometry and socio-demographic characteristics of study participants

The overall response rate in the current study was 210 (100%) among cases and 190 (90.5%) among controls.

Finally, the study included 400 women (210 women with UI as case subjects and 190 fertile women as control subjects). The two study groups were matched by age and BMI. The mean age of cases was 28.59 years with a SD of 5.22 years and a CI: of 27.87–29.3 years, while it was 28.44 years for controls (SD 4.95 years and a CI: of 27.73–29.15 years). The mean BMI values were 24.67 (SD 4.08 and CI: 24.11–25.22) and 24.41 (SD 4.38 and CI: 23.78–25.03) for the cases and controls, respectively. Besides, the two groups had almost the same mean value of the WHR [0.844 (SD 0.108 and CI: 0.829–0.859) for the cases and 0.837 (SD 0.114 and CI: 0.821–0.854) for the controls].

In addition to the above-mentioned anthropometry parameters, other sociodemographic characteristics of study participants, such as place of residence, marital status, education status, religious affiliation, and occupation, were checked. The only place of residence and marital status showed a statistically significant difference between the two study groups (Table 1). Wherein more women diagnosed with UI lived in rural areas than in urban areas (21.4% *vs* 11.1%, $P < 0.05$). Furthermore, when compared to controls, the vast majority of cases (99.1% *vs* 89.5%, $P < 0.05$) were married.

Meanwhile, the estimation of the effect sizes (Cramer's V) indicated a small association between female unexplained infertility (age, BMI, WHR, residence, education status, and religious affiliation) and a medium association between female unexplained infertility and (marital status and occupation).

Table 1 Characterization of selected anthropometry and socio-demographic variables of study participants, *n* (%)

Variable	Categories	Cases (*n* = 210), N%	Control (*n* = 190), N%	Effect size Cramer's V	*P* value
Age group (yr)	18-24	49 (23.3)	40(21.05)	0.08[3]	0.61
	25-29	87 (41.4)	91(47.9)		
	30-34	44 (21)	35(18.4)		
	35-39	23 (11)	21(11.05)		
	40-44	7 (3.3)	3(1.6)		
	mean ± SD	28.59 ± 5.22	28.44 ± 4.95	NA	NA
BMI (kg/m^2)	Underweight	1 (0.5)	4 (2.1)	0.07[3]	0.53
	Normal weight	127 (60.5)	111 (58.4)		
	Overweight	59 (28.1)	54 (28.4)		
	Obesity	23 (10.9)	21 (11.1)		
	mean ± SD	24.67 ± 4.08	24.41 ± 4.38	NA	NA
WHR	Less than 0.75	46 (22)	54 (28.4)	0.11[3]	0.08
	075-0.84	53 (25.2)	35 (18.4)		
	0.85-0.90	45 (21.4)	41 (21.6)		
	Greater than 90	66 (31.4)	60 (31.6)		
	mean ± SD	0.844 ± 0.108	0.837 ± 0.114	NA	NA
Residence	Rural	45 (21.4)	21 (11.1)	0.14[3]	**0.005**
	Urban	165 (78.6)	169 (88.9)		
Marital status	Married	208 (99.1)	170 (89.5)	0.21[4]	**0.001**
	Divorced[1]	2 (0.9)	13 (6.8)		
	Widow	0 (0)	7 (3.7)		
Education status	Diploma or above	89 (42.4)	85 (44.7)	0.05[3]	0.85
	Secondary edu	72 (34.3)	65 (34.2)		
	Primary edu	39 (18.6)	34 (17.9)		
	No formal edu[2]	10 (4.8)	6 (3.2)		
Religious affiliation	Muslim	198 (94.3)	184 (96.8)	0.06[3]	0.22
	Christian	12 (5.7)	6 (3.2)		
Occupation	Farmer	9 (4.3)	2 (1.1)	0.16[4]	0.06
	Governmental	34 (16.2)	29 (15.3)		
	Housewife	132 (62.9)	110 (57.9)		
	Private business	28 (13.3)	34 (17.9)		
	NGOs	1 (0.5)	6 (3.2)		
	Student	6 (2.9)	9 (4.7)		

[1]Information about, age, body mass index, and waist-to-hip ratio were reused from reference[81].
[2]Divorced women must have remained in regular sex in the last year intending to conceive.
[3]This includes the type of religious education (Khalwa). In the last column to the right, bold values indicate a significant level at $P < 0.05$. Cramer's V effect size was classified according to Kim[41], 2017 as small effect size.
[4]Medium effect size. Differences between groups were compared with chi-square test. Estimated effect sizes were calculated using Cramer's V statistic which is expressed as mean. Body mass index and waist-to-hip ratio status were follows World Health Organization and Centers for Disease Control and Prevention's classifications[36,37]. BMI: Body mass index; *n*: Sample size; NA: Not available; NGOs: Non-governmental organizations; SD: Standard deviation; WHR: Waist–hip ratio.

History and clinical characteristics of study participants

Table 2 shows the detailed history and clinical characteristics of the study participants. In this study, the median duration of unexplained infertility in cases was 3 years, with a range of 1–15 years and an interquartile range of 3.75, compared to the controls, who had a median of 4 for marriage duration, with a range of 3–20 years and an interquartile range of 2. These results were not compared since they were dissimilar.

Interestingly, fertile women had more previous miscarriages or abortions than infertile women (13.16% *vs* 5.71%, $P < 0.05$).

Furthermore, the current study found that infertile women had a higher proportion of infertility in their families than fertile women (28.1% *vs* 6.3%, $P < 0.05$). Similarly, the proportion of unexplained infertility in infertile women's families was higher than in controls (13.8% *vs* 1.6%, $P < 0.05$).

However, other clinical history variables did not show any significant association ($P > 0.05$) with unexplained female infertility and were reported as follows: The majority of study participants did not use contraceptives (64.8% for cases *vs* 62.6% for controls), none of the study participants have ever consumed alcohol, and the majority of them do not smoke (86.2% for cases and 91.6% for controls). Furthermore, the caffeine consumption patterns were almost the same in the two groups, whereby no consumption was 3.8% for cases *vs* 3.2% for controls, low consumption was 32.4% for cases *vs* 36.3% for controls, moderate consumption was 31.4% for cases *vs* 26.8% for controls, and high consumption was 32.4% for cases *vs* 33.7% for controls.

Moreover, the three (sedentary, moderately active, and very active) physical activity levels were at the same levels in the two groups, and the majority of the study participants had a sedentary lifestyle (50.5% for cases *vs* 48.1% for controls).

Finally, as can be seen in table 3, the estimation of the effect sizes (Cramer's V) indicated a small association between female unexplained infertility and (previous miscarriage or abortion, family history of infertility, family history of unexplained infertility, use of contraceptives, smoking, caffeine consumption, and physical activity levels) and a medium association between female unexplained infertility and the number of miscarriages or abortions.

Dietary diversity and consumption of different food groups by the study participants

This study examined the consumption patterns of the essential ten food groups described by the WHO and FAO for women of reproductive age and the number of daily meals based on a 7-d recall method. The results found that there was no significant association ($P > 0.05$) between unexplained infertility and the consumption patterns for each of the ten essential food groups and the number of daily meals consumed by the study participants.

Additionally, the estimation of the effect sizes (Cramer's V) of these associations was small for all studied variables.

With an in-depth look at the general features of these findings, we can conclude that almost half of the Sudanese women used to consume 3 meals per day (42.4% for cases *vs* 45.3% for controls). Besides, regarding the consumption pattern of specific food groups, grains, and pulses were the most consumed food groups by study participants, whereas nuts, meat (including red meat, poultry, and fish), and eggs were the least consumed food groups by both study groups equally. The fully detailed consumption patterns of different food groups by the study participants are described in Table 3 and Figure 1.

Factors associated with unexplained female infertility in the study participants

To identify the unexplained infertility predictor factors among Sudanese women, a binary logistic regression was used. The results found that from all the variables tested using the bi-variable binary logistic regression, only the place of residence, marital status, previous miscarriage or abortion, family history of infertility, family history of unexplained infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, dairy, dark green leafy vegetables, other vitamin A-rich fruits and vegetables, and other vegetables were found to have significant associations with unexplained female infertility.

However, after controlling for the effects of potentially confounding variables using multivariable logistic regression analysis, only marital status, family history of infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, other vitamin-A-rich fruits and vegetables, and other vegetables were found to be significant factors associated with unexplained infertility among Sudanese women. The variables eligible for the multivariable logistic regression model are described in Table 4 and Figure 2.

In the current study, married women were 0.073 times more likely to have unexplained female infertility in comparison to divorced and widowed women (AOR: 0.073, 95%CI: -4.431–0.803). Another vital factor significantly associated with unexplained female infertility was the family history of infertility, whereby any woman who has a history of infertility in her family was 3.257 times more likely to have unexplained female infertility compared with other women who don't have any family history of infertility (AOR 3.257, 95%CI: 0.175-2.186).

Meanwhile, the family history of unexplained infertility didn't show any significant associations with unexplained female infertility in the study participants. Women who did not use any contraceptives had

Table 2 History and clinical characteristics of study participants, *n* (%)

Variables	Categories	Cases (*n* = 210), N%	Controls (*n* = 190), N%	Effect size Cramer's V	*P* value
Duration of infertility/marriage duration (yr)		Median = 3; Range = 1-15; IQR (3.75)	Median = 4; Range = 3-20; IQR (2)	NC	NC
Previous miscarriage or abortion	Yes	12 (5.71)	25 (13.16)	0.13[3]	**0.02**
	No	198 (94.29)	165 (86.84)		
Number of miscarriages or abortions	Never	198 (94.29)	165 (86.84)	0.16[4]	0.08
	1	6 (2.86)	13 (6.84)		
	2	4 (1.9)	6 (3.16)		
	3	1 (0.48)	4 (2.1)		
	4	0 (0)	2 (1.05)		
	5	1 (0.48)	0 (0)		
Family history of infertility	Yes	59 (28.1)	12 (6.3)	0.29[3]	**0.001**
	No	151 (71.9)	178 (93.7)		
Family history of Unexplained infertility	Yes	29 (13.8)	3 (1.6)	0.23[3]	**0.001**
	No	181 (86.2)	187 (98.4)		
Use of modern contraceptives/yr	Never	136 (64.8)	119 (62.6)	0.09[3]	0.28
	0-3 yr	53 (25.2)	60 (31.6)		
	4-6 yr	16 (7.6)	9 (4.7)		
	More than 6 yr	5 (2.4)	2 (1.1)		
	Not in use now	0 (0)	0 (0)		
Alcohol consumption	Yes	0 (0)	0 (0)	NE	NE
	Not at all	210 (100)	190 (100)		
	Stopped taking	0 (0)	0 (0)		
Smoking[1]	Not at all	181 (86.2)	174 (91.6)	0.09[3]	0.54
	< 1 yr	1 (0.48)	1 (0.53)		
	1-2 yr	6 (2.9)	3 (1.6)		
	2-3 yr	9 (4.3)	5 (2.6)		
	> 4	13 (6.2)	7 (3.7)		
Caffeine consumption (cup/d)[2]	No consume	8 (3.8)	6 (3.2)	0.06[3]	0.72
	Low consume	68 (32.4)	69 (36.3)		
	Moderate consume	66 (31.4)	51 (26.8)		
	High consume	68 (32.4)	64 (33.7)		
Physical activity level	Very active	43 (22.6)	38 (18.1)	0.08[3]	0.26
	Moderately active	51 (26.9)	71 (33.8)		
	Sedentary	96 (50.5)	101 (48.1)		

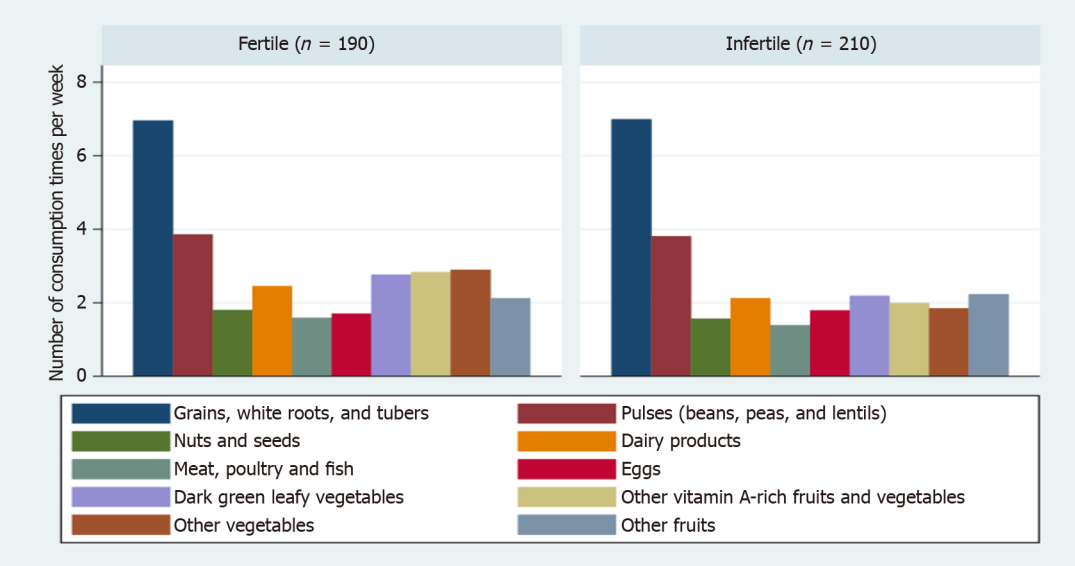
[1]The reported tobacco smoking in this study was in two forms [cigarettes, and shisha (water pipe)].
[2]Caffeine consumption was based only on liquid sources of caffeine (Tea, coffee, Sodas, and Energy Drinks).
[3]In the last column to the right, bold values indicate a significant level at $P < 0.05$. Cramer's V effect size was classified according to Kim (2017)(41) as small effect size.
[4]Medium effect size.
Differences between groups were compared with chi-square test. Estimated effect sizes were calculated using Cramer's V statistic which is expressed as mean. Physical activity level follows the WHO classification[38]. IQR: Interquartile range; *n*: Sample size; NC: Not Comparable due to dissimilar data; NE: Not evaluated in STATA due to zero value in one of the study groups.

Table 3 Frequency (Percentage) of dietary diversity and consumption patterns of different food groups by the study participants, *n* (%)

Variables	Categories	Cases (*n* = 210)	Controls (*n* = 190)	Effect size Cramer's V	*P* value
Meals consumed per day	2	50 (23.8)	44 (23.2)	0.04[1]	0.97
	3	89 (42.4)	86 (45.3)		
	4	42 (20)	34 (17.9)		
	5	24 (11.4)	21 (11.1)		
	6	5 (2.4)	5 (2.6)		
Grains, white roots, and tubers	No consume	0 (0)	0 (0)	NE	NE
	Low consume	0 (0)	0 (0)		
	Moderate consume	0 (0)	0 (0)		
	High consume	210 (100)	190 (100)		
Pulses (beans, peas, and lentils)	No consume	7 (3.3)	4 (2.1)	0.09[1]	0.36
	Low consume	27 (12.9)	36 (18.9)		
	Moderate consume	51 (24.3)	43 (22.6)		
	High consume	125 (59.5)	107 (56.3)		
Nuts and seeds	No consume	57 (27.1)	69 (36.3)	0.11[1]	0.18
	Low consume	88 (41.9)	77 (40.5)		
	Moderate consume	37 (17.6)	25 (13.2)		
	High consume	28 (13.3)	19 (10)		
Dairy products	No consume	37 (17.6)	38 (20)	0.09[1]	0.35
	Low consume	89 (42.4)	64 (33.7)		
	Moderate consume	42 (20)	42 (22.1)		
	High consume	42 (20)	46 (24.2)		
Meat, poultry and fish	No consume	74 (35.2)	65 (34.2)	0.09[1]	0.32
	Low consume	90 (42.9)	69 (36.3)		
	Moderate consume	37 (17.6)	43 (22.6)		
	High consume	9 (4.3)	13 (6.8)		
Eggs	No consume	44 (20.9)	49 (25.8)	0.13[1]	0.09
	Low consume	90 (42.9)	94 (49.8)		
	Moderate consume	60 (28.6)	36 (18.9)		
	High consume	16 (7.6)	11 (5.8)		
Dark green leafy vegetables	No consume	16 (7.6)	13 (6.8)	0.02[1]	0.97
	Low consume	101 (48.1)	89 (46.8)		
	Moderate consume	55 (26.2)	53 (27.9)		
	High consume	38 (18.1)	35 (18.4)		
Other vitamin A-rich fruits and vegetables	No consume	25 (11.9)	25 (13.2)	0.08[1]	0.47
	Low consume	87 (41.4)	75 (39.5)		
	Moderate consume	63 (30)	48 (25.3)		
	High consume	35 (16.7)	42 (22.1)		
Other vegetables	No consume	26 (12.4)	33 (17.4)	0.08[1]	0.49
	Low consume	83 (39.5)	66 (34.7)		
	Moderate consume	57 (27.1)	49 (25.8)		
	High consume	44 (20.9)	42 (22.1)		

Other fruits	No consume	28 (13.3)	30 (15.8)	0.04[1]	0.88
	Low consume	94 (44.8)	84 (44.2)		
	Moderate consume	56 (26.7)	46 (24.2)		
	High consume	32 (15.2)	30 (15.8)		

[1]The significance level was set at $P < 0.05$. Cramer's V effect size was classified according to Kim[41], 2017, as small effect size. Differences between groups were compared with chi-square test. Estimated effect sizes were calculated using Cramer's V statistic which is expressed as mean. The four classifications of the consumption levels of the different food groups per week followed FAO classifications[27]. *n*: Sample size; NE: Not evaluated in STATA due to zero value in one of the study groups.



DOI: 10.5662/wjm.v13.i3.98

Figure 1 Food groups consumed by the study participants (*n* = 400). Values are given as the mean.

significantly higher odds of developing unexplained female infertility compared with those who used them for any period of time (AOR 0.475, 95%CI: -0.987-0.502). In addition, smoking women had a significantly higher risk of developing unexplained female infertility than non-smoking women (AOR 1.276, 95%CI: 0.010–0.476).

This study also revealed that participants who had a sedentary lifestyle were more likely to have unexplained female infertility in comparison to participants with other lifestyles (AOR 0.423, 95%CI: 1.240–0.481). Furthermore, women who consumed more than two meals per day had a higher probability of developing unexplained infertility (AOR 1.606, 95%CI: -0.169-0.778). Additionally, women who didn't consume vitamin-A-rich fruits and vegetables and other vegetables had higher odds of having unexplained female infertility in comparison to those who consumed these two food groups in any amount. On the contrary, as caffeine consumption increases, the odds of having unexplained female infertility decrease (AOR 0.407, 95%CI: 0.168–0.514) (Table 4 and Figure 2).

DISCUSSION

The purpose of this thesis research was to identify factors associated with unexplained infertility in Sudanese women using the chi-square test to check the association and the logistic regression test to identify the unexplained infertility predictor factors. The results of the current study contribute to the limited knowledge of unexplained female infertility and have great significance for infertility program coordinators and policymakers as they design and implement effective strategies for preventing and managing female infertility. Also, the study data will be used as a baseline for other researchers who want to investigate further findings in this area.

Socio-demographic characteristics of the study participants

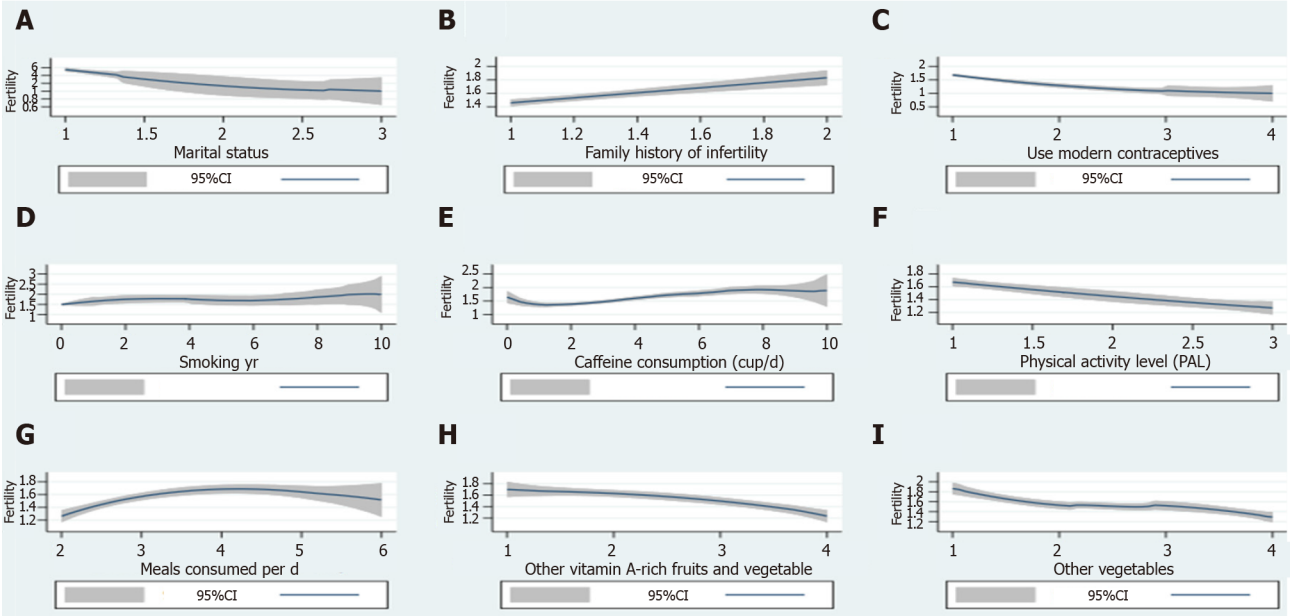
The current study's finding reveals that, from all studied sociodemographic variables, only the place of residence and marital status showed a significant association with unexplained infertility in Sudanese

Table 4 Binary logistic regression analysis of the factors associated with unexplained female infertility in the study participants

Predictors	COR, 95%CI	AOR, 95%CI
Residence		
Urban	Reference	Reference
Rural	2.195 (0.225-1.347)[a]	1.996 (-0.165-1.548)
Marital status		
Married	Reference	Reference
Divorced	0.126 (-3.576-0.571)[a]	0.073 (-4.431-0.803)[a]
Widow	0.419 (-0.922-0.386)[a]	
Previous miscarriage or abortion		
No	Reference	Reference
Yes	0.4 (-1.635 - 0.198)[a]	0.426 (-1.874-0.165)
Family history of infertility		
No	Reference	Reference
Yes	5.796 (1.1 - 2.415)[a]	3.257 (0.175-2.186)[a]
Family history of Unexplained infertility		
No	Reference	Reference
Yes	9.987 (1.095- 3.507)[a]	3.580 (-0.594-3.145)
Use of modern contraceptives/yr		
Never	Reference	Reference
0-3 yr	0.188 (-2.147-1.191)[a]	
4-6 yr	0.052 (-4.446-1.477)[a]	0.475 (-0.987-0.502)[a]
More than 6 yr	0.085 (-0.484-0.823)	
Smoking		
Not at all	Reference	Reference
< 1 yr	1.751 (-0.077-2.434)[a]	
1-2 yr	2.983 (-0.251-2.437)[a]	1.276 (0.010-0.476)[a]
2-3 yr	4.102 (0.117-2.706)[a]	
> 4	3.356 (0.176-2.245)[a]	
Caffeine consumption (cup/d)		
No consume	Reference	Reference
Low consume	0.254 (-2.521-0.219)[a]	
Moderate consume	0.648 (-1.586-0.718)[a]	0.407 (0.168-0.514)[a]
High consume	0.426 (-0.802-1.512)[a]	
Physical activity level (PAL)		
Sedentary	Reference	Reference
Moderately active	0.237 (-1.394- 0.464)[a]	0.423 (-1.240-0.481)[a]
Very active	0.292 (-2.291- 1.145)[a]	
Meals consumed per d		
2	Reference	Reference
3	4.172 (0.876-1.981)[a]	
4	5.293 (1.006-2.326)[a]	1.606 (0.169-0.778)[a]
5	5.286 (0.899-2.432)[a]	

6	2.917 (-0.253-2.394)	
Dairy		
No consume	Reference	Reference
Low consume	1.551 (-0.118-0.997)[a]	
Moderate consume	0.974 (-0.649-0.596)[a]	0.777 (-0.540-0.036)
High consume	0.674 (-1.015-0.226)[a]	
Dark green leafy vegetables		
No consume	Reference	Reference
Low consume	1.014 (-0.780-0.807)[a]	
Moderate consume	0.789 (-1.067-0.593)[a]	0.730 (-0.651-0.021)
High consume	0.346 (-1.948-0.176)[a]	
Other vitamin A-rich fruits and vegetables		
No consume	Reference	Reference
Low consume	0.673 (-1.078-0.287)[a]	
Moderate consume	0.469 (-1.467-0.047)[a]	0.540 (-0.948-0.283)[a]
High consume	0.131 (-2.837-1.232)[a]	
Other vegetables		
No consume	Reference	Reference
Low consume	0.177 (-2.543-0.920)[a]	
Moderate consume	0.169 (-2.614-0.940)[a]	0.466 (-1.087-0.441)[a]
High consume	0.064 (-3.623-1.866)[a]	

[a]Indicates a statistically significant effect at $P < 0.05$. Identification of female unexplained infertility predictors among clinical characteristics was done using a univariate and multivariate logistic regression analysis test. AOR: Adjusted odds ratio; CI: Confidence interval; COR: Crude odds ratio; n: Sample size.



