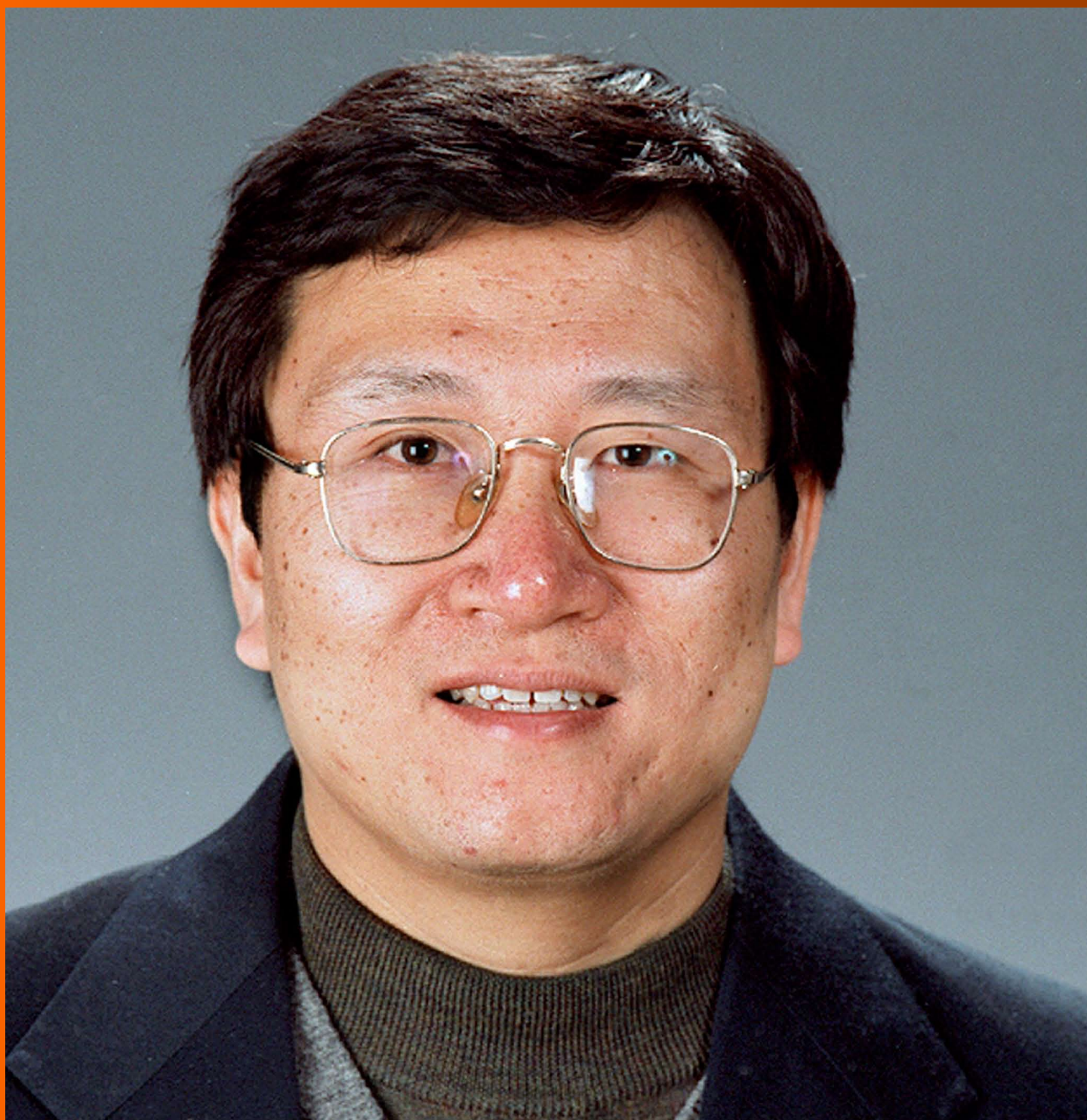


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Inhibitors of glucose transport and glycolysis as novel anticancer therapeutics