DOI: 10.5662/wjm.v13.i3.98

Figure 2 Variables eligible for the multivariable logistic regression model are described. A: The polynomial association between infertility in Sudanese women, and marital status; B: Family history of infertility; C: Use of modern contraceptives; D: Smoking; E: Caffeine consumption; F: Physical activity level; G: Number of meals consumed per day; H: Other vitamin A-rich fruits and vegetables; I: Other vegetables.

women (Table 2). Specifically, infertility with unknown causes (unexplained) was significantly associated with married women and women residents in rural areas. This might be due to the fact that, in rural areas of Sudan, there are not enough healthcare facilities providing primary healthcare services and consulting in general and specifically for obstetrics and gynecology[42,43]. This can lead to neglecting the initial symptoms of infertility, which exacerbates the infertility conditions and makes it difficult to diagnose and treat them.

Furthermore, sociocultural practices such as believing infertility is a curse and that you should accept it and not seek help from anyone other than God are still prevalent in many rural communities in Sudan. In support of that, in a recent cross-sectional study of infertility management strategies among Sudanese participants, it was discovered that nearly one-half of the participants strongly believed that the best strategy for infertility management is to use Qur'an and Sunna treatments[44]. Such beliefs make it very difficult to diagnose and treat infertility cases in these communities.

All the above-mentioned factors can partially explain the high prevalence of unexplained female infertility among married women in rural areas of Sudan compared with other women in urban areas. This is in line with the findings of other female infertility studies conducted in Iran[13], Pakistan[16], Cameroon[15], the Central African Republic[15], and Chad[15]. These countries have economic settings and religion-oriented communities similar to Sudan; therefore, it is logical to draw similar conclusions.

In this study, 95.5% of the participants were Muslims, so the religious affiliation wasn't comparable, thereby showing no significant association with unexplained infertility in Sudanese women. Meanwhile, other sociodemographic variables, like occupation and education status, were comparable between the two groups; nevertheless, they didn't show any significant association with unexplained infertility in Sudanese women.

This result corroborates other findings reported in Nigeria[24], Iran[13], Pakistan[16], Cameroon[15], the Central African Republic[15], and Chad[15].

On the other hand, in contrast with the findings of the current study, a study conducted on women in Yazd, Iran[23], found a significant association between education status and female infertility, whereby the infertility risk increases with high educational attainment. Interestingly enough, this conclusion went against all the currently available literature.

History and clinical characteristics of the study participants

The present study also investigated the clinical histories of the study participants (Table 3). Overall, the median duration of unexplained infertility among the case group was 3 years, ranging from 1 to 15 years. Furthermore, this study found a significant association between the family history of infertility (explained and unexplained) and unexplained infertility. This may indicate that there may be some genetic factors for unexplained female infertility, but due to the complexity of the reproduction process in females, this possible gene(s) is still unknown. Nevertheless, many studies found that most infertility causes and conditions, such as poor egg quality or low egg reserves and blocked or damaged fallopian tubes, can't be inherited and can happen to anyone, regardless of family history[45,46]. As a result, there is an urgent need for excessive research work to be done in the field of genetic infertility.

Although similar results to the findings of this study describing the association between family history of infertility and female infertility were reported in Iran[13,23,25], and Netherlands[47]. A case-control study conducted in Nigeria in 2020[24], with a smaller sample size, didn't find any significant association between female infertility and a family history of infertility.

It is important to note that, in the present study, the proportion of women with previous miscarriage and/or abortion was higher in fertile women compared with infertile women (13.16% *vs* 5.71%); however, the miscarriage proportion difference between the two groups is not significant.

This finding indicates that having a miscarriage and/or an abortion doesn't affect a woman's ability to get pregnant in the future. A substantial number of studies reached similar conclusions[8,13,48,49]. Meanwhile, this result disagrees with the findings of studies from Nigeria[24], Iran[23], and Germany [50], which found a positive association between secondary infertility and abortion.

In this study, none of the study participants had ever consumed alcohol in any form or amount before. As a result, alcohol consumption among the study participants cannot be compared and is associated with unexplained infertility. The low prevalence of alcohol consumption in Sudanese women [51-53] could be responsible for the zero prevalence of alcohol consumption in our study cohort. These findings indicate that alcohol consumption cannot be considered a risk factor for unexplained female infertility in the Sudanese population.

Interestingly, despite the established significant association between female infertility and physical activity level in the previous studies, whereby an active lifestyle and moderate exercise reduce the risk of infertility (explained and unexplained) and abortions and increase the pregnancy success rates among women who undergo any of the assisted reproduction technologies[20,21,54,55], the current study did not find a significant association between unexplained female infertility and the physical activity level in Sudanese women.

This might be related mainly to the similarity of lifestyle choices and occupation affiliations mentioned before and the generally inactive lifestyle among females in Sudan[56].

Dietary diversity and consumption of different food groups by the study participants

The present study examined the consumption patterns of the ten essential food groups described by the WHO and FAO for women of reproductive age and the number of daily meals based on a 7-d recall method (Table 4). This study didn't find any significant association ($P > 0.05$) between unexplained infertility and the consumption patterns for each of the ten essential food groups among the study participants.

This indicates that despite the fact that female reproductive performance is definitely influenced by incorrect food consumption patterns, which lead to disturbances in nutritional status, alterations in ovarian function, and a subsequent decrease in fertility as shown in previous studies[29,30,57-60], however, in Sudanese women (fertile and infertile), in line with previous studies done in the Sudanese population[61-65], which found a similarity in food consumption patterns among women in Sudan, there was no difference in food consumption patterns between the two study groups.

Risk factors of unexplained female infertility in Sudanese women

This study used binary logistic regression to assess the unexplained infertility predictor factors among Sudanese women. According to the findings in Table 4 and Figure 2, only the place of residence, marital status, previous miscarriage or abortion, family history of infertility, family history of unexplained infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, dairy, dark green leafy vegetables, other vitamin A-rich fruits, and vegetables. However, after controlling the effects of potentially confounding variables using multivariable logistic regression analysis, only marital status, family history of infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, other vitamin-A-rich fruits and vegetables, and other vegetables were found to be significant factors associated with unexplained infertility among Sudanese women.

In the present study, married women were 0.073 times more likely to have unexplained infertility in comparison to divorced and widowed women. This can be explained by the fact that most Sudanese women seeking infertility services are married women (99.1%), so it makes sense that the odds of unexplained infertility are greater among them.

Also, women with a family history of infertility were 3.257 times more likely to have unexplained female infertility compared with other women who didn't have any family history of infertility. Similar findings were reported in studies from Iran and France, which found that women with a history of infertility in their family were 3.88 times more likely to develop unexplained infertility compared with women who didn't have any of their close relatives who had infertility before[21,23].

In light of this finding, it is essential to note that there is a strong argument that can be made about the effect of inherited "unknown genes" on female infertility. However, this argument needs more supporting evidence to make definitive conclusions.

Women who did not use any contraceptives (prior or current) had significantly higher odds of developing unexplained female infertility compared with those who used them for any period of time. Nevertheless, it can't be said with complete confidence that the use of contraceptives has contributed to the development of unexplained infertility cases in Sudanese women. Because contraceptives are used in women to prevent pregnancy from unprotected vaginal sex during fertile days, there is no need to use them in infertile women; thus, women who suffer from infertility usually do not use contraceptive methods[12,26]. This result corroborates all existing findings[13,17,23,25,35,66].

In this study, approximately 11% of Sudanese women of reproductive age smoke nicotine products, which is in line with the overall prevalence of smoking in the Sudanese population.

When compared to non-smokers, smoking women had 1.276 times the risk of developing unexplained female infertility.

This indicates that smoking has adverse effects on the normal female reproductive process, whereby the evidence specifically suggests that the smoking process produces some toxins that affect the folliculogenesis and steroidogenesis in the ovary and the FSH and LH secretion from the pituitary gland[67]. As a result, women are advised not to smoke to increase their fertility and their ability to get pregnant.

This result is consistent with a recent committee opinion report of the American Society for Reproductive Medicine[67], and other comprehensive studies about the negative effect of smoking on female fertility[14,18,68,69].

Women who had a sedentary lifestyle were more likely to have unexplained female infertility in comparison to participants with other lifestyles. This is consistent with recent findings that increasing physical activity level has a negative significant association with unexplained female infertility, with sedentary women 3.61 times more likely than moderately and very active women[21]. This finding may be explained by two main mechanisms. Sedentary physical activity in women may lead to an increase in leptin expression levels in the hypothalamus-pituitary-ovarian (HPO) axis, which has a negative effect on normal HPO axis function and may result in poor-quality eggs. Hence, an increase in physical activity leads to a decrease in leptin expression levels in the HPO axis and the resumption of ovulation by regulating HPO axis activity[70-73].

Additionally, high physical activity combined with the consumption of a healthy diet in women with infertility can decrease insulin and free androgen levels, leading to the restoration of the normal

function of HPO by increasing the ovarian sensitivity to gonadotropins (LH and FSH) and resulting in recovery from infertility[20,74,75].

Women who consumed more than two meals per day had a 1.606 greater probability of developing unexplained infertility compared with other women who consumed three or more meals per day. In addition, women who didn't consume vitamin-A-rich fruits and vegetables and other vegetables had higher odds of having unexplained female infertility in comparison to those who consumed these food groups in any amount.

The current finding is corroborated by the findings of the American Society for Reproductive Medicine about the relationship between diet and female fertility[76].

The three food groups mentioned—vitamin A-rich fruits, vegetables, and other vegetables—are the main source of micronutrients (vitamin A, vitamin C, vitamin E, magnesium, zinc, phosphorus, and folic acid) in Sudanese women's diet; therefore, the lack of consumption of these food groups without compensation from an external source led to a deficiency in these micronutrients. Several studies have proven that vitamins A, C, and E, magnesium, zinc, phosphorus, and folic acid play an essential role in the normal female reproductive process; any deficiency in these substances leads to disturbances in the normal female reproductive process, resulting in infertility[60,76-79].

It is important to note that insufficient nutrient intake, in general, can contribute to female infertility. This may be due to the fact that unquestionably women of reproductive age are often nutritionally vulnerable because of the dynamic way in which their bodies work and the high physiological demands, especially during the menstrual cycle. Thus, they require a more nutrient-dense diet[28].

Finally, this study showed that as caffeine consumption increases, the odds of having unexplained female infertility decrease. Studies on the effect of caffeine consumption on female fertility are inconsistent, with several studies showing negative effects of caffeine on fertility, while other studies show no association or even a significant improvement in female fertility with caffeine consumption, as summarized in a systematic review done by Bu and his colleagues in 2020[80].

The findings of the current study can be explained in light of the fact that there may be some sort of unknown negative interaction between caffeine consumption (external causes) and the female reproductive system (internal causes), which increases the risk of infertility. Although this conclusion is not conclusive, we recommend that any woman who desires to have a baby consume caffeine wisely.

To recapitulate all the above, married women with a family history of infertility who smoke and consume a high amount of caffeine, who have a sedentary lifestyle, and who consume more than two meals free of vitamin-A-rich fruits and/or vegetables and/or other vegetables per day are at the highest risk of developing unexplained infertility. Therefore, any woman who intends to get pregnant and has one or more of these risk factors needs to get an immediate consultation from a certified fertility doctor to find out early if she has unexplained infertility or not and to know the best strategies to deal with it. Also, those women need to change their diet and lifestyle to decrease the likelihood of developing unexplained infertility.

Strengths and limitations of the study

This matched case-control study provided a useful characterization of unexplained female infertility in Sudan; this, along with the control of confounding factors and low loss to follow-up, contributes to the current study's strength.

Additionally, the study outcomes and arguments were based on relatively large sample size. Hence, its conclusion could be generalizable to other settings. We were constrained by the objectives of the significant study from which this study emanated. As such, some clinical details of the women with unexplained infertility were not captured. The data used for this study were obtained through patient-reported interviews, and some responses appear retrospective; thus, the participants' responses could not be validated. However, efforts were made as much as possible to validate the information reported by participants through the patient records, especially for the participants who were not first-time visitors to the study recruitment clinics.

CONCLUSION

In conclusion, the median duration of unexplained infertility in this study was 3 years, with a range of 1–15 years. Furthermore, married women with a family history of infertility who smoke and consume a high amount of caffeine, who have a sedentary lifestyle, and who consume more than two meals free of vitamin-A-rich fruits and/or vegetables and/or other vegetables per day are at the highest risk of developing unexplained infertility.

ARTICLE HIGHLIGHTS

Research background

Unexplained infertility remains one of the mysteries in the reproductive health field, where the diagnostic evidence is still weak and the proposed treatments still work with unknown methods.

Research motivation

Unexplained infertility remains one of the mysteries in the reproductive health field, where the diagnostic evidence is still weak and the proposed treatments still work with unknown methods.

Research objectives

The objective of this study was to characterize and identify factors associated with unexplained infertility in Sudanese women.

Research methods

A matched (age and body mass index) case-control study was conducted from March 2021 to February 2022. The study samples were 210 women with unexplained infertility and 190 fertile women of reproductive age who were attending the maternity hospitals and fertility clinics in Khartoum, Sudan. The risk factors for unexplained infertility were identified using a structured, pre-tested questionnaire.

Research results

Infertile women had a significantly higher proportion of family history of infertility (explained and unexplained) compared with controls. Also, only marital status, family history of infertility, use of modern contraceptives, smoking, caffeine consumption, physical activity level, meals consumed, other vitamin-A-rich fruits and vegetables, and other vegetables were found to be significant ($P < 0.05$) factors associated with unexplained infertility among Sudanese women.

Research conclusions

Married women with a family history of infertility who smoke and consume a high amount of caffeine, who live a sedentary lifestyle, and who consume more than two meals free of vitamin-A-rich fruits and/or vegetables and/or other vegetables per day are at the highest risk of developing unexplained infertility.

Research perspectives

More interventional studies regarding the main factors, such as physical activity and dietary intake, for infertile women with unexplained causes, need to be done.

FOOTNOTES

Author contributions: Abdullah AA, Ahmed M, and Oladokun A analyzed and interpreted the data, and drafted the manuscript; Abdullah AA and AO designed the study and directed implementation and data collection; Abdullah AA, Ahmed M, and Oladokun A edited the manuscript for intellectual content and provided critical comments on the manuscript; All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Institutional review board statement: The study protocol and procedures were approved by the University of Ibadan/University College Hospital (UI/UCH) Ethics Committee (Ref. No; UI/EC/20/0438), and the Federal Ministry of Health, Sudan republic (Ref. No; 4-12-20).

Informed consent statement: Informed consent was obtained from all the study participants, and all necessary information regarding the study (objectives, requirements of the participants, and duration of the study) was given to the prospective study participants on an information sheet in Arabic to ensure an informed decision to participate in the study. Then, the full case histories of the participants were obtained through clinical examinations and laboratory investigations. Ethical principles such as discretion and confidentiality, the interviewees' free consent, and beneficence and nonmaleficence to participants were strictly adhered to.

Conflict-of-interest statement: The authors declared no potential conflict of interest concerning the research, authorship, and/or publication of this article.

Data sharing statement: The datasets used during this study are available from the principal investigator upon reasonable request.

STROBE statement: The authors have read the STROBE Statement – checklist of items, and the manuscript was

prepared and revised according to the STROBE Statement – checklist of items.

Country/Territory of origin: Sudan

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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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World Journal of Methodology

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World J Methodol 2023 June 20; 13(3): 118-126

DOI: 10.5662/wjm.v13.i3.118

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Epidemiological trends in acute pancreatitis: A retrospective cohort in a tertiary center over a seven year period

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Specialty type: Medical laboratory technology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Gong F, China; Litvin A, Russia; Mao EQ, China; Trna J, Czech Republic

Received: March 24, 2023
Peer-review started: March 24, 2023
First decision: April 27, 2023
Revised: May 4, 2023
Accepted: June 6, 2023
Article in press: June 6, 2023
Published online: June 20, 2023

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Abstract

BACKGROUND

Acute pancreatitis (AP) remains a major cause of hospitalization and mortality with important health-related costs worldwide. Using an electronic database of a large tertiary center, we estimated the incidence, etiology, severity and costs of hospitalized AP cases in southern Romania.

AIM

To estimate the incidence, cost and tobacco usage of hospitalized AP cases in southern Romania and to update and upgrade the knowledge we have on the etiology, severity (in regard to Revised Atlanta Classification), outcome, morphology and local complications of AP.

METHODS

We performed an electronic health care records search on AP patients treated at Emergency University Hospital of Bucharest (Spitalul Universitar de Urgență București) between 2015 and 2022. The incidence, etiology, and severity were calculated; potential risk factors were evaluated, and the hospitalization costs of AP were documented and analyzed. The cohort of this study is part of the BUCharest - Acute Pancreatitis Index registry.

RESULTS

A total of 947 consecutive episodes of AP where the patients were hospitalized in the gastroenterology department were analyzed, with 79.45% as 1st episode and the rest recurrent. The majority of the patients were males (68.9%). Alcoholic (45.7%), idiopathic (16.4%) and biliary (15.2%) were the main causes. The

incidence was estimated at 29.2 episodes/100000 people. The median length of stay was 7 d. The median daily cost was 747.96 RON (165 EUR). There was a high prevalence of active tobacco smokers (68.5%). The prevalence of severe disease was 11.1%. The admission rate to the intensive care unit was 4.6%, with a mortality rate of 38.6%. The overall mortality was 5.5%.

CONCLUSION
We estimated the incidence of AP at 29.2 episodes that required hospitalization per 100000 people. The majority of our cases were found in males (68.9%) and were related to alcohol abuse (45.7%). Out of the cases we were able to find data regarding tobacco usage, the majority were active smokers (68.5%). Most patients had a mild course (54.4%), with a mortality rate of 5.5%. Interstitial AP prevailed (45.3%). The median daily cost of hospitalization was 747.96 RON (165 EUR).

Key Words: Acute pancreatitis; Epidemiology; Revised Atlanta Classification; Mortality; Outcome; Cost

Core Tip: Retrospective cohort study on 947 consecutive hospitalized episodes (in 829 patients) of acute pancreatitis (AP) with the aim of estimating the incidence, cost and tobacco usage in AP cases and to update & upgrade the knowledge we have on the etiology, severity (as stated in Revised Atlanta Classification), outcome, morphology and complications of AP. Out of our study resulted that: The majority of patients were males (68.9%); alcoholic etiology prevailed (45.7%); estimated incidence: 29.2 episodes/100000 people; median daily cost: 165 EUR; median hospitalization 7 d; majority active tobacco smokers (68.5%); 11,1% severe disease; admission to intensive care unit 4.6%; overall mortality 5.5%.

Citation: Ghiță AI, Pahomeanu MR, Negreanu L. Epidemiological trends in acute pancreatitis: A retrospective cohort in a tertiary center over a seven year period. *World J Methodol* 2023; 13(3): 118-126
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/118.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.118

INTRODUCTION

Acute pancreatitis (AP) remains one of the main conditions treated in gastroenterological departments worldwide and is a common cause of hospitalization. With an incidence of 3.8-74.8 cases per 100000 in Europe[1]. AP remains a burden for health care system expenditures, although approximately 75%-80% of patients will develop only a mild clinical course[2]. Positive diagnosis is based on at least 2 positive criteria from the following: Lypasemia (or amylasemia) higher than 3 times the normal range, a clinical presentation (upper abdominal pain that radiates to the back, nausea, vomiting, *etc*.) or imaging criteria (ultrasound, contrast-enhanced computed-tomography, contrast-enhanced magnetic resonance imaging)[3].

Updated population-based studies on AP in Romania are lacking. Our aim was to evaluate the current data for AP in a tertiary center in Bucharest. The objective of this paper is to estimate the incidence, cost and tobacco usage of hospitalized AP cases in southern Romania and to update and upgrade the knowledge we have on the etiology, severity (in regard to Revised Atlanta Classification), outcome, morphology and local complications of AP. Although most of those issues have already been addressed in other populations, there is a high degree of heterogeneity in the worldwide and time-related AP demographics, etiologies, management practices, and outcomes[4]. A previous study conducted in 2005 on a similar population[5] of patients from a gastroenterological services found a dominance of alcohol-related pancreatitis. However, that study had a relatively small number of patients and could not estimate the incidence and stratify etiologies other than alcoholic, biliary, post-endoscopic retrograde cholangiopancreatography and hypertriglyceridemia.

MATERIALS AND METHODS

Patients

For this observational retrospective study, we inquired about the Spitalul Universitar de Urgență București (Emergency University Hospital of Bucharest) digital database for cases of AP [International Classification of Diseases 10 code: K85, B25.2, B26.3], in which adult patients were admitted to gastroenterological wards between 1 June, 2015 and 1 April, 2022.

The inquiry obtained 1074 consecutive episodes (Table 1). All of the patients were screened by the authors for miscoding, revealing that all 1074 fulfilled at least 2 criteria for AP (as they are mentioned in the Revised Atlanta Classification)[6], out of which 126 were chronic pancreatitis, so we excluded them from this study. We collected the following data: Sex, month of admission, age, number of days of admission, number of days of admission to the intensive care unit (ICU) (if it were the case), outcome at discharge, type of severity according to the Revised Atlanta Classification[6], type of morphology according to the Revised Atlanta Classification, probable etiology, urban-rural residence, county of origin, previous history of pancreatitis, smoking habits and cost of admittance.

Morphology was assessed according to the Revised Atlanta Classification by the authors' consensus from the available imagistic investigation. We took into consideration (arranged by the power of evidence) abdominal ultrasound, endoscopic ultrasound, contrast-enhanced computer tomography, and contrast-enhanced magnetic resonance.

This cohort is represented only by the patients admitted to the gastroenterology department and represents the BUCharest - Acute Pancreatitis Index (BUC-API) 1 - Gastroenterology cohort. For the aforementioned cohort, we took into consideration demographic, clinical, biological and imagistic data obtained from the electronic database of Emergency University Hospital of Bucharest. The population of this cohort is represented by 918 patients, with 1074 episodes of AP, recurrent AP and acute-on-chronic pancreatitis involving patients who were admitted to our department from 1 June, 2015 to 1 April, 2022 with AP. Details regarding the number of unique patients are from the BUC-API 1 - Gastroenterology cohort can be found in Table 1.

Statistical analysis

The database was organized using Microsoft Excel 2019©. For the statistical analysis of the data, we used crosstab analysis, frequency analysis, linear regression, ANOVA, χ^2 test, Fisher exact test, and goodness of fit run on the statistical program IBM SPSS Statistics version 29.0.0.0©.

RESULTS

Estimated incidence of AP

Our hospital serves as a tertiary referral center for a population of approximately 950 thousand inhabitants, and we are admitting half of the AP patients in our hospital as the other half being admitted to surgical wards. Our search identified 1074 episodes, of which 126 were miscoded as AP, being in fact acute-on-chronic pancreatitis. The remaining 947 consecutive episodes were AP to which the patients were admitted to the gastroenterological wards of our hospital in the timespan of 6 years and 10 mo, between 1st of June 2015 and 1st of April 2022. Based on the aforementioned statistics, we managed to estimate an incidence of AP in southern Romania of 29.2 episodes per 100000 people. This incidence means that we estimate approximately 5900 hospitalizations for AP annually at the country level.

Demographics

We found a total of 947 consecutive episodes that fulfilled at least 2 out of the 3 diagnostic criteria and were not chronic pancreatitis. Of them, 75.39% (n = 714) of the patients did not have any history of AP, and the others had at least one previous episode of AP but without signs of chronic disease and/or pancreatic malignancy.

In total, 68.88% (n = 652) of the cases were in male patients, and the median age was 54 years (± 15.9). By type of residence, 73.1% (n = 692) of the patients were from cities, 25.4% (n = 241) were from the countryside, and the remaining 1.4% (n = 14) did not have a fixed residence within Romania.

Etiology

We have defined the etiology of AP in regard to 16 possible causes and another 18 possible intricate etiologies, based on how they were defined as predisposing conditions in Sleisenger and Fordtrans - Gastrointestinal and Liver diseases - 10th edition[7]. We have defined some of the etiologies as follows: (1) Alcohol-related[8]: Regular alcoholic consumption (obtained through anamnesis) and/or indirect elements in cases without an apparent etiology, such as macrocytosis, icterical cholestasis, DeRitis ratio [9] > 2 in middle-aged men, Dupuytren contracture, *etc.* We could not quantify the usage of CAGE Questionnaire[10] from the medical records we reviewed; (2) Biliary: Imagistic findings (ultrasonographic, computer tomography or magnetic-resonance) with elevated aminotransferases (alanine aminotransferase or aspartate aminotransferase)[8]; (3) Hypertriglyceridemia: Triglycerides > 750 mg/dL, we sought to use a threshold formed from an average between 1000 mg/dL[8], and the one recommended for treatment of hypertriglyceridemia by ATP III guideline (500 mg/dL)[11]; (4) Trauma: Anamnesis, a high creatine kinase; and (5) Diabetes mellitus: No apparent etiology and at least one of the following: Hemoglobin A1c > 7.5% or glycemia > 250 mg/dL at two consecutive findings (without prior history of diabetes mellitus).

Table 1 Number of unique patients in the BUCharest – Acute Pancreatitis Index 1 – gastroenterology cohort

Type of disease	Number of episodes	Number of unique patients
AP & RAP	947	829
Acute-on-chronic pancreatitis	126	89
Total BUC-API 1 gastroenterology	1074	918

BUC-API: BUCharest – Acute Pancreatitis Index; AP: Acute pancreatitis; RAP: Recurrent acute pancreatitis.

All other single etiologies were classified by the authors' consensus. We found 45.7% (n = 433) of the cases to be related to alcohol consumption and 15.2% (n = 144) were related to gallstones. Among other remarkable etiologies, we found 16.4% (n = 155) idiopathic, 3.5% (n = 33) hypertriglyceridemia-related, 3% (n = 28) diabetes mellitus-related, and 2.5% (n = 25) pharmacological (Table 2).

Tobacco usage

We were able to identify tobacco usage in 40.5% of the patients (n = 384), out of which 68.5% (n = 263) were active smokers and another 22.4% (n = 86) ceased smoking tobacco more than 4 wk prior to hospitalization. We could not objectively quantify the number of pack-years from the medical records.

Severity and outcome

At discharge, we found that 54.4% (n = 515) had mild AP, 34.5% (n = 327) had a moderately severe course of disease, and the latter 11.1% (n = 105) had severe disease. A total of 4.6% (n = 44) were admitted to the ICU, with a mortality rate of 38.6% (n = 17) and a median length of stay within the ICU of 4 d (± 0.8). Regarding the entire population, the mortality rate observed was 5.5% (n = 52), with a healing rate of 83.2% (n = 788). The outcome is presented in detail in Table 3.

Regarding morphology, we retrieved information from the medical records in 73.4% of the patients. The most frequently encountered morphology was 45.3% (n = 429) who had interstitial edema, followed by 11.3% (n = 107) with a normal pancreas and 7.4% (n = 70) with acute peripancreatic collections. Necrosis as understood by acute necrotic collection and walled-off necrosis was encountered in 3.9% (n = 37) of the patients. Table 4 shows the available details about the morphology.

Hospitalization and estimated costs

The length of hospitalization varied greatly, with a median of 7 d (± 6.05) and a maximum of 101 d. Regarding the month of hospitalization, most of the patients were hospitalized in May (11.4%, n = 108), and the fewest were in hospitalized in February (n = 62). All the cases by month of hospitalization are shown in Figure 1. The median total cost was 5177.5 RON (± 6238.89) (approximately 1100 EUR), with a maximum of 100762 RON (approximately 22400 EUR). The median daily cost, was calculated to be 747.96 RON (± 411) (approximately 165 EUR). Considering the data, we were able to calculate the cost of hospitalization of the entire population included in this study at 4958226.84 RON (approximately 1 million EUR) and to estimate the annual cost of hospitalization for this disease in Romania at 30890748 RON (approximately 6.3 million EUR).

DISCUSSION

Previous reports estimated that the incidence of AP varied across Europe between 4.6 and 100 cases/100000 people annually[1,2,5,12]. We have estimated an incidence of 29.2 cases/100000 people, or approximately 5900 episodes annually throughout the entire country, which is an expected and moderate profile of incidence. We could not find any specific data about incidence in our country, so this is most likely the first attempt to estimate the incidence of AP in Romania.

To the best of our knowledge, this is the first attempt to estimate the cost of hospitalization in Romania. We observed a median total cost of 5177.5 RON (approximately 1100 EUR) and a median daily cost of 747.96 RON (approximately 165 EUR). Comparing it to other studies[12,13], we found a median daily cost similar to that in Spain (143 EUR) but far lower than the median total cost of 10069 USD in the United States in 2010. A possible limitation resides in the fact that all our patients were hospitalized in public-owned facilities, so it is possible that some of the costs were underestimated.

Smoking might be an independent risk factor for AP severity and evolution[14-16]. In our study, we were able to find that more than two-thirds of the patients smoked actively, while another 22.4% were former smokers. These data show us a higher percentage of active smokers in the AP population than those reported in the general population of Romania (68.5% *vs* 30%, as stated by the 2021 Eurobarometer). We will soon try to observe if there is any correlation between smoking tobacco products and

Table 2 Frequency of etiologies

Cause	Number of cases	Percent (%)
Alcohol	433	45.7
Idiopathic	155	16.4
Biliary	144	15.2
Hypertriglyceridemia	33	3.5
Diabetes mellitus	28	3.0
Pharmacological	25	2.6
Mixed (alcohol & biliary)	21	2.2
Mixed (alcohol & diabetes mellitus)	21	2.2
Mixed (alcohol & hypertriglyceridemia)	18	1.9
Mixed (hypertriglyceridemia & diabetes mellitus)	17	1.8
Ischemic	14	1.5
Extra pancreatic anomalies	13	1.4
Other (trauma, IBD, intrapancreatic anomalies *etc.*)	25	2.6

IBD: Inflammatory bowel disease.

Table 3 Outcome at discharge

Outcome	Number of cases	Percent (%)
Healed	788	83.2
Discharge at will	78	8.2
Deceased	52	5.5
Transferred	26	2.7
Stationary	3	0.3

Table 4 Morphology

Morphology	Number of cases	Percent (%)
Interstitial	429	45.3
Normal pancreas	107	11.3
Acute peripancreatic fluid collection	70	7.4
Pseudocyst	52	5.5
Acute necrotic collection	33	3.5
Walled off necrosis	4	0.4
N/A	252	26.6

N/A: Not applicable.

AP in another paper.

The median length of stay in AP varies in the literature from 4 d in Finland[17] to 9 d in Chile[18] and 10 d in Spain[12]. We found a length of stay of 7 d, which is similar to other previously published studies. Regarding the seasonality of AP, we found a peak in incidence in May, which is somewhat similar to the findings from a Chinese study and might be related to cultural habits[19].

Previous papers found a high prevalence of alcohol-related AP with a gallstone-to-alcohol ratio of 0.39 in 2005 in Romania[2,5], similar in trend to other Eastern and Northern European countries[17,20].

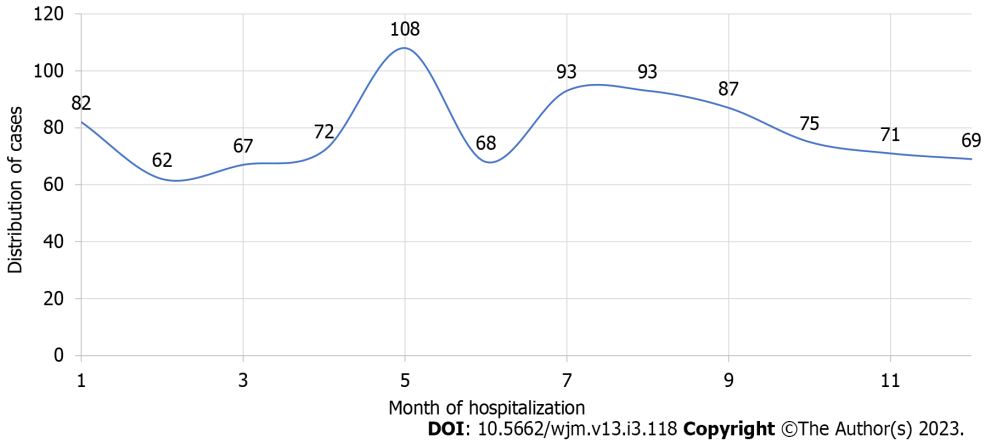

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Figure 1 Distribution of cases by month of hospitalization.

In India, the gallstone-to-alcohol ratio seems to be very close to 1 (0.95), with other etiologies being negligible[21]. In Spain and the Americas[12,18,22], the gallstone etiology seems to prevail, while in China[23], although the gallstone cause prevails, hypertriglyceridemia appears to be a highly important cause (probably due to lower rates of alcoholism in that region). Globally, it seems that gallstone-related AP is the most common[24]. In our cohort of patients, alcoholic etiology was most prevalent (45.7%), with a lower than previously reported gallstone-to-alcohol ratio of 0.33 and an overall relative lower rate of all the main four etiologies (alcohol, biliary, idiopathic and hypertriglyceridemia).

In regard to the outcomes, we observed lower rates of mild AP than those of other studies that have stratified severity in regard to the Revised Atlanta Classification[18,25]. We observed a lower (4 *vs* 8 d) median length of stay in the ICU than those observed in China[26] or Australia[27] but higher mortality rates than those of China[28], Finland[17] or Germany[29]. Nevertheless, similar mortality and severity rates were observed in populations that are geographically, culturally, culinary, and genetically similar to the Portuguese[30].

CONCLUSION

We estimated the incidence of AP at 29.2 episodes that required hospitalization per 100000 people. The majority of our cases were found in males (68.9%) and were related to alcohol abuse (45.7%). Out of the patients we were able to find data regarding tobacco usage, a vast majority of the patients were active smokers (68.5%). Most of our patients had a mild course (54.4%), and the total mortality rate was 5.5%. Interstitial AP prevailed in our cohort (45.3%). 747.96 RON (approximately 165 EUR) was the median daily cost of hospitalization. This study's main strengths are based on the fact that is a large cohort study with over 1000 episodes and with a low bias risk of population selection regarding the fact that the episodes taken into account were consecutive AP cases of our department. The weaknesses of this study resides in the fact that is a retrospective, unicentric study that is based on medical-chart reviews that is prone to data loss between discharge and study analysis and also the lack of surgical patients. There is a need to extend this study to patients admitted in surgical departments to correctly evaluate prognosis and severity.

ARTICLE HIGHLIGHTS

Research background

Acute pancreatitis (AP) is a global burden, especially in Eastern Europe and former Soviet space. Romania although is part of Eastern Europe lacks quality epidemiological studies regarding the topics we aim in this study, like: Estimation of incidence, stratification of: Etiology, severity, outcome, morphology, estimation of cost regarding hospitalization of AP and tobacco usage prevalence in our country regarding AP cases.

Research motivation

From this study we aim to estimate the incidence, cost and tobacco usage in hospitalized AP cases and to upgrade and update former knowledge regarding: Etiology, severity, outcome, morphology of AP. Once this aim is fulfilled the data from this paper should be of use for: Medical practitioners from our

country and countries that have large Romanian diaspora, medical researchers and healthcare policy-makers from our country or any other international organization with a focus on this topic.

Research objectives

Main objective: Estimating the incidence was fulfilled although we were able to do that only in regard to southern Romania. Secondary objectives achieved: First estimation of AP costs in our country, first attempt to find the prevalence of tobacco usage in AP in our country. Also, we were able to update the knowledge regarding stratification. This study should be of use for a nationwide metanalysis of smaller regional studies or for a European or international metanalysis regarding this topic. This study should be also expanded with surgical patients.

Research methods

Cases drawn from BUCharest - Acute Pancreatitis Index cohort of AP and Acute-on-Chronic Pancreatitis cases, which to the best of our knowledge is the largest analysed in Romania to this date. The entire team that worked on this study, did that remotely due to coronavirus disease 2019 pandemic restriction with the help of several online applications like: Adobe Reader, Microsoft 365 (formerly known as Microsoft Excel), Google Teams, Zoom, SPSS statistical package. We analysed data from Electronic Health Records of our facility, based on International Classification of Diseases (ICD)-10 coding of diagnostics. For the statistical analysis we used IBM SPSS v. 29.0.0.0 and we run the following tests depending on the type of variable of interest: Crosstab analysis, frequency analysis, χ^2 test.

Research results

We managed to show that AP incidence total annual cost in Romania might be overestimated as stated in Global Burden of Disease study 2019 (no other recent data publicly available), this should be of great interest for healthcare policy makers in our country or other international organization interested by this topic. In the selection of cases, we also observed that ICD-10 does not have a particular code for Acute-on-Chronic Pancreatitis, this was a limitation that was dealt trough authors screening and exclusion but we consider that a code for this particular situation would be of great necessity.

Research conclusions

Our study proposes a first, as far we know, estimation regarding costs of AP in our country and also a first glance, to the best of our knowledge, regarding tobacco usage prevalence in this disease. We also managed to make a reasonable first, as far as we know, grassroots estimation of incidence of AP in our region based on the data we have at this moment.

Research perspectives

We seek in a near future to expand this study also on surgical cases and territorially as a multicentric study to be able to better estimate the current status of AP outside southern Romania.

ACKNOWLEDGEMENTS

Ana Stemate and Narcis Zărnescu (local registry coordinators); Irina Diaconu, Dalia Constantinescu, Dana Corbu, Daniela Grigore, Julia Elena Fuseiller and Melissa Kabamba Kalonji (data base and charts analysis).

FOOTNOTES

Author contributions: Ghiță AI and Pahomeanu MR contributed to the collection of data, input of data, primary statistical analysis and drafting of the article; Negreanu L contributed to the article writing and final corrections; and all authors approved the final version of the article.

Institutional review board statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Emergency University Hospital of Bucharest (Spitalul Universitar de Urgență București).

Informed consent statement: Participants gave general informed consent at admittance for medical data to be used in medical research and consent was not obtained in particular for this study but the presented data are anonymized and risk of identification is low.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix and dataset available from the corresponding author at

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STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhao S

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 127-141

DOI: 10.5662/wjm.v13.i3.127

ISSN 2222-0682 (online)

SYSTEMATIC REVIEWS

Acceptability and strategies for enhancing uptake of human immunodeficiency virus self-testing in Nigeria

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Specialty type: Medical laboratory technology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Liu D, China; Zhu C, China

Received: January 5, 2023
Peer-review started: January 5, 2023
First decision: March 15, 2023
Revised: April 1, 2023
Accepted: April 27, 2023
Article in press: April 27, 2023
Published online: June 20, 2023

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Abstract

BACKGROUND

In 2019, the Nigerian Ministry of Health published the first operational guidelines for human immunodeficiency virus self-testing (HIVST) to improve access to human immunodeficiency virus (HIV) testing services among undertested populations in the country. Also, as part of the campaign to increase HIV testing services in Nigeria, the Nigerian Ministry of Health developed standard operating procedures for using HIVST kits.

AIM

To systematically review the acceptability and strategies for enhancing the uptake of HIVST in Nigeria.

METHODS

The systematic review was conducted and reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. Different databases were searched to get the necessary materials needed for this review. Standardized forms developed by the authors were used for data extraction to minimize the risk of bias and ensure that the articles used for the study were properly screened. Identified articles were first screened using the titles and their abstracts. The full papers were screened, and the similarities of the documents were determined. Qualitative, quantitative, and mixed-method studies were evaluated using the Critical Appraisal Skills Programme and Critical Appraisal Framework criteria.

RESULTS

All the publications reviewed were published between 2015 and 2022, with 33.3% published in 2021. Most (77.8%) of the studies were cross-sectional, 43.3% were conducted in Lagos State, and 26.3% were conducted among young people. The study revealed a high level of acceptability of HIVST. Certain factors, such as gender, sexual activity, and previous testing experience, influence the acceptability of HIV self-testing, with some individuals more likely to opt-out. The cost of the kit was reported as the strongest factor for choosing HIVST services, and this ranged from 200 to 4000 Naira (approximately United States Dollar 0.55-11.07), with the majority willing to pay 500 Naira (approximately United States Dollar 1.38). Privately-owned, registered pharmacies, youth-friendly centres, supermarkets, and online stores were the most cited access locations for HIVST. The least influential attribute was the type of specimen needed for HIVST. Strategies addressing cost and preferred access points and diverse needs for social media promotion, local translation of product use instructions, and HIVST distribution led by key opinion leaders for key populations were found to significantly enhance HIVST uptake and linkage to care.

CONCLUSION

HIVST acceptability is generally high from an intention-to-use perspective. Targeted strategies are required to improve the acceptability of HIV self-testing, especially among males, sexually active individuals, and first-time testers. Identified and proposed uptake-enhancing strategies need to be investigated in controlled settings and among different populations and distribution models in Nigeria.

Key Words: Acceptability; HIV self-testing; Uptake; Intention-to-use; Regulation; Linkage to care

Core Tip: This is the first systematic literature review on the acceptability and uptake of human immunodeficiency virus self-testing (HIVST) in Nigeria. The findings suggested that the acceptability of HIVST is high in Nigeria. However, the actual use of HIVST in programmatic implementation was lower than expected. The use of key opinion leaders among key populations successfully increased the acceptability and uptake of HIVST. However, cost was a major barrier to the acceptability of HIVST. More studies are required to evaluate how the uptake of HIVST compares in routine programs *vs* real-life settings in the absence of support and resources that enhance HIVST uptake.