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Abstract

Metabolic reprogramming and altered energetics have become an emerging hallmark of cancer and an active area of basic, translational, and clinical cancer research in the recent decade. Development of effective anticancer therapeutics may depend on improved understanding of the altered cancer metabolism compared to that of normal cells. Changes in glucose transport and glycolysis, which are drastically upregulated in most can-

cers and termed the Warburg effect, are one of major focuses of this new research area. By taking advantage of the new knowledge and understanding of cancer's mechanisms, numerous therapeutic agents have been developed to target proteins and enzymes involved in glucose transport and metabolism, with promising results in cancer cells, animal tumor models and even clinical trials. It has also been hypothesized that targeting a pathway or a process, such as glucose transport or glucose metabolism, rather than a specific protein or enzyme in a signaling pathway may be more effective. This is based on the observation that cancer somehow can always bypass the inhibition of a target drug by switching to a redundant or compensatory pathway. In addition, cancer cells have higher dependence on glucose. This review will provide background information on glucose transport and metabolism in cancer, and summarize new therapeutic developments in basic and translational research in these areas, with a focus on glucose transporter inhibitors and glycolysis inhibitors. The daunting challenges facing both basic and clinical researchers of the field are also presented and discussed.

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Key words: Cancer metabolism; Warburg effect; Glycolytic enzymes; Glucose transporters; Translational research

Core tip: Reprogramming of metabolism has been recognized at the beginning of 21st century as an emerging hallmark of cancer. The Warburg effect is one of the major focuses in the reprogramming. We cannot fully understand or more effectively treat cancer without a better understanding of cancer metabolism. Targeting cancer metabolism, particularly glucose transport and glycolysis, has been shown to be effective in inhibiting cancer growth. This review summarizes recent progresses in developments of therapeutics inhibiting glucose transporters and glycolytic enzymes, provides key

information associated with each inhibitor, discusses their promises and problems as well as future challenges and directions of the basic and translational research of the field.

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INTRODUCTION

Cancer has long been considered a group of diseases caused by genetic mutations and genetic mutations only. However, in recent decades, extensive biochemical and biological studies have convincingly demonstrated that cancers exhibit significantly reprogrammed metabolism, which plays important roles in tumorigenesis^[1-6]. In some cases, altered metabolism may be not only the consequence of genetic mutations, but also a contributing factor or cause of tumorigenesis^[7-9]. Cancer metabolic reprogramming and altered energetics have been recognized now as a hallmark of cancer^[10].

The importance of metabolism in cancer was actually recognized long time ago. In the 1920s, the German biochemist Otto Warburg, studied glucose metabolism in cancer tissues. He found that, unlike in normal tissue, incubated cancer samples always switched from mitochondrial oxidative phosphorylation (OXPHOS) to cytosolic glycolysis even when oxygen was abundant^[11]. This phenomenon of so-called aerobic glycolysis has been known as the Warburg effect^[12-15]. Warburg went so far as to claim that the altered glucose metabolism was the cause of cancer. This hypothesis is called the Warburg theory of cancer. He speculated that due to some mitochondrial dysfunctions, mitochondria could not synthesize ATP and thus cells must switch to cytosolic glycolysis, leading to cancer formation^[14,16]. Biological studies in recent decades have found that Warburg's view on the cause of the switch was largely incorrect: many cancers switch to glycolysis even without any mitochondrial defects. New biological and biochemical studies in the past decades revealed that the switch from OXPHOS to glycolysis is not just for ATP synthesis but also for biomass synthesis^[15,17], production of NADPH^[15,18], a reducing agent needed to remove reactive oxygen species (ROS) generated by cancer cells' accelerated metabolism, as well as synthesis of amino acids^[15,19]. The Warburg effect appears to be a strategic move made by cancer cells to deal with multiple requirements for growth, survival, and proliferation in a microenvironment with numerous constraints.