Citation: Adepoju VA, Umebido C, Adelekan A, Onoja AJ. Acceptability and strategies for enhancing uptake of human immunodeficiency virus self-testing in Nigeria. *World J Methodol* 2023; 13(3): 127-141
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/127.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.127

INTRODUCTION

Nigeria is ranked second among the countries with a high burden of human immunodeficiency virus (HIV) in the world[1]. In 2018, the national prevalence of HIV was 1.5%, with an estimated 1.9 million people living with HIV/acquired immune deficiency syndrome (AIDS), of which only 30.0% were on antiretroviral therapy[2]. According to the Nigeria HIV/AIDS Indicator and Impact Survey, the national HIV prevalence rate in the group of 15-49 years of age was 1.4%, with a population of 201 million in 2019[3]. In Nigeria, 53000 people died of HIV/AIDS in 2018, while the rate of HIV/AIDS-related deaths appears to have remained constant in recent years, owing to the ongoing problem of advanced HIV disease[4].

Despite increased scientific and medical advancement in the understanding and management of HIV, a large number of those infected remains untested and unaware of their serostatus[5]. One of the reasons for the poor coverage of conventional health facility-based counselling and testing is the refusal to test due to the fear of societal stigma and discrimination that may result from a positive HIV test result[6] and the fear of long-term treatment, which may affect the quality of life of those infected[7].

The World Health Organization recommends HIV self-testing (HIVST) as a tool for improving the uptake of HIV testing services and achieving the Joint United Nations Programme on HIV and AIDS 90-90-90 target[8]. HIVST is an unconventional and innovative strategy to reach the first 90% goal of the United Nations Programme on HIV and AIDS by facilitating access to testing for early detection and prevention of HIV transmission[9]. Evidence shows that the deployment of HIVST has improved the uptake of HIV testing among men[10-13] in several countries implementing HIVST in Sub-Saharan

Africa including South Africa, Zimbabwe, and Botswana[10-12].

Nigeria has identified the need to increase HIV counselling and testing, including the potential of a self-testing methodology[14]. In 2019, the national AIDS and sexually transmitted diseases control programme under the Federal Ministry of Health developed the operational guidelines for the delivery of HIVST in Nigeria. The document provides guidance for the operationalization of HIVST in Nigeria including the different service delivery and distribution models, procurement and supply chain management, monitoring, and evaluation among others[5]. HIVST addresses the gap in HIV testing, especially in clinical settings. Surveys conducted among diverse populations in Malawi, Spain, the United States, and Nigeria showed varying interest in HIVST and acceptability ranges between 22% and 88%[15-19]. There is no study that systematically documented evidence either on the acceptability of HIVST or the proposed strategies to enhance its uptake in Nigeria. HIVST, as an innovative tool, is still a growing intervention in Nigeria with potential barriers to its acceptance among populations and settings. This study, therefore, aimed to systematically review the acceptability, existing regulations, and strategies for enhancing the uptake of HIVST in Nigeria.

MATERIALS AND METHODS

Design

A systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Figure 1).

Search strategy

Different databases were searched to get the necessary materials needed for this review. A scientific literature search was performed using Elsevier, Google Scholar, EMBASE, PubMed, Ovid, and Scopus databases. Grey literature was also searched from Google and Google literature, the largest databases for grey literature. Additionally, literature was systematically searched from ResearchGate, Cochrane library, and Directory of Open Access Journal. For studies that may have been missed in the electronic search, cross reference was undertaken using reference lists of all identified articles. The first search was conducted between April 4-8, 2022, while the second took place between April 15-20, 2022. Detailed inclusion and exclusion criteria were cautiously developed to match the review questions and have sufficient details to pinpoint all relevant studies and exclude irrelevant studies[20]. The literature search combined specific keywords with Boolean operators (Table 1). Although the Reference Citation Analysis tool was available for use, it was not utilized for this review. This decision was formed based on the nature of the research question and the inclusion and exclusion criteria developed for the review, which ensured that all relevant studies were identified through the comprehensive search strategy described above.

Inclusion criteria

Both qualitative and quantitative studies on HIVST in Nigeria were included in this study.

Exclusion criteria

Articles were excluded if no data was found for the desired outcome. Editorials and short commentaries were also excluded. Papers that were not peer-reviewed and those that the full text could not be assessed were also excluded.

Data extraction

Standardized forms developed by the authors were used for data extraction to minimize the risk of bias. One of the authors extracted data from the included studies, while the other authors checked these datasets. Discrepancies were resolved by referring to the original studies. Data on acceptability, existing regulatory context, and preference level for HIVST in Nigeria were extracted. Other data extracted include the level of uptake, linkage to treatment, and strategies for enhancing the uptake of HIVST in Nigeria. Adelekan A and Adepoju VA independently evaluated the potential eligibility of each of the abstracts and titles from the retrieved citations after requesting full-text versions of these potentially eligible studies. Onoja AJ and Umebido C independently assessed the full text of the potentially eligible publications. Disagreements were resolved by consensus. Discrepancies were discussed between authors until a 100.0% agreement was achieved. The following information was extracted from the included studies: Authors, title, study population, study state, study objective(s), study design, and study findings.

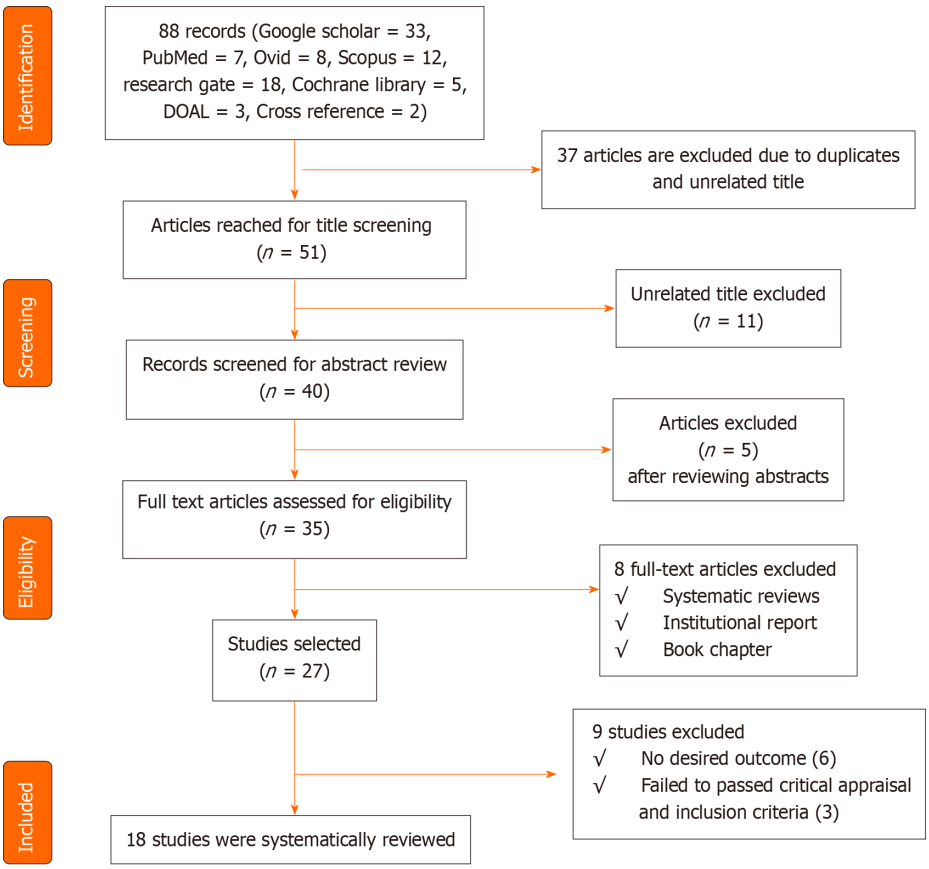
Quality assessment

The articles used for the review were properly screened. They were first screened using the titles and their abstracts. The full papers were screened, and the similarities of the papers were determined by reading the title, author(s), and abstract. The papers were then de-duplicated. The quality assessment of

Table 1 Search terms used in the literature search on human immunodeficiency virus self-testing uptake in Nigeria

Search terms	And	And
HIV self-testing	Acceptability	Nigeria
HIV regulatory	Nigeria	Self-testing
HIV self-testing	Preference	Nigeria
Nigeria HIV uptake	Self-testing Nigeria	
HIV self-testing	Nigeria treatment	Linkage

HIV: Human immunodeficiency virus.



DOI: 10.5662/wjm.v13.i3.127

Figure 1 Preferred reporting items for systematic reviews and meta-analyses flow chart of the study. DOAJ: Directory of Open Access Journals.

each study selected was based on set criteria[21,22].

Critical appraisal

The qualitative, quantitative, and mixed-method research were evaluated using the critical appraisal skills programme instrument[23] and critical appraisal framework criteria[24,25].

Data analysis

In compiling and summarizing the findings of the included studies, the researchers employed a variety of methodologies. Cleaning of data in the extraction sheet was an important step before analysis. The researchers structured the data from the extraction sheet into a format that analytical tools could read. The analysis was divided into qualitative and quantitative. Quantitative data analysis involved descriptive and narrative data. This technically followed the process of classification and tabulations. Content analysis technique was used for the qualitative data analysis.

List of papers reviewed

The search results are shown in Figure 1, along with a synopsis of the papers consulted (the PRISMA flow chart). Although the databases contained 88 research articles, only 18 met the inclusion criteria for this systematic review (Table 2).

RESULTS

The included studies were published between 2015 and 2022, with 33.3% published in 2021 (Figure 2). Majority (77.8%) of the studies were cross-sectional in design (Figure 3), 43.3% were carried out in Lagos State (Figure 4), and 26.3% were conducted among young people (Table 3).

Acceptability of HIVST in Nigeria

This review operationally defined the acceptability of HIVST as an intention to use, willingness to use, actual collection, and interest in use. The findings of most studies from Nigeria[5,7,26-31] revealed a high level of acceptability of HIVST compared to what was reported by a study from Northern Nigeria [15] (Figure 5). Brown *et al*[27] reported that 54.8% of the respondents supported having HIVST in Nigeria. Adebimpe *et al*[7] also reported that 86.0% of the respondents agreed that they would accept HIVST if kits were available, and 84.0% agreed that they would be willing to introduce and recommend HIVST to others. In another qualitative study by Dirisu *et al*[28] it was observed that most participants were willing to use oral HIVST kits.

Iliyasu *et al*[15] found that 70.4% of university students in northern Nigeria were willing to self-test for HIV and pay for the test kits. Specifically, 55.9% of the participants were willing to pay for the test kits themselves, and 14.5% were willing to pay for the test kits if they were cheaper. Additionally, 61.4% of the participants were willing to self-test with a sexual partner[15]. Also, Ugwu *et al*[29] reported that 61.3% of the health workers working in primary health care centres preferred HIVST over the facility-based testing modalities. In 2021, a study also reported that 59.3% of the respondents were interested in HIVST[30]. In another study, it was observed that nearly all (99.5%) of the pregnant women enrolled in the study preferred conventional HIV testing service at booking. However, 83.8% of these pregnant women were keen to learn how to self-test for HIV, and 85.7% of the respondents were willing to repeat the HIV test during pregnancy, of which 29.3% were willing to self-test[31]. Similarly, 94.6% of respondents were willing to retest for HIV after delivery, of which 27.4% were willing to self-test.

However, another study among young people in Nigeria noted that more than half (69.9%) of the participants indicated they would prefer a physician to administer the HIV test. In comparison, the proportions of those who preferred HIV tests administered by a nurse and self-administered HIV tests were 15.7% and 4.8%, respectively. Another study from Southern Nigeria reported that 23.4% of the respondents accepted HIVST, of which 33.3% of the clients were assisted[5]. In terms of preference of oral *vs* blood-based HIVST in Nigeria, another study by Obiezu-Umeh *et al*[32] reported that oral-based HIVST was preferred by most of the young participants when compared to blood-based HIVST.

Ong *et al*[33] used a discrete choice experiment to design HIVST services for young people in Nigeria. The authors reported that male individuals (compared with female individuals), those who never had sex (compared with sexually active), and those who had never tested for HIV before (compared with those who had previously tested) were more likely to opt-out of using an HIVST kit.

Existing regulatory context for HIVST

Only one study examined the regulatory context for HIVST in Nigeria. The study by Dirisu *et al*[34] examined the regulatory framework for HIVST in Nigeria, revealing several issues. Of the providers who marketed HIVST kits, 94.0% of community pharmacists (CPs) and 33.0% of patent proprietary medicine vendors (PPMVs) claimed to be authorized to sell them[34]. Despite the existence of a National Drug Policy and an automated product registration system administered by the National Agency for Food and Drug Administration and Control, the process for authorizing, manufacturing, and distributing medical products was reported as cumbersome, time-consuming, and costly[34]. Furthermore, the National Drug Distribution Guideline was not implemented, leading to an uncoordinated supply chain. The study also found that less than half (45.6%) of PPMVs and CPs had a standard operating manual for administering HIVST, and about one-third had standard guidelines for HIV testing services. While 77.0% of providers offered counselling before selling HIVST, only 23.0% of CPs and 13% of PPMVs that sold HIVST were accredited HIV counselling and testing centres[34]. These findings demonstrate the need for improved regulatory oversight and support for HIVST implementation in Nigeria.

Uptake of HIVST and linkage to care services

A cohort study on the uptake of HIVST revealed that 97.0% had used self-testing kits. Among these, almost a quarter (22.7%) tested themselves the same day they received the kit, and 49.4% tested within 1 week. About one-quarter (23.5%) reported that they had someone else present while they tested. Of

Table 2 List of papers reviewed

Ref.	Title	Study population	State of study	Objectives/research question	Study design	Findings
Adebimpe *et al*[7], 2019	How acceptable is the HIV/AIDS self-testing among women attending immunization clinics in Effurun, Southern Nigeria	All women of reproductive age (15-49 year) attending the immunization clinic (for their children) in Ekpan General Hospital	Delta	Assess the knowledge and acceptability of HIVST among women of childbearing age attending immunization clinics in Effurun, Southern Nigeria	Descriptive cross-sectional study	The study respondents' high knowledge levels and acceptability of HIVST lend support to the fact that the procedure should be promoted in the stakeholders' efforts to improve HIV testing among the general population
Adeoti *et al* [30], 2021	Sexual practices, Risk perception, and HIV Self-Testing acceptability among long-distance truck drivers in Ekiti State, Nigeria	Adult male long-distance truck drivers in Ado-Ekiti, Southwestern Nigeria	Ekiti	Examined the sexual practices, risk perception, and HIVST acceptability among long-distance truck drivers in Ekiti State, Nigeria	Cross-sectional study	Many long-distance drivers were engaged in unsafe sexual practices and were at risk for HIV transmission. Increasing testing using HIVST has the potential to bridge the gap in the diagnosis of HIV among long-distance drivers who are willing to be tested
Brown *et al* [27], 2015	HIVST in Nigeria: Public opinions and perspectives	Researchers, academics, journalists, community advocates, activists, and HIV policy-makers and programmers, including those working in the development sectors, enlisted on the new HIV vaccine and microbicide advocacy society listserv	All states	Obtained perspectives of informed members of the Nigerian public on the use of the HIVST	Cross-sectional study	Cost-based pricing can be based on and directly tied to current product experiences and information as well as how crucial product monitoring is when pricing a product
Dirisu *et al* [28], 2020	'I will welcome this one 101%, I will so embrace it': A qualitative exploration of the feasibility and acceptability of HIV self-testing among MSM in Lagos, Nigeria	MSM	Lagos	Explored MSM perceptions of oral HIVST and potential barriers to and facilitators of HIVST use. In addition, it sought to identify operational and contextual issues that might affect the distribution of HIVST kits to MSM in the Nigerian context and the potential for linkage to care	Qualitative descriptive study	The potential of HIVST to increase the uptake of HIV testing among MSM in Nigeria was supportive of HIVST. Privacy and convenience offered by HIVST address concerns about stigma and waiting times associated with facility-based testing
Iliyasu *et al* [15], 2020	Acceptability and correlates of HIV self-testing among university students in northern Nigeria	University students	Kano	Examine the acceptability of HIVST and identify factors associated with the uptake of HIVST services among university students in Kano, Nigeria	Cross-sectional study	HTS uptake was low among a sample of university students in northern Nigeria, but most university students were willing to self-test for HIV
Iwelunmor *et al*[36], 2020	The 4 youth by youth HIV self-testing crowdsourcing contest: A qualitative evaluation	All young people between the ages of 10 year to 24 year in Nigeria	All states	Describe the responses to a crowdsourcing contest aimed at soliciting ideas on promoting HIVST among young people in Nigeria	Qualitative study	The study informed the development of innovative youth implementation strategies to increase the uptake of HIVST among adolescents and youth at risk for HIV
Agada *et al* [5], 2021	Reaching out to the hard-to-reach populations with HIV self-testing services in South-south Nigeria	General population	Cross River and Akwa-Ibom	Assess the impact of the total market approach deployed in Cross River and Akwa Ibom States in South-south Nigeria to enhance the demand for HIVST to ensure product equity, accessibility, and sustainability	Retrospective cross-sectional study	The HIVST model demonstrated the potential to be a vital tool in expanding HIV testing services and linking HIV care services to populations who would otherwise not have been tested
Dirisu *et al* [34], 2020	Exploring the regulatory context for HIV self-testing and PrEP market authorisation and use in Nigeria	PPMVs and CPs	Abuja, Rivers, Imo, Lagos, and Ogun	Assess HIVST/PrEP availability and market authorization; determine the facilitators and barriers to access; and identify existing systems that support the availability, appropriate use,	Cross-sectional study	About 63% of CPs and 27% of PPMVs sold HIVST kits, while 15% of CPs and no PPMV sold PrEP in their facilities. Most CPs (94%) and 33% of PPMVs who sold HIVST kits reported that their facilities were

				affordability, and accessibility in the private sector in Nigeria		authorized to sell HIVST kits
Nwaozuru *et al*[26], 2019	Preferences for HIV testing services among young people in Nigeria	Youth aged 14–24 year	Lagos	Assessed preferences for HIV testing options among young people in Nigeria	Cross-sectional study	HIV testing services was optimized to reach young people in various options to meet their unique preferences
Ong *et al* [33], 2021	Designing HIV Testing and Self-Testing Services for young people in Nigeria: A discrete choice experiment	Nigerian youth (14-24 year)	Lagos	Examine the strength of Nigerian youth preferences related to HIV testing and HIVST	Discrete choice experiments	There could be demand for HIVST for Nigerian youth, who prefer HIVST kits that integrate testing for other STIs and is accessed from community health centres
Obiezu-Umeh *et al* [32], 2021	Young people's preferences for HIV self-testing services in Nigeria: A qualitative analysis	Young people (14–24 year)	Lagos	Use qualitative methods to examine HIVST preferences among Nigerian youth	Cross-sectional study	HIVST preferences among Nigerian youth appeared to be influenced by several factors, including lower cost, less invasive testing method, location of testing, and linkage to care and support post-testing. Findings underscored the need to address young people's HIVST preferences as a foundation for implementing programs and research to increase the uptake of HIVST
Obiezu-Umeh *et al* [51], 2020	Development of HIVST services through youth engagement: A qualitative evaluation of a health designation in Nigeria	Young people (14–24 year)	Lagos	Explore strategies for HIVST delivery developed at a designations contest in Nigeria	Cross-sectional study	Designations were a feasible method of facilitating meaningful youth engagement to develop deployable strategies to increase the uptake of HIV testing in young people in Nigeria
Durosinmi-Etti *et al* [38], 2021	Communication needs for improved uptake of PrEP and HIVST services among key populations in Nigeria: A mixed-method study	MSM, FSWs, and key influencers of the KP groups (health providers, peer educators, HIV program officers)	Akwa Ibom, Cross River, and Lagos	Identify the communication needs and preferences of the KP groups as evidence for developing strategies and interventions to increase awareness and use of HIVST and PrEP services among the KPs in Nigeria	Cross-sectional study	KPs effectively networked to increase awareness and access to PrEP and HIVST services in Nigeria. They will make the peers receptive to the interventions and help them reach other peers in their network, especially the hard-to-reach
Sekoni *et al* [37], 2022	Operationalizing the distribution of oral HIVST kits to MSM in a highly homophobic environment: the Nigerian experience	MSM and KOL	Lagos	Explore the operationalization of using KOLs to distribute HIVST kits to MSM	Cross-sectional study	This study showed the practical steps involved in operationalizing the KOL support system distribution of HIVST that positively influenced the testing experience for the participants irrespective of their HIV status and engagement in care. KOLs were a reliable resource to leverage for ensuring that HIVST kit was utilized, and HIV-positive individuals were linked to treatment and care in homophobic environments
Iwelunmor *et al*[52], 2022	Enhancing HIVST among Nigerian youth: Feasibility and Preliminary Efficacy of the 4 youth by youth study using crowdsourced youth-led strategies	Youth (14-24 year)	Lagos, Enugu, Ondo, and Oyo	Examine the feasibility and efficacy of crowdsourced youth-led strategies to enhance HIVST and STI testing	Quasi-experimental	The study provided promising evidence of efficacy that youth-led, crowdsourced strategies led to higher uptake of HIV and STI testing
Tun *et al* [35], 2018	Uptake of HIVST and linkage to treatment among MSM in Nigeria: A pilot programme using key opinion leaders to reach MSM	Males (17-59 year)	Lagos	Assess the feasibility, acceptability, uptake of HIVST, and linkage to HIV treatment among MSM through KOLs in Lagos, Nigeria	Cohort study	HIVST distribution through KOLs was feasible, and oral self-testing was highly acceptable among this urban MSM population. This study showed that linkage to treatment could be achieved with active follow-up and access to a trusted MSM-

						friendly community clinic that offers HIV treatment. HIVST should be considered an additional option to standard HIV testing models for MSM
Ugwu *et al* [29], 2020	HIVST: Perspectives from primary healthcare workers in Enugu State, Southeast Nigeria	Health workers in the primary health facilities	Enugu	Assess issues surrounding the HIVST from the perspectives of the primary healthcare workers in Enugu State	Cross-sectional study	Most of the primary healthcare workers in Enugu State had poor knowledge of HIVST
Iliyasu *et al* [31], 2022	HIVST and repeat testing in pregnancy and postpartum in Northern Nigeria	Pregnant women	Kano	Determine the predictors of willingness to self-test for HIV when retesting in pregnancy and postpartum among antenatal clients at a large teaching hospital in Northern Nigeria	Cross-sectional	The acceptability of HIVST for repeat testing in pregnancy and postpartum was low, but most respondents desired to be trained to self-test for HIV

AIDS: Acquired immune deficiency syndrome; CPs: Community pharmacists; HIV: Human immunodeficiency virus; HIVST: HIV self-testing; MSM: Men who have sex with men; HTS: HIV testing services; FSWs: Female sex workers; KOL: Key opinion leader; KP: key population; PPMVs: Patent and proprietary medicine vendors; PrEP: Preexposure prophylaxis; STI: Sexually transmitted infection.

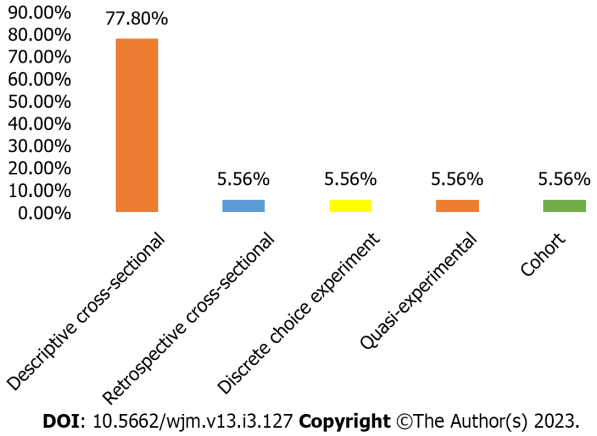


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Figure 2 Study design.

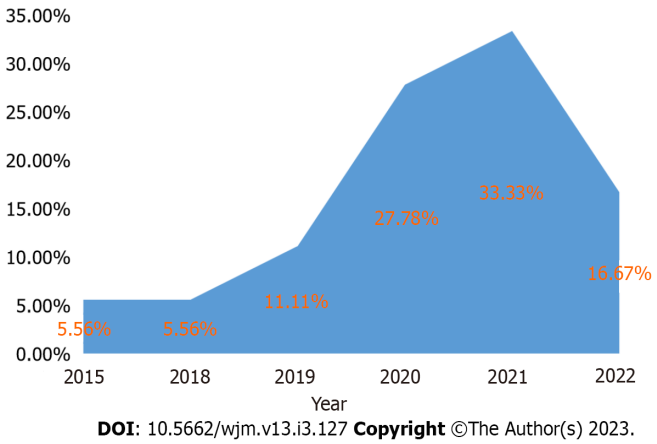

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Figure 3 Year of publication.

these, 55.0% tested with a friend, 21.7% tested with a family member, 16.7% tested with a sex partner, and 6.7% tested with a key opinion leader (KOL)[35]. Another study revealed that 9.0% of the respondents reported previous HIVST[15] (Figure 6).

Regarding linkage to care after HIVST, it was reported that in Cross River State 14 of the 15 clients who reported reactive results (93.3%) were linked to confirmatory testing. Of the 14 linked to confirmatory testing, 13 (91.0%) were confirmed positive, and all (100.0%) were linked to HIV treatment

Table 3 Target population for the included studies

Population	*n*	Frequency, %
Young people aged 15-24 yr	5	26.3
MSM	3	15.8
Key population influencers	2	10.5
Women of reproductive age	1	5.3
Long distance drivers	1	5.3
Professionals[1]	1	5.3
Student at university	1	5.3
General population	1	5.3
PPMV and CP	1	5.3
FSW	1	5.3
Males aged 17-59 yr	1	5.3
Pregnant women	1	5.3

[1]Ethicists, researchers, those in the academia, journalists, community advocates, activists, and policymakers.
CP: Community pharmacists; FSW: Female sex workers; MSM: Men who have sex with men; PPMV: Patent proprietary medicine vendors.

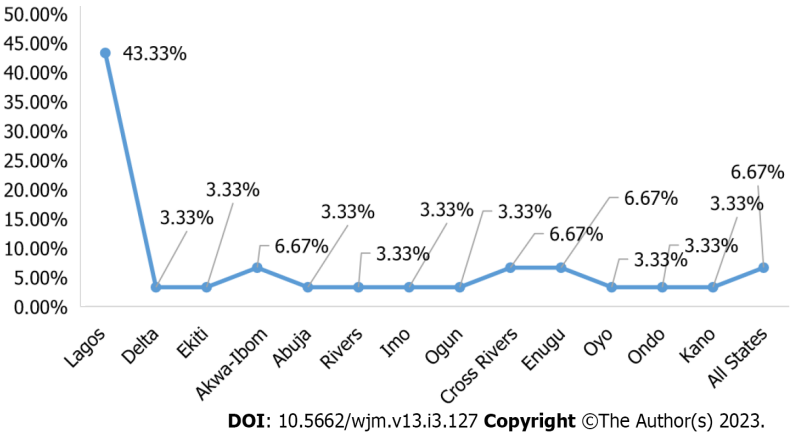

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Figure 4 Study area in Nigeria.

[5]. Of the 24 who reported reactive results in Akwa Ibom State, 87.5% had confirmatory testing, 100.0% reported confirmed HIV-positive results, and 100.0% were successfully linked to HIV care and treatment[5]. Another study reported that the 14 participants who had a reactive HIV self-test sought post-test counselling and had confirmatory HIV testing at the community health centre[35].

Dirisu *et al*[34] highlighted barriers to linkage to care, including concerns around post-test counselling services and linkage to confirmatory HIV testing services following reactive HIVST results. For instance, men who have sex with men (MSM) were concerned that because self-testers would be testing alone, many would deny their HIV-positive test results and may not seek HIV treatment. In another interview that sought the opinions of the public and that of stakeholders and policy makers on the introduction of HIVST in Nigeria, participants expressed concerns about how to link individuals who tested HIVST reactive to confirmatory HIV testing services and care and treatment services as in the facility-based testing model[27]. Obiezu-Umeh *et al*[32] highlighted the motivations to seek a confirmatory HIV test in the event of a reactive HIVST result to include encouragement from peers, family members, or healthcare workers, denial about the initial HIVST test result, lack of satisfaction with the test result, and the possibility of living longer if initiated on treatment and care.

Strategies for enhancing uptake of HIVST in Nigeria

Studies conducted among young people in Nigeria by Obiezu-Umeh *et al*[32] and Iwelunmor *et al*[36] reported that the cost of HIVST was the strongest determinant for choosing HIVST services. The cost of HIVST ranged from 200 to 4000 Naira (approximately United States Dollar 0.55-11.07). However, the

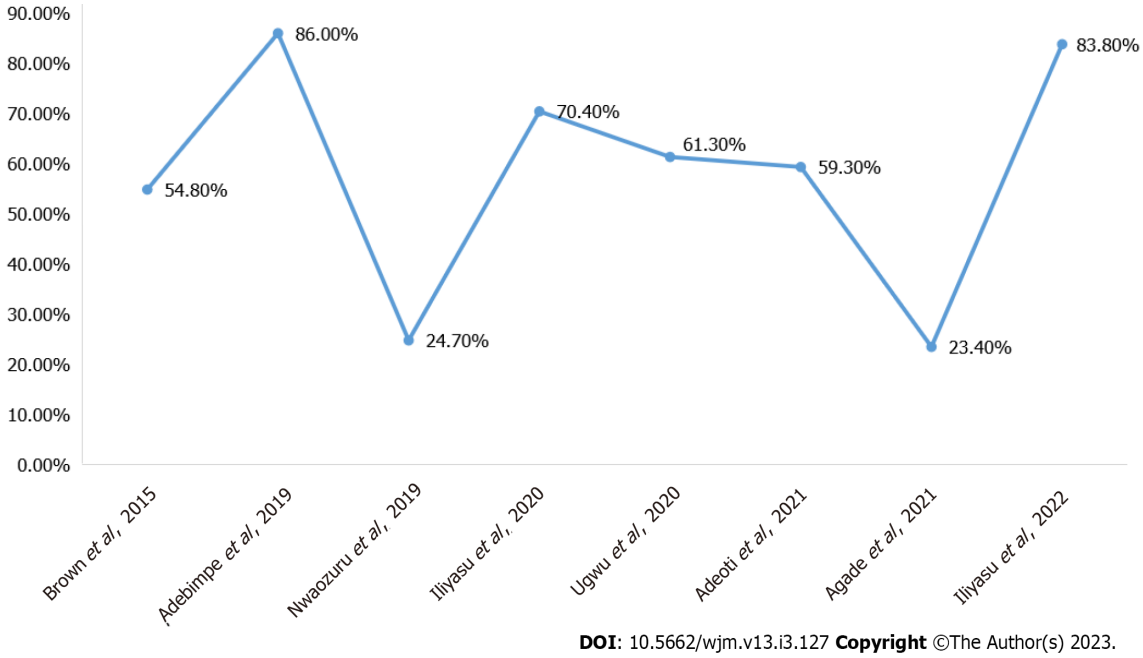

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Figure 5 Acceptability of human immunodeficiency virus self-testing in Nigeria.

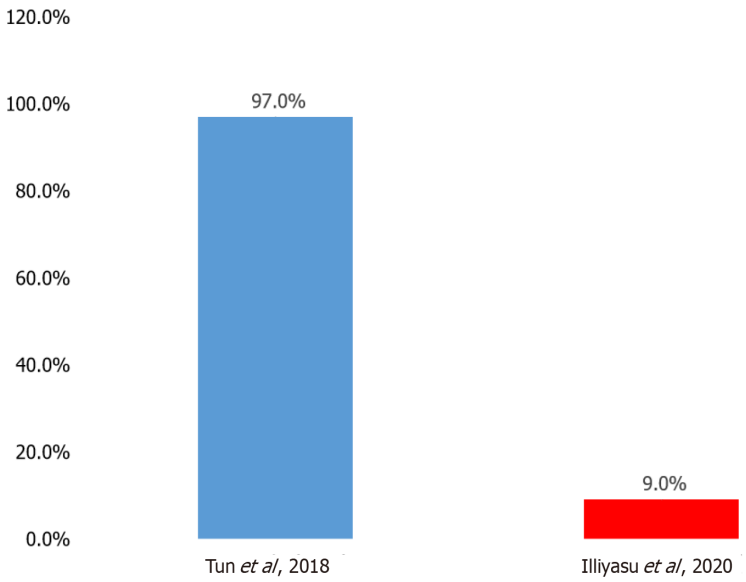

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Figure 6 Uptake of human immunodeficiency virus self-testing.

majority of young people suggested 500 Naira (approximately United States Dollar 1.38) as the preferred cost of the kit. Young people argued that the high cost of HIVST remained a major barrier to uptake since most young people might not be willing to purchase the kit given that HIVST kits are available in some hospitals and non-governmental organizations either free of charge or at a subsidized rate[32].

Ong *et al*[33] and Obiezu-Umeh *et al*[32] reported access location as a major driver and the most influential driver, respectively, of HIVST uptake. Obiezu-Umeh *et al*[32] reported privately-owned facilities, registered pharmacies, youth-friendly centres, supermarkets, and online stores as the most cited preferred locations to access HIVST kits. Ong *et al*[33] added that the least influential driver of HIVST uptake was the type of specimen needed for HIVST. Obiezu-Umeh *et al*[32] and Iwelunmor *et al*[36] suggested making HIVST more appealing to young Nigerians. This could be achieved by repackaging existing HIVST products with colours, taglines, designs, and youth-friendly animations[32,36]. Iwelunmor *et al*[36] also found that providing instruction for use translated into the three most common Nigerian languages (Igbo, Hausa, and Yoruba) would further enhance the appeal and uptake of the product among diverse segments of youths in Nigeria.

Studies among young people, MSM, and KOLs recommended using social media (Facebook/SMS and WhatsApp, *etc.*) and bulk SMS messages to enhance the uptake of HIVST[36,37]. In contrast, a quantitative study among key populations in Nigeria reported that 85% of female sex workers and 68% of MSM preferred an in-person modality of receiving information on HIVST services[37]. Furthermore, Sekoni *et al*[37] further suggested using KOLs to distribute and enhance the uptake of HIVST kits among MSM. Similarly, Iwelunmor *et al*[36] opined that recruiting local celebrities to join HIVST online campaigns and endorse HIVST-related hashtags could generate high demand for HIVST services and promote its uptake among their teeming fans who are mostly young people.

DISCUSSION

HIVST is a rapidly growing HIV testing strategy that is gradually gaining acceptability globally. However, the level of acceptability and strategies to enhance the uptake of HIVST varies across different populations and settings. In this systematic review, we examined the acceptability of HIVST and strategies to enhance the uptake of HIVST in Nigeria. Our findings revealed a high level of acceptability of HIVST in most of the studies included in this systematic review with many citing privacy and convenience as key factors in their willingness to use the service[5,7,15,27-31,38]. These findings are consistent with previous studies, which reported high acceptability and ease of use of HIVST in other settings such as United States, Tanzania, and Zimbabwe[39-41].

For instance, a recent cross-sectional study conducted in Tanzania among medical students showed a high level of knowledge, acceptability, and willingness to use oral fluid HIVST[39]. Similarly, a recent survey among people who use illicit drugs in the United States reported high acceptability of an HIVST program, and 77% of study participants were willing to use HIVST kits regularly if available[40]. In addition, a campus-based distribution of oral HIVST was highly acceptable among young adult students in Zimbabwe, with 97.1% of participants indicating willingness to use oral HIV self-tests[41]. However, some studies have reported low acceptability for HIVST among specific populations, such as MSM in Brazil, where less than half (47.3%) were willing to use HIVST[42].

Cost was a significant barrier to HIV testing among young people in Nigeria, and they preferred free or low-cost testing services[26]. The cost of HIVST in the private sector, especially in low-income contexts, may contribute to an unwillingness to use HIVST, which justifies the need for free distribution to key and priority groups in the public sector. Additionally, the fear of getting a positive result without being appropriately linked to a health service could also contribute to low acceptance of HIVST, thus emphasizing the importance of a peer navigator to support clients across the continuum of care.

The uptake of HIVST and linkage to care services varies across different population groups and countries. This systematic review found a high uptake of HIVST among MSM in Nigeria, similar to findings from Bangkok, Thailand[35,43], while low uptake of HIVST has been reported in South Africa and China[44,45]. This variation in findings could be attributed to differences in the level of awareness about HIVST among the studied populations. Factors such as education level, age, marital status, and knowledge about HIV can also influence the uptake of HIVST[27,28,35,44-46].

A study conducted in the Republic of Congo reported a high linkage to HIV care services (82.2%) among individuals with a reactive result from HIVST, which is consistent with the findings of another study in Nigeria[47]. However, studies have highlighted several barriers to linkage to care, such as social stigma, lack of communication about the benefits of testing, and the referral process after testing, as well as a lack of a supportive peer network to encourage linkage after testing[27,28,48]. Therefore, it is important to identify and address these barriers to linkage to care to ensure that HIVST programs are effective in reducing the burden of HIV and promoting early diagnosis and treatment.

Further research is needed to develop interventions that address the barriers to uptake and linkage to care. The systematic review on strategies to enhance HIVST uptake shows the importance of accessible points of HIVST distribution and involvement of young people in the development and design of HIVST services to address their preferences, which include privacy, confidentiality, convenience, and assurance of accuracy in order to enhance uptake[32,33,36]. These findings are supported by studies from Rwanda[49,50] which suggested that involving the target population in program design could improve HIVST uptake. The co-creation process that involved men in Rwanda identified the need for a comprehensive health education program to address barriers to HIVST uptake. Key stakeholders emphasized the need for community engagement, regulatory frameworks, and sustained political commitment to promote the increase of HIVST.

Furthermore, this systematic review also highlighted additional strategies to enhance HIVST uptake in Nigeria, including increasing awareness, regulating the sale of self-test kits, subsidizing the cost of self-test kits, maintaining consistent availability of self-test kits, and documenting HIVST standards and policies[35,38,51,52]. The articles also suggested that mobilization campaigns, training for people involved in implementation, and engaging key stakeholders such as religious and community leaders, employers, KOLs, celebrities, and health workers could accelerate HIV testing and promote uptake and linkage to care services.

Overall, the findings suggested that tailored communication strategies that address misinformation, misconceptions, and mistrust about HIVST and pre-exposure prophylaxis are needed for improved uptake of HIVST among key populations in Nigeria[38]. The involvement of stakeholders and the target population can lead to the design of HIVST programs that address the unique needs and preferences of each population, ultimately improving HIV testing and linkage to care services[49].

CONCLUSION

In summary, the landscape of HIVST in Nigeria is still in its infancy with a limited evidence base. Therefore, there is a compelling need for more high-quality research such as randomized clinical trials to advance our understanding of HIVST. This study revealed a shortage of implementation science research, despite the various self-testing activities ongoing in Nigeria. The investigators also noted lack of studies evaluating other HIVST distribution models, such as workplace, community distribution, and distribution among facility providers, and sub-populations, like pregnant women, people who inject drugs, and female sex workers. Only one study from Southern Nigeria evaluated programmatic HIVST distribution data among CPs. While the acceptability of HIVST is generally high in Nigeria when measured from the intention-to-use perspective, actual use in programmatic implementation was lower, primarily due to the cost barrier among pharmacy retail outlets. Therefore, innovative financing approaches targeting different population segments are necessary for effective scaling and growth of the HIVST market in Nigeria using demand side subsidy financing, total market approach, and social marketing.

More controlled implementation studies are required to test the acceptability of HIVST. The use of KOLs among key populations has been successful in increasing the acceptability and uptake of HIVST. The uptake of HIVST was generally high among reported studies, except for reported HIVST results. Therefore, more studies are needed to evaluate factors responsible for poor uptake of HIVST result retrieval and how uptake will compare in routine programs *vs* real-life settings in the absence of support and resources that enhance HIVST uptake. In conclusion, despite limitations, this is the first systematic literature review of HIVST in Nigeria, providing valuable insights into the evidence base on the acceptability and uptake of HIVST in the country.

ARTICLE HIGHLIGHTS

Research background

Nigeria has a high burden of human immunodeficiency (HIV)/acquired immune deficiency syndrome, and a significant proportion of infected individuals remain untested due to fear of stigma and discrimination. HIV self-testing (HIVST) is recommended by the World Health Organization as a tool for improving testing uptake and achieving the United Nations Programme on HIV and acquired immune deficiency syndrome 90-90-90 target. However, HIVST is still a growing intervention in Nigeria, and there is a need to systematically review its acceptability and uptake in the country.

Research motivation

To systematically review the acceptability, existing regulations, and strategies for enhancing the uptake of HIVST in Nigeria.

Research objectives

To fill a crucial gap in understanding the HIVST landscape in Nigeria and provide insights into the evidence base on the acceptability and uptake of HIVST in the country.

Research methods

A systematic literature review was conducted, and 18 articles were included in the analysis.

Research results

The study found that the acceptability of HIVST is generally high in Nigeria from the intention-to-use perspective. However, the actual use of HIVST in programmatic implementation was lower than expected. The study recommends more controlled implementation studies to test the acceptability of HIVST and to explore factors responsible for poor uptake. The use of key opinion leaders among key populations has been found to be successful in increasing the acceptability and uptake of HIVST. However, cost remains a major barrier to the acceptability of HIVST among pharmacy retail outlets.

Research conclusions

The present study provided crucial understanding of the HIVST landscape in Nigeria, which is young

and evolving. The study highlighted the need for further high-quality research in this area and recommended innovative financing approaches targeting different population segments for effective scaling of HIVST under the total market approach.

Research perspectives

More studies are required to evaluate how the uptake of HIVST compares in routine programs *vs* real-life settings in the absence of support and resources that enhance HIVST uptake. Overall, this study contributed to the current knowledge base on HIVST in Nigeria and highlighted the need for further high-quality research in this area.

ACKNOWLEDGEMENTS

The investigators want to acknowledge all authors who shared the full text of their manuscripts with us. The investigators also wish to acknowledge Dr. Akintayo Ogunwale and Dr. (Mrs.) Omotayo Sindiku for their time and intellectual contribution while reviewing this manuscript.

FOOTNOTES

Author contributions: Adepoju VA and Adelekan A conceived the study; Onoja AJ provided overall guidance to the study; Adelekan A, Umebido C, and Adepoju VA conducted screening and led data extraction; Adelekan A and Onoja AJ drafted the manuscript; All authors read and approved the final version.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Liu XF
L-Editor: Filipodia
P-Editor: Ju JL

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 142-152

DOI: 10.5662/wjm.v13.i3.142

ISSN 2222-0682 (online)

SYSTEMATIC REVIEWS

Preferences for oral- *vs* blood-based human immunodeficiency virus self-testing: A scoping review of the literature

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Specialty type: Medical laboratory technology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kocazeybek B, Turkey

Received: January 18, 2023
Peer-review started: January 18, 2023
First decision: April 20, 2023
Revised: April 22, 2023
Accepted: May 24, 2023
Article in press: May 24, 2023
Published online: June 20, 2023

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Abstract

BACKGROUND

The evidence on preferences for oral- *vs* blood-based human immunodeficiency virus self-testing (HIVST) has been heterogenous and inconclusive. In addition, most evaluations have relied on hypothetical or stated use cases using discreet choice experiments rather than actual preferences among experienced users, which are more objective and critical for the understanding of product uptake. Direct head-to-head comparison of consumer preferences for oral- versus blood-based HIVST is lacking.

AIM

To examine the existing literature on preferences for oral- *vs* blood-based HIVST, determine the factors that impact these preferences, and assess the potential implications for HIVST programs.

METHODS

Databases such as PubMed, Medline, Google Scholar, and Web of Science were searched for articles published between January 2011 to October 2022. Articles must address preferences for oral- *vs* blood-based HIVST. The study used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist to ensure the quality of the study.

RESULTS

The initial search revealed 2424 records, of which 8 studies were finally included in the scoping review. Pooled preference for blood-based HIVST was 48.8% (9%-78.6%), whereas pooled preference for oral HIVST was 59.8% (34.2%-91%) across all studies. However, for male-specific studies, the preference for blood-based

HIVST (58%-65.6%) was higher than that for oral (34.2%-41%). The four studies that reported a higher preference for blood-based HIVST were in men. Participants considered blood-based HIVST to be more accurate and rapid, while those with a higher preference for oral HIVST did so because these were considered non-invasive and easy to use.

CONCLUSION

Consistently in the literature, men preferred blood-based HIVST over oral HIVST due to higher risk perception and desire for a test that provides higher accuracy coupled with rapidity, autonomy, privacy, and confidentiality, whereas those with a higher preference for oral HIVST did so because these were considered non-invasive and easy to use. Misinformation and distrust need to be addressed through promotional messaging to maximize the diversity of this new biomedical technology.

Key Words: Human immunodeficiency virus self-testing; Preferences; Oral human immunodeficiency virus self-testing; Blood-based human immunodeficiency virus self-testing

Core Tip: We conducted a scoping review of the literature to determine the preferences for oral- *vs* blood-based human immunodeficiency virus self-testing (HIVST) and related factors. We searched PubMed, Medline, Google Scholar, and Web of Science databases for articles published between January 2011 and October 2022 that addressed preferences for oral- *vs* blood-based HIVST. The pooled preferences for blood- and oral-based HIVST were 48.8% and 59.8%, respectively. For male-specific studies, the preference for blood-based HIVST was higher than for oral. These results highlight the need to address misinformation and distrust through promotional messaging to maximize the diversity of this new biomedical technology.

Citation: Adepoju VA, Imoyera W, Onoja AJ. Preferences for oral- *vs* blood-based human immunodeficiency virus self-testing: A scoping review of the literature. *World J Methodol* 2023; 13(3): 142-152
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/142.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.142

INTRODUCTION

The Joint United Nations Programme on human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (UNAIDS) has set 95:95:95 as a strategy to end HIV/AIDS by 2030. Although much progress has been made in achievement of the first 95 (*i.e.* 95% of individuals with HIV should test and know their HIV status), progress has been slow among hard-to-reach populations such as men, key populations, adolescents, and young persons. Men living with HIV perform less than women, with only 82% of men living with HIV knowing their HIV status[1]. Compared to women living with HIV, there are 740000 more men living with HIV who do not know their HIV status, 1.3 million more men who are not on treatment, and 920000 more men who are not virally suppressed[2]. The World Health Organization (WHO) released the first normative guideline on HIV self-testing (HIVST) in 2016[3]. WHO recommended HIVST as an additional approach to HIV testing services and recently added that both oral- and blood-based options should be provided. HIVST is safe, private, confidential, and convenient with the potential to improve access to testing for hard-to-reach populations such as men, adolescents, and young people as well as key populations. ST, being the first step in the care continuum, presents an enormous opportunity to close the HIV testing gap and achieve the global 95:95:95 fast track target set by UNAIDS. Self-testing empowers consumers to control when, where, and how they test for any of these diseases. Given the challenges in accessing traditional, provider-led testing services such as long distance from facilities, limited operating hours of conventional clinics, competing client priorities such as job and schooling, stigma, high cost, poor awareness, and dearth of culturally competent healthcare workers[4,5], ST as an alternative testing model, is a useful tool to expand access to testing for HIV, especially among vulnerable groups.

As of August 2022, six HIVST have been prequalified by the WHO (one using oral fluid and five using whole blood), *i.e.* Wondfo, Mylan, Insti, Check Now, Sure Check, and OraQuick[6,7]. However, evidence on preferences for oral- *vs* blood-based options has been heterogenous and inconclusive[8,9]. In addition, most evaluations have relied on hypothetical or stated use cases using discreet choice experiments[10,11] rather than actual use preferences from experienced end-users, which are more

objective and critical for uptake. Two main types of HIVST are available: oral- and blood-based tests. While both tests have demonstrated high sensitivity and specificity, the preferences of individuals for one test type over the other remain unclear. Understanding these preferences is crucial to promoting the widespread adoption and usage of HIVST.

The purpose of this scoping review is to provide a comprehensive overview of the literature on preferences for oral- *vs* blood-based HIVST, identify factors influencing these preferences, and explore the implications of these preferences for the promotion and implementation of HIVST programs. By synthesizing existing evidence, this review aims to inform policy-makers, healthcare providers, and other stakeholders involved in the design and implementation of HIVST programs, in order to maximize uptake and improve overall public health outcomes.

MATERIALS AND METHODS

The scoping review adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Figure 1). These reviews follow explicit, pre-specified and reproducible methods in order to identify, evaluate, and summarize the findings of all relevant individual studies (Grant and Booth, 2009)[12].

Search strategy

One of the authors (Adepoju VA) searched for eligible studies between October 15 and 20, 2022. The Arksey and O'Malley[13] (2005) methodological framework guided the scoping of the published data. The scoping review conducted in this study was not registered in a registry such as PROSPERO. We chose not to register this scoping review, as registration is not a mandatory requirement for scoping reviews and our primary aim is to provide a broad overview of the literature rather than conduct a systematic assessment of the evidence. Although the Reference Citation Analysis tool was available for use, it was not utilized for this review. This decision was made based on the nature of the research question and the inclusion and exclusion criteria developed for the review, which ensured that all relevant studies were identified through the comprehensive search strategy described above.

Data sources

Searched databases included PubMed, MEDLINE, Web of Science, and Google Scholar. For studies that may have been missed in the electronic search, we used reference lists of all the articles identified for cross-referencing. The first search took place between October 1 and 6, 2022, whereas the second took place between October 8 and 14, 2022. Detailed inclusion and exclusion criteria were developed with caution, to make sure that they matched the review questions and involved sufficient details to help point out all relevant studies and exclude irrelevant ones. The researchers then embarked on a two-stage process in which two reviewers independently screened the titles and abstracts for eligibility to be included in the final selection of papers. A combination of terms was used in the database searches; specifically: "HIV self-testing," OR "HIVST" OR "HIV self-screening" OR "HIVSS" AND "preferences" AND "values" AND "oral- and blood-based" OR "oral and fingerstick HIVST" OR Oral and capillary" OR "oral- *vs* blood-based." Specific keywords were combined with Boolean operators in the literature search (Table 1).

Study selection

The systematic searches for eligible articles retrieved 2424 studies and 1454 duplicates were eventually removed. The authors (Adepoju VA, Imoyera W) independently screened the titles and abstracts for eligibility with the condition that if one or both authors identified the article as relevant, then the full-text review would be carried out. The researchers solved any disagreements *via* discussions and reached a consensus. After the title and abstract screening, two reviewers (Adepoju VA, Imoyera W) independently screened the full text of selected articles. Disagreements were resolved through discussions with a third reviewer (Onoja AJ) for final inclusion. The articles were selected in several parts, which allowed the reviewers to have a regular discussion of the eligibility criteria, ensuring the same understanding of the criteria, and the criteria remaining the same throughout the article selection phase. The researchers did not assess the risk of bias of the included studies. As in many scoping reviews, the goal was to describe preferences for oral- *vs* blood-based HIVST.

Inclusion and exclusion criteria

Studies published in peer-reviewed journals between January 2011 and October 2022 and focusing on preferences of oral- *vs* blood-based HIVST among actual users were reviewed.

Inclusion criteria were: primary studies with participants aged 15 years or more with no geographic or population limitations; studies reporting on user preferences for HIVST with only two comparison groups (*i.e.* oral- *vs* blood-based HIVST); studies that adapted HIV Point of Care for HIVST for research purpose only; and studies that included actual users of oral- and blood-based HIVST. Exclusion criteria were studies comparing either oral- or blood-based HIVST with facility-based test or any other HIV

Table 1 Search terms used in the literature search on preference for oral- *vs* blood-based human immunodeficiency self-testing in Nigeria

Search terms	OR	AND	AND
HIVST	HIV self-testing	HIV self-screening	
HIV self-testing			
HIVSS			HIV self-screening
HIV self-screening			
Blood-based HIVST	Fingerstick HIVST		Capillary HIVST
Fingerstick HIVST			
Capillary HIVST			Oral HIVST
Oral HIVST			
Preference			

Please note that the terms in the "OR" column are combined with an "OR" operator, whereas the terms in the "AND" columns are combined with an "AND" operator. The search strategy is designed to identify studies focusing on preferences for different HIV self-testing methods within the Nigerian context. HIV: Human immunodeficiency virus; HIVSS: HIV self-screening; HIVST: HIV self-testing.

testing approaches (*e.g.*, Voluntary Counselling and Testing, mail-in, Dry Blood Sample); studies evaluating user preferences for one type of HIVST only (*i.e.* oral- or blood-based specimen); studies where comparison group for preferences was not clear, not stated or measured qualitatively; and studies including hypothetical users rather than actual users of HIVST. Also excluded were articles published before January 2011, conference papers, books, studies with no full-text available, and magazines. This is because HIVST was not popular before this period and publications on this subject matter were either scarce or non-existent before this period. In accordance with PRISMA guideline 16b, we have cited and explained the exclusion of studies that appeared to meet the inclusion criteria but were ultimately excluded. The reasons for their exclusion are provided in the results and appendix section (Supplementary Table 1) ensuring transparency in the review process.

Data extraction

The authors extracted relevant data using a standard excel-based template. Two authors (Adepoju VA, Imoyera W) independently extracted the data, and the results were reviewed and verified by both authors for quality and clarity. Two authors (Adepoju VA, Onoja AJ) separately and independently assessed the full text of the potentially eligible publications. Disagreements were resolved by consensus. Initial agreement was obtained on 90% of the items, and discrepancies were discussed between authors until 100% agreement was obtained. The following information was extracted from the included studies: author name and year of study, study design, type of specimen, product type, population and age, prevalence of preference for oral- and blood-based HIVST and major findings (Table 2). After extracting relevant information from the studies, the authors constructed a more specific classification for preferences of oral- *vs* blood-based HIVST.

List of papers reviewed

The search results are shown in Figure 1, along with a summary of the papers consulted (the PRISMA flow chart). Although 2424 research articles were retrieved initially from the databases, only 8 met the inclusion criteria for this scoping review.

RESULTS

During the study selection process, we identified several studies that initially appeared to meet our inclusion criteria but were ultimately excluded upon closer examination and based on the predefined inclusion and exclusion criteria. We have provided a comprehensive list of these excluded studies and the reasons for their exclusion in the Supplementary Table 1. By documenting this information, we aim to ensure transparency and reproducibility in our review and study selection process and to demonstrate compliance with PRISMA 16B.

Geographic and population distribution of the included articles

The total number of participants across the 8 studies was 7129 (40-4496). Of the eight studies reviewed,

Table 2 Summary of the included studies, *n* = 8

Ref.	Country	Study design	Type of specimen	Product type	Population, age in yr	Preference for oral, %	Preference for blood, %	Other findings
Tonen-Wolyec *et al*[14], 2020	The Republic of Congo	Cross-sectional	Oral *vs* Fingerstick	Oraquick, Exacto	General population,18-49	85.6	78.6	Comparable accuracy. University education and higher risk increases BB preference
Trabwongwitaya *et al*[15], 2022	Thailand	Cross-sectional	Oral *vs* Fingerstick	Oraquick, INSTI	Young adult KP,18-24	34.4	65.6	Performance and interpretation, O-93.3%, 100%; B-89.5%,98%
Cassell *et al*[19] , 2022	Cambodia	Cross-sectional	Oral *vs* Fingerstick	Oraquick, CombokitsAbbot	KPs, 15+	88.5	11.5	Assisted-98.6%; Unassisted-1.4%
Shapiro *et al*[20], 2020	South Africa	Cross-sectional	Oral *vs* Fingerstick	Oraquick, Atomo	Adult men, 18+	42	58	10% and 90% will prefer different and the same kit for repeat tests, respectively
Lippman *et al* [17], 2018[1]	South Africa	Cross-sectional	Oral *vs* Fingerstick BB	Oraquick, Atomo	MSM	34.2	64.6	97% will use HIVST again if available in the future
Lee *et al*[16], 2022	Australia	Randomized Clinical Trial (RCT)	Oral *vs* Fingerstick	Oraquick, Atomo	MSM,18+	41	58	O-not swabbing both gum, placing buffer on stand; BB-filling test channel, squeezing finger for blood drop
Ritchwood *et al* [18], 2019	South Africa	Qualitative	Oral *vs* Fingerstick	Not stated	Young adult,18-24	80	20	Post-test opinion change on ease of use and trust in result
Gaydos *et al*[21], 2011	United State	Crosssectional	Oral *vs* Fingerstick	Oraquick, Unigold	Emergency department, 18-64	91	9	'Trust in result' O-similar for initial HCW-led and client ST (91%); B-client BBST result more (91.7%) than HCW provided HIV test (77.8%)

[1]Indicates that it is a subsequent publication by the same author(s) in the same year. B: Blood; BBST: Blood-based self-testing; HCWs: Healthcare workers; HIV: Human immunodeficiency; KP: Key Population; MSM: Men who have sex with men; O: Oral; ST: Self-testing.

three studies were from South Africa and one each was from Cambodia, the United States, Thailand, Australia, and the Democratic Republic of Congo Figure 2. Three studies involved the general population (*n* = 3)[14-16], four involved the key population (*n* = 4)[14-17], and one involved young people (*n* = 1)[18]. A total eight studies, *i.e.* 6 quantitative studies[14,15,17,19-21], 1 randomized control trial[16], and 1 qualitative[18], were included in the study.

Year of publication of included studies

Out of the eight articles included, three were published in 2022[15,16,19], two in 2020[14,20], one in 2018 [17], one in 2019[18], and one in 2011[21] (Figure 3).

Preference for oral- vs blood-based HIVST

One hundred percent of the studies reported preference based on the actual use of HIVST, and 50% reported usability. Four of the eight studies (50%) reported a higher preference for blood-based HIVST [16,18-20], whereas four of the eight studies (50%) reported a higher preference for oral HIVST[14,15,17, 21]. Pooled preference for blood-based HIVST was 48.8% (9%-78.6%), whereas pooled preference for oral HIVST was 59.8% (34.2%-91%) across all studies. However, for male-specific studies[16,18-20], preference for blood-based HIVST (58%-65.6%) was higher than that for oral (34.2%-41%). The four studies that reported a higher preference for blood-based HIVST were in men, and participants considered blood-based HIVST to be more accurate and rapid whereas studies reporting higher preference for oral HIVST did so because they were considered non-invasive and easy to use with few false-negative results.

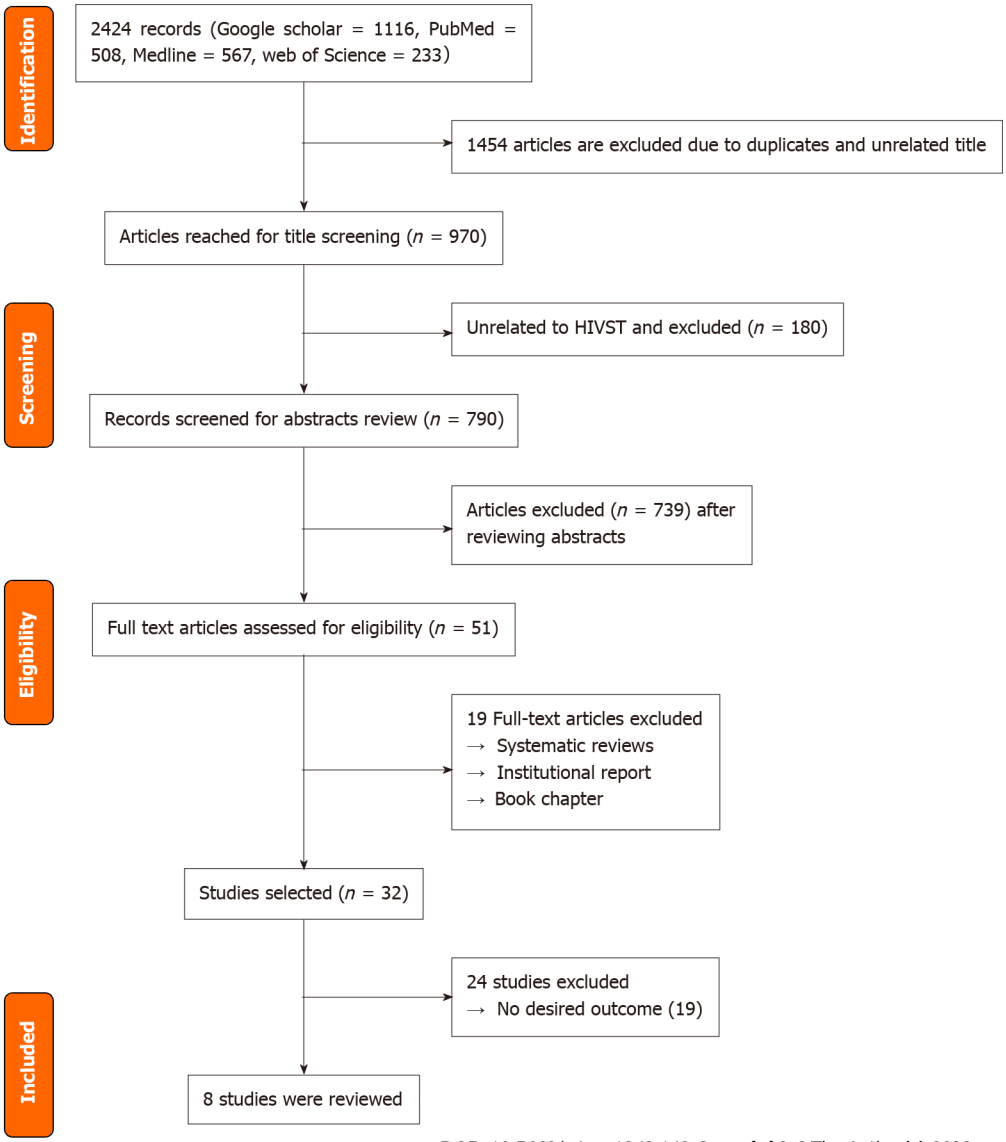

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Figure 1 Preferred reporting items for systematic reviews and meta-analyses flowchart: Study selection process for the scoping review on preferences for oral- *vs* blood-based human immunodeficiency virus self-testing.

DISCUSSION

Overall, the study observed a slightly higher preference for oral than fingerstick HIVST. Similar to this finding, in studies among pregnant women in India[22], primary healthcare attendees in South Africa [23], female sex workers in China[24], and young people in Nigeria[25], participants who chose oral HIVST (over blood-based) cited ease of use and ability to avoid needle prick as reasons for choosing oral HIVST. Those who did not choose oral HIVST distrusted its capacity to detect HIV in saliva specimens. The distrust in HIV detection in saliva may have stemmed from HIV messaging that has historically emphasized that HIV can neither be acquired nor transmitted through kissing and oral sex[26,27]; hence, clients have questioned the scientific basis for HIV detection in oral fluid.

Furthermore, a significantly higher preference for blood-based HIVST than oral HIVST was noted in male-specific studies in this scoping review. Consistent with this finding, preferences for blood-based HIVST in men who have sex with men (MSM) in the United Kingdom and heterosexual men in Singapore were higher due to its accuracy, rapidity of results, and minimal false-negative results[28-30]. Preferences were also associated with certain factors such as previous testing, type of product used for recent testing, and presence of high-risk sexual behavior, indicating that these factors may influence individual preferences[31-33]. For instance, a study previously highlighted that individuals reporting recent high-risk sexual behaviors (*e.g.*, unprotected sex, sex when drunk) were less likely to use oral HIVST[32], whereas the likelihood of using blood-based HIVST increased when offered with information on other sexually transmitted infections[33]. Men's greater preference for blood-based HIVST was influenced by perceived higher risk, desire for accuracy, and perception of having lesser

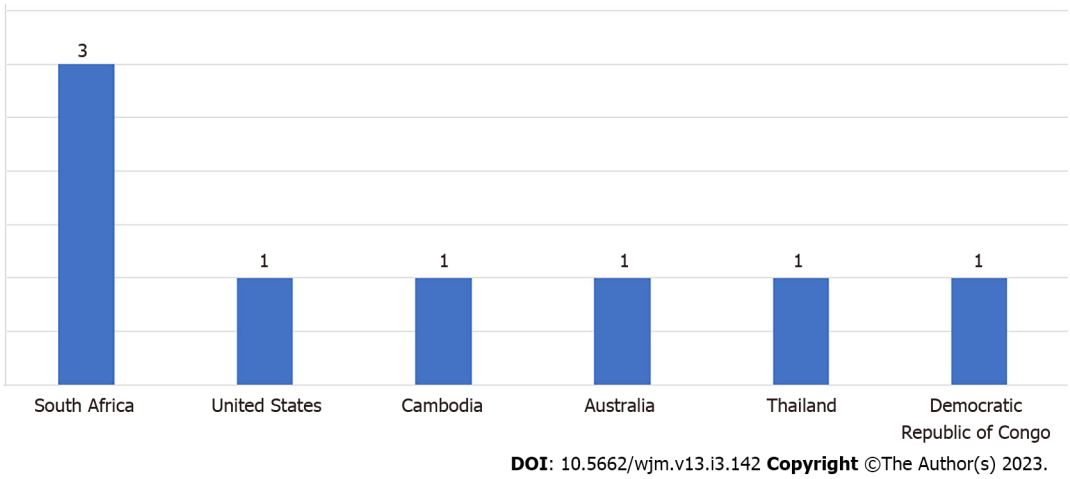

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Figure 2 Number of included studies by country.

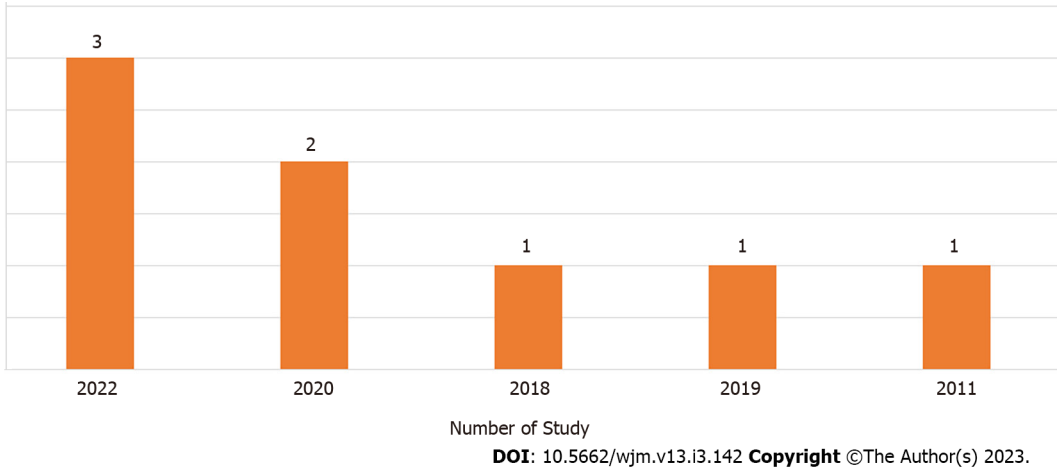

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Figure 3 Number of included studies by year of publication.

false-negative results[30]. Previous studies have also suggested that the accuracy of blood-based self-tests is higher than that of oral-fluid self-tests due to the lower quantity of HIV antibodies in oral fluid compared with whole blood[34] and reduced sensitivity for oral fluid testing for antibody detection (compared with blood testing) when specimen was obtained early after HIV infection[35]. Moreover, evaluation report of the third-generation blood-based HIVST showed very high sensitivity of 100% and high specificity of 99.9% and the ability of this product to detect HIV infections 7 d sooner than second-generation tests (*i.e.* from day 21 of infection instead of 28 d associated with most second-generation oral- and blood-based HIVST)[36]. One would expect usability of blood-based testing to be a major barrier, especially among men where preference was high. By contrast, a usability index average of 92.8% (92.2%-95.5% for oral HIVST; 84.2%-97.6% for blood-based HIVST) was reported in a study that evaluated the usability of seven WHO Prequalified HIVST kits (five blood-based and two oral HIVST) in South Africa[37]. Since both oral- and blood-based HIVST are complementary, a choice-based approach is therefore needed to optimize HIV testing programs and close the gaps between HIV testing and treatment.

There are several limitations to consider when interpreting our findings. First, we only used four databases to search the literature and may have missed articles not embedded. That notwithstanding, these databases are the basic sources of public health literature. Also, by not including conference abstracts, more recent unpublished articles may have been missed. Moreover, by reviewing citations of scoping and systematic reviews, the chances of incorporating the full breadth of the research through our search strategies were increased. We are convinced of having reached saturation with our methods. The real strength of the study lies in the inclusion of studies that offered both oral- and blood-based HIVST to actual users in real-world situations rather than experimental studies. This has removed the generalizability bias often seen in studies that offer only one type of HIVST or measure preferences from an intention-to-use perspective[38,39].

CONCLUSION

The scoping review consistently showed that men preferred blood-based HIVST than oral HIVST due to a higher risk perception and desire for a test that provides higher accuracy coupled with autonomy, rapidity, privacy, and confidentiality. The UNAIDS 2021 report showed a huge gap in knowledge of HIV status among general men and MSM, whereas AIDS-related death was higher in men than women due to late diagnosis, hence providing a blood-based HIVST option that can facilitate acceptability and the earlier diagnosis of HIV in men.

Similarly, the scoping review highlighted the diversity in preferences for oral- and blood-based HIVST and found that a single type of self-test kit is unlikely to cater for the preferences of diverse population and achieve high testing coverage. Integrating novel biomedical instruments into standard clinical and community procedures can occasionally prove difficult, as evidenced by the adoption of oral and injectable preexposure prophylaxis along with contemporary contraceptive methods. That notwithstanding, Ministries of Health and country programs should consider both blood and oral HIVST options. Offering choices among multiple kits may be the best way to maximize uptake and reach populations who may not otherwise test for HIV. Offering broader choices for HIVST could have a greater impact on testing uptake, but more research is needed to address misconceptions that drive HIVST and identify effective, population-specific dissemination channels needed to promote HIVST choices so people can make appropriately informed choices.

ARTICLE HIGHLIGHTS

Research background

Human immunodeficiency virus self-testing (HIVST) has been shown to increase testing rates and improve early HIV diagnosis. However, there are different testing modalities, including oral- and blood-based HIVST, and little is known about the preferences for these different types of HIVST.

Research motivation

Identifying preferences for oral- *vs* blood-based HIVST is crucial for the development and implementation of effective HIVST programs. Understanding the factors that influence these preferences can also inform strategies for increasing uptake of HIVST.

Research objectives

The main objective of this scoping review was to provide a comprehensive overview of the literature on preferences for oral- *vs* blood-based HIVST. Specific objectives included identifying factors that influence preferences, exploring the implications of these preferences for the promotion and implementation of HIVST programs, and highlighting gaps in the literature.

Research methods

A scoping review methodology was used to identify and synthesize relevant literature on preferences for oral- *vs* blood-based HIVST. The review included studies published in English between 2011 and 2021 that focused on actual and not hypothetical users of HIVST.

Research results

The search yielded 2424 records, of which 8 studies were included in the review. Across all studies, pooled preference for oral HIVST was 59.8%, whereas for blood-based HIVST, it was 48.8%. However, in studies specific to men, the preference for blood-based HIVST (58%-65.6%) was higher than oral (34.2%-41%). Men favored blood-based HIVST because of its perceived accuracy and rapidity, whereas oral HIVST was preferred for being non-invasive and easy to use.

Research conclusions

Preferences for oral- *vs* blood-based HIVST are influenced by various factors, including user characteristics such as sex, testing context, and perceived test accuracy. Programs promoting HIVST should consider these factors when designing and implementing HIVST programs. Further research is needed to explore the impact of these preferences on HIV testing rates and to identify effective strategies for increasing the uptake of HIVST.

Research perspectives

Future research should focus on identifying effective strategies for increasing the uptake of HIVST, particularly among populations that may have unique preferences or barriers to testing. Longitudinal studies could also help to explore the impact of these preferences on HIV testing rates and linkage to care. Additionally, studies should continue to explore the accuracy and feasibility of new HIVST techno-

logies.

ACKNOWLEDGEMENTS

We would like to acknowledge all of the authors who shared the full text of their manuscripts with us.

FOOTNOTES

Author contributions: Adepoju VA conceptualized the study, designed the review methodology, conducted the initial literature search, contributed to the data analysis, and wrote the first draft of the manuscript; Imoyera W was involved in the study design, literature search, and data analysis, and contributed to writing and revising the manuscript including reviewing and synthesizing the data; Onoja AJ was involved in the literature search, data analysis, and manuscript revisions.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Li L
L-Editor: Filipodia
P-Editor: Li L

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2023 June 20; 13(3): 153-165

DOI: 10.5662/wjm.v13.i3.153

ISSN 2222-0682 (online)

META-ANALYSIS

Microvessel density in patients with gastrointestinal stromal tumors: A systematic review and meta-analysis

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Guo F, China; Shi H, China

Received: January 14, 2023
Peer-review started: January 14, 2023
First decision: April 13, 2023
Revised: April 30, 2023
Accepted: May 16, 2023
Article in press: May 16, 2023
Published online: June 20, 2023

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Abstract

BACKGROUND

Gastrointestinal stromal tumors (GISTs) are considered the most common mesenchymal tumors of the gastrointestinal tract. Microvessel density (MVD) constitutes a direct method of vascularity quantification and has been associated with survival rates in multiple malignancies.

AIM

To appraise the effect of MVD on the survival of patients with GIST.

METHODS

This study adhered to Systematic reviews and Meta-Analyses guidelines and the Cochrane Handbook for Systematic Reviews of Interventions. Electronic scholar databases and grey literature repositories were systematically screened. The Fixed Effects or Random Effects models were used according to the Cochran Q test.

RESULTS

In total, 6 eligible studies were identified. The pooled hazard ratio (HR) for disease free survival (DFS) was 8.52 (95%CI: 1.69-42.84, $P = 0.009$). The odds ratios of disease-free survival between high and low MVD groups at 12 and 60 mo did not reach statistical significance. Significant superiority of the low MVD group in terms of DFS was documented at 36 and 120 mo (OR: 8.46, $P < 0.0001$ and OR: 22.71, $P = 0.0003$, respectively) as well as at metastases rate (OR: 0.11, $P = 0.0003$).

CONCLUSION

MVD significantly correlates with the HR of DFS and overall survival rates at 36

and 120 mo. Further prospective studies of higher methodological quality are required.

Key Words: Vascularity; Microvessel density; Gastrointestinal stromal; Survival; Meta-analysis

Core Tip: This systematic review and meta-analysis summarize all available data regarding the prognostic role of microvessel density (MVD) in gastrointestinal stromal tumors (GISTs). MVD measurement affects long term GIST survival. However, further prospective studies are necessary.

Citation: Perivoliotis K, Baloyiannis I, Samara AA, Koutoukoglou P, Ntellas P, Dadouli K, Ioannou M, Tepetes K. Microvessel density in patients with gastrointestinal stromal tumors: A systematic review and meta-analysis. *World J Methodol* 2023; 13(3): 153-165
URL: https://www.wjgnet.com/2222-0682/full/v13/i3/153.htm
DOI: https://dx.doi.org/10.5662/wjm.v13.i3.153

INTRODUCTION

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal tumours of the gastrointestinal (GI) tract. According to existing literature, the average GIST incidence is estimated at 10 to 15 cases per million, ranging from 4.3 to 22/1000000 between different geographical locations[1,2]. Furthermore, although the age of reported cases spans from 10 to 100 years, the median GIST presentation appears during the mid-60 years of age, with no discrepancies in terms of gender allocation [1,2].

Based on recent studies, the origin of these tumours can be traced to the interstitial cell of Cajal, a myenteric plexus pacemaker[2-4]. The most frequent GIST locations are the stomach (55.6%), small (31.8%) and large intestine (6%). Further primary sites include the oesophagus, the omentum, the mesentery and the retroperitoneum[1-2,5]. Regarding morphological characteristics, GISTs are classified in spindle cell, epithelioid cell and mixed type histological subgroups[6].

The majority of GISTs have been found to express KIT, a proto-oncogene protein[7]. Specifically, KIT or c-kit is positive through immunohistochemical staining in almost 95% of all GISTs[6], while KIT-negative GISTs have been demonstrated to harbour mutations of platelet-derived growth factor receptor-alpha[6,8,9]. Alteration of the function of these receptor tyrosine kinases is considered of major importance in the GIST oncogenesis, through the RAS-RAF-MAPK and PI3K-AKT-mTOR pathways[6]. Surgical excision is considered the gold standard treatment for GISTs. However, kinase inhibitor adjuvant therapy (*i.e.* imatinib and sunitinib) has been introduced for treatment of advanced and metastatic disease[10-15], improving the overall survival (OS) and time to progression rates. Despite this, treatment resistance and disease recurrence rates still remain a significant problem[11,13].

To prognose the therapy outcomes, various risk grading systems have emerged, including those proposed by Fletcher *et al*[16] and Miettinen *et al*[17]. Several clinical and histopathologic factors been investigated such as tumor size, mitotic activity, anatomical origin, tumor rupture, tumor mutation type, predominant cell type, cellular density, p53, Ki-67, neutrophil to lymphocyte ratio and blood vessel invasion[6,11,13,18-20].

Microvessel density (MVD) assessment technique, based on the original work of Weidner *et al*[21], constitutes a direct method of vascularity quantification, since it represents the number of small blood vessels in tumoral tissue. Estimation of the vasculature is achieved through the application of various immunohistochemical endothelium labelling stains, such as cluster of differentiation (CD) 31, CD34, CD105 and von Willebrand Factor (vWF). The correlation between tumoral MVD and overall survival outcome in GIST patients has been extensively researched[8-9,22-25]. However, to the best of our knowledge, there is still no study assessing overall prognostic value of MVD in these neoplasms.

Considering the above, a systematic literature review and meta-analysis of the reported outcomes was designed to estimate the pooled effect of tumor vascularity on survival of GIST patients, based on MVD measurements.

MATERIALS AND METHODS

Study protocol

The present meta-analysis was conducted based on the Cochrane Handbook for Systematic Reviews of

Interventions and Systematic reviews and Meta-Analyses (PRISMA) guidelines[26]. The study was not registered in current electronic databases.

Primary endpoint

The primary endpoint of the present meta-analysis was considered the Hazard Ratio (HR) of Disease-Free Survival (DFS) between low and high MVD measurements in patients suffering from GISTS. Pooled HR > 1 denoted a higher risk of death in patients with high MVD, compared to patients with low MVD.

Secondary endpoints

The secondary endpoints included pooled odds ratios (ORs) of DFS between high and low MVD measurements, at four specific time points (12, 36, 60 and 120 mo) of follow-up. Moreover, the pooled OR between high and low MVD tumours of the presence of metastases in GIST patients was estimated. A pooled OR > 1 suggested superiority of low MVD tumours when compared to respective high MVD tumours, in terms of survival endpoints. On the contrary, concerning the metastases endpoint the opposite applied.

Eligibility criteria

Eligible studies were prospective or retrospective trials with a study population consisting of GIST patients, whose outcomes of interest were reported in English and were retrievable. Specifically, the study design must have incorporated a primary tumor MVD assessment.

Exclusion criteria consisted of studies written in a language other than English, with no endpoint of interest, insufficient survival data and no human studies. Furthermore, studies in the format of a letter, conference abstract, expert opinion or duplicate trials were not incorporated in the meta-analysis.

Literature search

A systematic literature search in electronic scholar databases (MEDLINE, CENTRAL, Scopus and Web of Science) and grey literature repositories (OpenGrey.eu and medRxiv) was performed to identify eligible studies. The last search date was December 2022. The literature search included the following search keywords: 'gist', 'gastrointestinal stromal tumor', 'stromal tumor', 'mvd', 'microvessel density' and 'microvascular density'.

Study selection and data collection

The first step of screening included removing duplicate entries. Subsequently, titles and abstracts of the remaining studies were assessed based on the inclusion criteria. A full text review of accepted entries was then performed, to validate consistency with the eligibility criteria. The electronic database screening, study selection, data extraction as well as methodological and quality assessment were performed in duplicate and blindly by two independent investigators (K.P. and P.K.). To reach consensus, disagreements were resolved by mutual revision and discussion. If discrepancies were not resolved, the opinion of a third investigator (K.D) was considered.

The Newcastle-Ottawa Scale (NOS)[27] was utilized to perform rigorous quality and methodological evaluation of eligible studies. NOS evaluates non-RCT trials in certain endpoints, such as selection and comparability of study groups and confirmation of the exposure. Each included study was rated with a score ranging from 0 to 9. Cohen's k statistic was also calculated.

Statistical analysis

Data analysis and statistical computations were performed using Cochrane Collaboration RevMan version 5.3 and IBM SPSS version 23. The primary and secondary endpoints were reported in the form of HR and OR, respectively. Results of the analyses were presented with the corresponding 95% Confidence Interval (95%CI).

If eligible trials did not directly provide the HR or OR in the article results, they were estimated based on the algorithm proposed by Parmar *et al*[28] and Tierney *et al*[29]. Specifically, required data for the meta-analysis of trials endpoints were reconstructed from the Kaplan-Meier curves provided[30]. The precision of extracted coordinates was enhanced through utilization of a digitizing software (Digitizelt)[31].

If included trials did not provide the mean and standard deviation (SD) of continuous variables, they were estimated from the median and the Interquartile Range (IR) based on the algorithm described by Hozo *et al*[32]. Given a sample size of >25, the mean was considered equal to the median. For a sample of < 70, the SD was regarded as IR/4. In the other case, the SD was calculated as IR/6.

The statistical method applied was Mantel-Haenszel (MH) and the Inverse Variance for OR and HR, respectively. Both the Fixed Effects and Random Effects (RE) model were calculated. The final model that was estimated was based on the Cochran Q test. If statistically significant heterogeneity was present (Q test $P < 0.1$), then the RE model was applied. A further quantification of the heterogeneity was performed through the calculation of I^2. Statistical significance was considered at the level of $P < 0.05$.

Risk of bias across studies

To estimate the publication bias of included studies, the funnel plot of the primary outcome was visually inspected. Regarding the primary outcome, Egger's test was also performed.

RESULTS

Study selection

Through the above-mentioned search algorithm (Figure 1), 994 citations were retrieved (MEDLINE: 412, Web of Science: 526, Scopus: 31, CENTRAL: 1, OpenGrey.eu: 15, medRxiv: 9). The next step included the removal of 340 duplicate records. A total of 654 records underwent title and abstract screening, resulting in the exclusion of 631 entries (17 reviews/ meta-analyses, 2 conference abstracts, 4 paediatric studies, 608 irrelevant records). Examination of compliance with eligibility criteria extended to the full text articles of the previously accepted records. In total, 5 studies with inadequate survival data and 12 irrelevant studies were excluded. Subsequently, 6 studies[8-9,22-25] were included in the present meta-analysis.

Study characteristics

Table 1 summarizes the characteristics of included studies. Regarding study type, all trials had a retrospective design. Furthermore, all except one trial[9] were single centre, with sample size ranging from 53 to 124. More specific information regarding the analysis and total specimen sample are reported in Table 1. Mean patient age and gender allocation are also displayed in Table 1. Mean follow-up period extended from 2.5 years in the study by Waengertner *et al*[24], up to 81.7 mo in the study by Takahashi *et al*[23].

Concerning the MVD assessment method that was applied, the majority of included trials described the use of light microscopy and immunochemistry, implementing the technique proposed by Weidner *et al*[21] (Table 2). Exceptions to this were trials by Imamura *et al*[8] and Waengertner *et al*[24] which reported the application of a modified Horak technique and Chalkley method, respectively. Despite the fact that the majority of eligible trials used CD31 antibodies, Zhao *et al*[25] utilized the CD34 antibody. Heterogeneity was identified in the reported level of magnification. More specifically, the applied magnification spanned from 40X up to 400X. Furthermore, non-uniformity was discovered in the number of spots examined, which ranged from 3 to 10 spots. Only two trials[8,9] confirmed blinded estimation of microvessel density by two independent observers, and none provided information about the existence of separate count for intratumoral and peritumoral vessels. All researchers except Zhao *et al*[25] included the MVD cut-off value in their study articles.

Table 3 summarizes the data regarding the risk classification of included tumours. Moreover, the localization of GISTs included: 9 in the oesophagus, 284 in the stomach, 127 in the small intestine and 28 in the anatomic area of the colon and rectum. According to Table 4, only the study group of Chen *et al* [22] recorded tumor complications like necrosis (37%) and haemorrhage (72.6%). Table 4 incorporates histopathologic characteristics, such as the mitotic count and the tumor size of included GISTs. From the eligible trials, tumor cell type categorization was performed in only 3[8,24,25] studies. In total, 29 epithelioid, 222 spindle and 25 mixed tumours were identified. Finally, inconsistent data were provided by the included trials in terms of the operation performed and chemotherapy type administered.

Risk of bias within studies

Regarding the assessment based on the NOS scale, most studies achieved a 5-star score. The trial by Chen *et al*[22] was an exception, as it appointed a 6 star score. Inter-rater agreement was estimated to be in a very good level (Cohen's k statistic: 86.8%, $P < 0.001$)

Primary endpoint

Data regarding the HR of DFS were extracted from 4 studies (Figure 2). Meta-analysis of these data showed a statistically significant ($P = 0.009$) hazard ratio of DFS (HR: 8.52, 95%CI: 1.69-42.84), in favour of the low MVD group. Since heterogeneity was significant (Q test $P < 0.001$, $I^2 = 90\%$), a RE model was applied.

Due to the high heterogeneity level, further statistical investigation was performed. The first step included a sensitivity analysis for the effect of each study separately. The overall heterogeneity level was not affected by any study. Meta-regression (Supplementary Tables) for the variables sample size, age and follow-up duration did not identify any statistically significant factor. Subgroup analysis regarding the number of study centres and the antibody used were identical to the above-mentioned sensitivity analysis. Analysis of studies implementing the Weidner MVD assessment method showed a statistically significant hazard ratio. Similarly, exclusion of the two studies which did not report blinded MVD evaluation did not influence heterogeneity. Further explanatory analyses (Supplementary Tables) included meta-regression of the primary outcome with the number of spots examined, the percentage of high-risk tumours, gastric and small intestine tumours, large size tumours (≥ 5 cm) and spindle cell

Table 1 Study characteristics, *n* (%)

Ref.	Type of study	Country	Centre	Sample (patients)	Analysis sample	Specimens	Age	Gender (male/female)	Follow-up
Chen *et al*[22], 2005	Retrospective	Taiwan	Single centre	62	59 (3 cases lost to follow-up)	62	24 (38.7) ≤ 61 yr; 38 (61.3) > 61 yr	34 (54.8)/28 (45.2)	50.5 (31) mo for 59 cases
Imamura *et al* [8], 2007	Retrospective	Japan	Single centre	95	95 (80 from the K-M curves)	95	64 (11.667) yr	48 (50.5)/47 (49.5)	48.4 (26.1833) mo for 80 cases
Takahashi *et al* [23], 2003	Retrospective	Japan	Single centre	53	53	53	59.5 (13.3) yr	32 (60.3)/21 (39.6)	81.7 (63.2) mo
Waengertner *et al*[24], 2011	Retrospective	Brazil	Single centre	79	79	79	58.9 (13) yr	42 (53.2)/37 (46.8)	2.5 (2.8) yr
Wang *et al*[9], 2009	Retrospective	China	Multicentre	68	68	68	56.8 (14.75) yr	38 (55.9)/30 (44.1)	42.9 (14) mo for 64 patients
Zhao *et al*[25], 2012	Retrospective	China	Single centre	124	124	124	54.6 (11.667) yr	64 (51.6)/60 (48.4)	52 (32.333) mo

Table 2 Microvessel density assessment

Ref.	MVD assessment method	Antibody	Magnification used	Spots examined	Blinded reading	Observers	Separate count for intra/peritumoral vessels	MVD cut off
Chen *et al*[22]	Light microscopy, immuno-histochemistry	CD31	10X; 20X; 100X	3	N/A	N/A	N/A	15/HPF
Imamura *et al* [8]	Light microscopy, immuno-histochemistry, slight modification of Horak *et al* technique	CD31	40X; 200X	10	Yes	2	N/A	7/0.95 mm²
Takahashi *et al*[23]	Light microscopy, immuno-histochemistry	CD31	40X; 100X; 400X	3	N/A	N/A	N/A	19/HPF
Waengertner *et al*[24]	Light microscopy, immuno-histochemistry, modified Chalkley method	CD31	200X	3 to 5	N/A	N/A	N/A	6 vessels
Wang *et al*[9]	Light microscopy, immuno-histochemistry	CD31	200X	4	Yes	2	N/A	10.54/200HPF
Zhao *et al*[25]	Light microscopy, immuno-histochemistry, Weidner technique	CD34	100X; 200X	5	N/A	N/A	N/A	N/A

MVD: Microvessel density; N/A: Not applicable.

malignancies. A significant correlation was not confirmed with any of the previously mentioned variables.

Secondary endpoints

In total, 3 studies provided data concerning the comparison between high and low MVD groups for DFS at 12 mo (Figure 3). Meta-analysis of these data showed no statistically significant difference (P = 0.13) of DFS (OR: 1.91, 95%CI: 0.83-4.41) at 12 mo between the two study groups. However, a statistically significant difference (P < 0.001) of DFS (OR: 8.46, 95%CI: 3.54-20.19) in favour of the low MVD group was estimated at 36 mo. Although there was no difference (P = 0.58) of DFS rates (Figure 4) at 60 mo (OR: 2.31, 95%CI: 0.12-44.82), the low MVD group displayed a higher (P = 0.0003) DFS rate (Figure 3) at 120 mo (OR: 22.71, 95%CI: 4.11-125.57).

Finally, two studies provided data concerning the development of metastases (Figure 3). Meta-analysis of these data showed a statistically significant (P = 0.0003) lower ratio of metastases (OR: 0.11, 95%CI: 0.03-0.36) in the low MVD group. Heterogeneity was not significant in this analysis (Q test P = 0.29, I^2 =10).

Table 3 Tumor classification, *n* (%)

Ref.	Risk				Location				
	Very low risk	Low risk	Intermediate risk	High risk	Stomach	Small intestine	Colon	Rectum	Esophagus
Chen *et al*[22]	0 (0)	31 (50)	0 (0)	31 (50)	41 (66)	18 (29)	3 (4.8)	0 (0)	0 (0)
Imamura *et al*[8]	7 (7.3)	22 (23.2)	38 (40)	28 (29.5)	64 (67.4)	31 (32.6)	0 (0)	0 (0)	0 (0)
Takahashi *et al*[23]	16 (30.1)		10 (18.8)	27(50.9)	53 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Waengertner *et al*[24]	12 (15.4)	11 (13.8)	18 (23.1)	38 (47.7)	36 (45.6)	30 (38)	0 (0)	0 (0)	0 (0)
Wang *et al*[9]	0 (0)	20 (29.4)	0 (0)	48 (70.6)	28 (41.2)	20 (29.4)	11 (16.2)	0 (0)	0 (0)
Zhao *et al*[25]	6 (4.8)	20 (16.1)	37 (29.8)	61 (49.3)	62 (50)	28 (22.6)	14 (11.3)		9 (7.3)

Risk of bias across studies

Visual inspection of the funnel plot suggested that studies by Wang *et al*[8] and Waengertner *et al*[23] lie beyond the 95%CI limits. Based on Egger's test, there was no statistically significant publication bias (P = 0.517). Exclusion of the above-mentioned trials resulted in a statistically significant HR (7.71 95%CI: 4.02-14.8, P < 0.001) in favour of the low MVD group, though with a limited degree of heterogeneity (Q test P = 0.64, I^2 = 0%).

DISCUSSION

Since GISTs are the most frequently occurring parenchymal neoplasms of the GI tract, research is focused on improving prognosis, introducing novel chemotherapeutic agents and refining current surgical approaches[11-15,33]. Since a few decades ago conventional chemotherapy and radiotherapy did not yield satisfactory results, a R0 resection of the tumor was considered the only therapeutic option for adequate long-term survival[33]. The discovery of the c-kit proto-oncogene mutation and ligand independent activation of the KIT receptor tyrosine kinase in GISTs, resulted in subsequent development of the tyrosine kinase inhibitors imatinib and sunitinib. This led to the onset of targeted molecular therapy of these neoplasms[11,13,33]. In a cohort study by Guller *et al*[34], the Surveillance, Epidemiology and End Results database was screened, with 5,138 GIST patients included. Data analysis revealed that recent advancements in treatment resulted in a significant increase in survival rates of both metastatic (3-year OS: 54.7%, cancer-specific survival: 61.9%) and non-metastatic disease (3-year OS: 88.6%, cancer-specific survival: 92.2%)[34].

It must be noted that despite the above-mentioned novelties, the mortality rate – particularly for the metastatic group - remains high. As a result, various risk grading tools have been developed to quantify the risk and provide accurate prognosis regarding survival endpoints. The study group of Fletcher *et al*[16] proposed the use of primary tumor size and mitotic count as grading parameters, which classified GISTs in four successive categories based on risk of aggressive behaviour. Due to a discrepancy in the metastatic risk between gastric and intestinal GISTs of different grading scores, the primary tumor location was also incorporated[17]. Exporting data from the SSG XVIII trial and using the Z9001 study as a validation tool, Joensuu *et al*[18] suggested that high tumor mitotic count, non-gastric location, large size and tumor rupture were significantly and independently related to a suboptimal recurrence-free survival (RFS).

Besides these grading tools, various independent tumor histopathological factors have been studied for their prognostic value. Specifically, GISTs with an epithelioid or mixed cell type have been associated with a significantly lower 5-year recurrence free survival, when compared with the respective spindle cell tumours (23% *vs* 49%)[35]. Moreover, according to Martin *et al*[36], high tumor cellularity was characterized as a significant poor RFS prognostic factor. Overexpression of Ki67, a nuclear marker abundant in proliferating cells, was found to have an increased incidence in the high risk group[19]. On the contrary, expression levels of p53 in GISTs were not significantly associated with clinical outcomes[37,38]. A pooled analysis from Luo *et al*[20] showed that an elevated neutrophil to lymphocyte ratio was associated with decreased DFS/RFS (HR: 2.18, 95%CI: 1.30-3.67). Furthermore, blood vessel invasion in the primary tumor was suggested as a predictor of liver metastasis and an aggressive behaviour[39].

Angiogenesis in GISTs is considered of the utmost importance for the neoplasm growth and metastasis process[8]. Proliferation of tumor vasculature is achieved *via* the paracrine release of angiogenic molecules and growth factors from tumor and stromal cells[8]. In a recent study by Zhao *et al*[25], the altered expression and secretion of proliferating and angiogenic agents like PI3K, Akt, PTEN, MMP9 and VEGF were directly associated with the DFS in GIST patients. Regarding VEGF, higher serum VEGF values were found in GIST patients when compared to healthy controls, while a positive

Table 4 Tumor and treatment characteristics, *n* (%)

Ref.	Necrosis		Hemorrhage		Mitotic count		Tumor size		Pcna index		Cell type			Treatment			
	Yes	No	Yes	No					≤ 10%	> 10%	Epithelioid	Spindle	Mixed	Surgery	Surgery type	Chemotherapy	Chemotherapy type
Chen *et al*[22]	23 (37)	39 (63)	45 (72.6)	17 (27.4)	36 (58) < 2/10 HPF	26 (42) ≥ 2/10 HPF	32 (51.6) < 5 cm	30 (48.4) ≥ 5 cm	32 (51.6)	30 (48.4)	N/A	N/A	N/A	Yes	Subtotal gastrectomy, complete tumor resection or segmental enterectomy	Yes (some of them)	See comments
Imamura *et al*[8]	N/A	N/A	N/A	N/A	55 (57.9) < 5/50 HPF	40 (42.1) ≥ 5/50 HPF	39 (41.05) < 5 cm	56 (58.95) ≥ 5 cm	N/A	N/A	1 (1.05)	92 (96.85)	2 (2.1)	Yes	Resection with negative margins	N/A	N/A
Takahashi *et al*[23]	N/A	N/A	N/A	N/A	33 (62.2) < 3/50 HPF	20 (37.7) ≥ 3/50 HPF	21 (39.6) ≤ 3 cm	32 (60.3) > 3 cm	N/A	N/A	N/A	N/A	N/A	Yes	Surgical resection	N/A	N/A
Waengertner *et al*[24]	N/A	N/A	N/A	N/A	N/A	N/A	N/A, varies from 0.5 to 25 cm (median 4.8 cm)		N/A	N/A	N/A	57 (72.2%)	N/A	N/A	N/A	Yes	Adjuvant therapy with tyrosine kinase inhibitors (400mg/daily) for no longer than 3 months
Wang *et al*[9]	N/A	N/A	N/A	N/A	45 (66.2) < 2/10 HPF	23 (33.8) ≥ 2/10 HPF	24 (35.3) ≤ 5 cm	44 (64.7) > 5 cm	N/A	N/A	N/A	N/A	N/A	Yes	N/A	No	No
Zhao *et al*[25]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28 (22.58)	73 (58.87)	23 (18.55)	Yes	Only biopsy, palliative resection, radical resection	Yes	Postoperative

N/A: Not applicable.

VEGF expression rate was found in high risk groups[9]. A considerable number of clinical trials have correlated high VEGF levels with poor prognosis[9,23,25,40]. Another angiogenic factor, PDGF, has been related to GIST vasculogenesis at both theoretical and clinical levels[41,42]. As tumor angiogenesis often progresses through a hypoxic drive, researchers have correlated the expression levels of respective markers (*e.g.* HIF-1α) with survival outcomes[22,43]. Finally, vasculogenic mimicry (VM) which is a novel pattern of angiogenesis and defined as the formation of fluid conducting channels by highly invasive and dysregulated tumor cells, has also been studied in GISTs[44,45]. MMP-2 and MMP-9 were found to be contributing factors in VM; a significant association between VM, a high mitotic rate and liver metastases was confirmed[44].

Microvessel density is a direct method of quantifying and assessing intratumoral vasculature, and consequently angiogenesis potential. Due to the above-mentioned correlation between tumor vascularity and clinicopathological endpoints, various trials investigated GIST MVD. According to Imamura *et al*[8] and Waengertner *et al*[24], a statistically significant difference of survival rates in favour of low MVD GISTs was reported. Furthermore, Wang *et al*[9] stated that higher MVD values

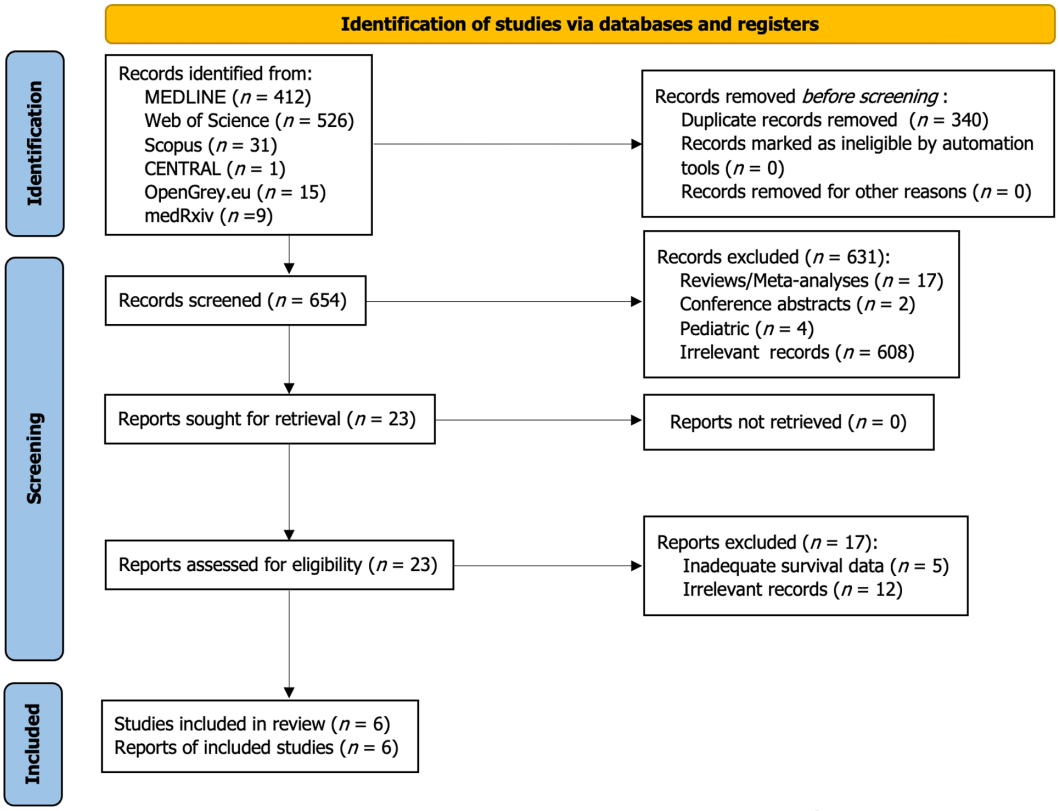

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Figure 1 PRISMA flow chart.

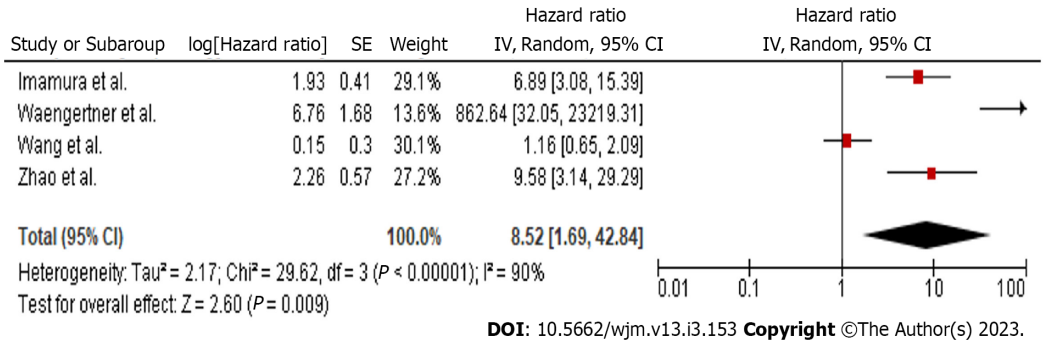

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Figure 2 Hazard ratio of disease free survival.

were found in high mitotic count and recurrence groups. Similar results were published by Zhao *et al* [25], where a significant hazard ratio for DFS was found. A retrospective study by Takahashi *et al*[23] suggested that while high MVD displayed a significant relationship with liver metastases, it did not influence the survival outcome at 10 years.

The results of our meta-analysis validated the significance of the MVD value effect on survival. Specifically, higher intratumoral MVD measurements were associated with a lower DFS rate at 36 and 120 mo of follow-up. These were not confirmed at the intermediate endpoints of 12 and 60 mo. The enhanced malignant potential of high vascularized GISTs was also depicted by the significant association among metastatic rate and MVD values.

The usefulness of these results involve extensive approaches in the clinical outcome prognosis[8-9,23, 25,43]. Consolino *et al*[46] showed that in dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), imatinib-resistant tumors had an increased vessel density and permeability, with these attributes significantly correlated with MVD and MDD, respectively. Contrast enhanced endoscopic ultrasound has also demonstrated the ability to assess GIST vascularity, and subsequently, malignant potential[47]. Furthermore, since MVD is a direct tumor vasculature marker, it has been used as an indicator of the angiogenesis inhibition, as well as the overall response to novel medical treatment[48].

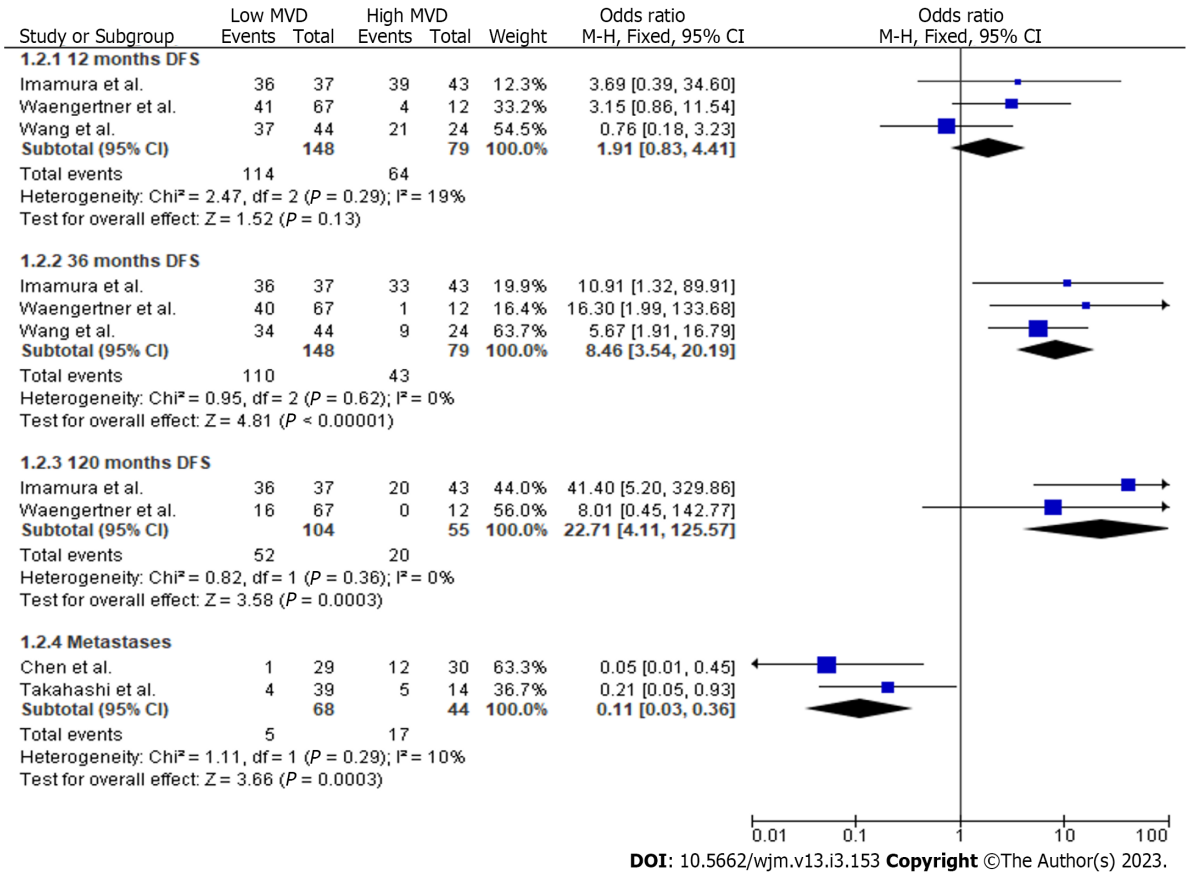

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Figure 3 Odds ratios of disease free survival and metastases.

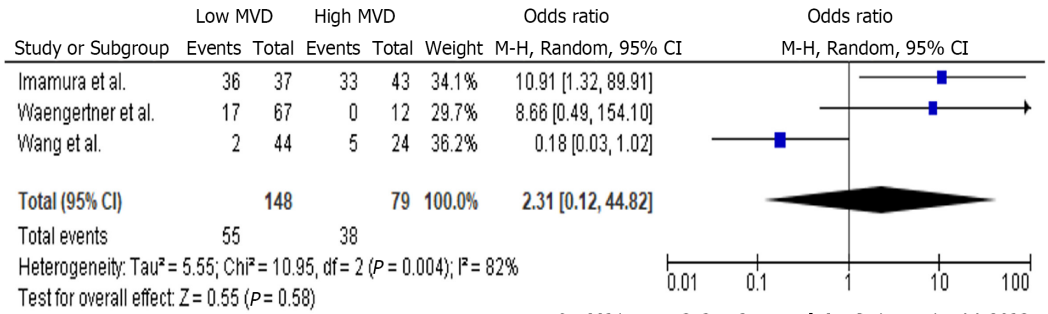

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Figure 4 Sixty months disease free survival.

Besides GIST, MVD assessment has been extensively researched as a means of solid tumor vasculature quantification. Researchers have attempted to identify and estimate the presence of a correlation between microvessel density and survival outcomes in malignancies of the prostate[49], cervix[50], ovaries[51], breast[52], pancreas[53], kidney[54] and lung[55]. Moreover, in two recent meta-analyses from our study group concerning cutaneous melanoma and patients with differentiated thyroid cancer, high intratumoral MVD was related to poor survival outcomes[56,57]. According to current literature, the majority of studies validate the presence of a significant correlation between intratumoral MVD and prognosis in solid tumors[58]. However, a discrepancy exists since a minority of publications question the significance of the above-mentioned correlation[58].

Heterogeneity of various clinicopathological endpoints (survival, metastasis, local recurrence, response to treatment, *etc*.) in the reported results has been widely attributed to certain methodological variations[58]. Among these, selection of the hot-spot examination technique is the most important, due to the variability rates and dependence on the assessor training and experience[59,60]. Furthermore, the MVD assessment technique includes various modifications, such as Weidner's hot-spot method[21], the lumen method[61], Chalkley's method[62] and the computerized image analysis system[63]. Another field of methodological diversity is considered the selection of the endothelial marker, where a variety

of choices such as pan-endothelial cell markers (CD31, CD34, vWF) and selective for the activated endothelium factors (CD105) are described. Finally, technical discrepancies are also reported in other methodological fields, such as type of fixative, vasculature estimation, the MVD cut-off value, level of magnification and overall field size[58]. Our study highlighted this heterogeneity; the use of different assessment methods and definitions of high and low MVD tumours prohibited the calculation of a pooled cut-off point.

Certain limitations should be taken into consideration, prior to appraising results of the present meta-analysis. Firstly, significant levels of heterogeneity were identified; despite conducting explanatory analyses, the validity of study conclusions may be compromised. Furthermore, all eligible studies were designed using a retrospective methodology and included a small sample size, thus allowing the introduction of bias. Moreover, diversity among included studies regarding methodological characteristics of the MVD assessment technique should be also acknowledged. The implementation of different assessment methods and different cut-off points prohibited the strict definition of high and low MVD GISTs. Furthermore, heterogeneity in terms of tumor location, risk classification, histopathological characteristics and cell subtype jeopardized the significance of our outcomes. Inconsistency in surgical or medical treatment could also be an influencing factor on survival endpoints. Finally, since in most trials the raw survival data had to be extracted and reconstructed from the provided Kaplan-Meier curves, a certain amount of bias was introduced, although this procedure has been extensively described and applied in the literature.

CONCLUSION

To the best of our knowledge, the present study is the first attempt to provide an overall estimation of the impact of MVD on survival rates of GIST patients. According to the pooled results of the meta-analysis, GIST allocation between high and low MVD values significantly influenced the DFS hazard ratio. Moreover, high MVD GISTs demonstrated a statistically significant lower DFS at 36 and 120 mo of follow-up, while no difference was found at 12 and 60 mo. Moreover, high MVD tumours were associated with a significantly higher rate of metastases. Based on the above-mentioned results and given several limitations, further studies with a larger sample size and adequate methodology are required.

ARTICLE HIGHLIGHTS

Research background

Several clinical and histopathologic factors have been investigated as prognostic indicators of survival in patients with gastrointestinal stromal tumours (GISTs).

Research motivation

Microvessel density (MVD) has been extensively applied as a direct method of tumour vascularity assessment.

Research objectives

This meta-analysis attempted to estimate the pooled effect of tumoral vascularity based on MVD assessment on the survival of patients with GISTs.

Research methods

The present meta-analysis adhered to the Systematic reviews and Meta-Analyses guidelines and the Cochrane Handbook for Systematic Reviews of Interventions.

Research results

Low vascularized tumours were associated with improved pooled disease-free survival. GISTs with lower MVD values displayed a reduced risk of metastases.

Research conclusions

MVD is significantly associated with the survival outcomes of GIST patients.

Research perspectives

Further prospective randomized controlled trials are required to delineate the exact correlation between MVD and prognosis outcomes in GIST patients.

FOOTNOTES

Author contributions: Perivoliotis K, Ntellas P, Baloyiannis I contributed to study conception and design; Dadouli K, Koutoukoglou P contributed to acquisition of data; Perivoliotis K, Ntellas P, Samara A contributed to analysis and interpretation of data; Perivoliotis K, Ntellas P contributed to drafting of manuscript; Ioannou M, Tepetes K contributed to critical revision.

Conflict-of-interest statement: The authors declare no potential conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Liu JH
L-Editor: A
P-Editor: Zhang XD

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