Altered cancer metabolism has also been recognized as a potential target for cancer therapeutics. Glucose transport and glucose metabolism are significantly up-regulated in cancer as revealed by the PET scan and

other detection methods^[20-24]. The reliance of cancer cells on glucose indicates that they are addicted to the Warburg effect or glucose^[25-27]. As a result, cancer cells are more sensitive than normal cells to changes in glucose concentration and will die before normal cells^[25-28]. The recognition of this vulnerability in cancer cells has led to targeting glucose transport and metabolism as a new anticancer strategy. Furthermore, although targeted anticancer drugs inhibit one or more proteins or enzymes, cancers demonstrate the ability to escape inhibition using redundant signaling pathway(s). It has been proposed that targeting a signaling pathway or a metabolic process, rather than a protein in a pathway, may be more effective in preventing drug resistance and prolonging treatment effectiveness^[29,30]. Potential targets for this proposed new approach include glucose transport and glycolysis, the predominant glucose metabolic changes found in cancer cells.

It should be emphasized that targeting cancer metabolism is not an entirely novel strategy. Some of the earliest chemotherapy drugs, such as methotrexate, also target metabolism and show significant efficacy^[31-33]. As we have accumulated more knowledge about cancer metabolism, we should be able to develop more successful anti-cancer-metabolism drugs. In the following sections, recently developed glucose transport and glycolysis inhibitors will be described.

GLUCOSE TRANSPORT AND GLUCOSE METABOLISM IN CANCER CELLS—THE WARBURG EFFECT

In normal cells under aerobic conditions, OXPHOS is used to make ATP, the universal energy currency in all living organisms^[34]. OXPHOS is used because it is the most efficient way for making ATP. For each molecule of glucose, approximately 34 molecules of ATP can be produced by OXPHOS^[34]. However, OXPHOS can proceed only when oxygen is present and abundant, a condition called normoxia. When oxygen is lacking, a condition called hypoxia, cells are forced to shift to anaerobic glycolysis to maintain ATP synthesis and energy metabolism^[35]. Due to rapid growth and proliferation, a large proportion of the cancer cells in a tumor are in a hypoxic condition and thus use glycolysis to make ATP and other essential biomass molecules such as ribonucleotides. The phenomenon of OXPHOS-to-glycolysis shift in cancer cells is called the Warburg effect^[12-16]. Although the Warburg effect was observed more than 80 years ago, its interpretation is still controversial and evolving. Warburg thought that the effect was caused by mitochondrial dysfunctions and the effect is a forced alternative strategy for ATP synthesis. However, research in recent decades largely disagrees with this interpretation. Recently, it has been found that the switch in cancer cells is primarily for the synthesis of biomass (*e.g.*, of RNA precursor and others)^[17], the reducing agent NADPH^[18], which is need-

ed for clearing ROS, and the amino acid serine^[19]. ATP synthesis seems not to be a rate-limiting factor. This conclusion is very different from Warburg's and is based on the observation that although cancer cells upregulate all glycolytic enzymes, they switch pyruvate kinase (PK), the last enzyme in the glycolytic pathway, from a form with higher activity (PKM1) to that with lower activity, PKM2^[36-39]. This change suggests that cancer cells do not want all the glucose obtained from the upregulated glucose transport to be converted to pyruvate, but rather diverts some glucose metabolic intermediates to other connected metabolic pathways, such as pentose phosphate pathway (PPP) for synthesis of biomass and reducing agents^[17-19,40]. This also suggests that ATP synthesis is not the top priority of the upregulation of glucose transport and metabolism. On the other hand, since glycolysis is about 18 times less efficient compared to OXPHOS, cancer cells must drastically upregulate glycolysis to compensate for the low ATP production.

ANTICANCER THERAPEUTICS TARGETING GLYCOLYSIS AND ITS CONNECTED PATHWAYS

Currently, the Warburg effect is a very active cancer research area^[13]. Targeting glucose metabolism and transport, has been proposed as an effective anticancer strategy^[1,3]. Glycolysis, the key process of increased glucose metabolism in cancer cells, has been targeted both *in vitro* and *in vivo*^[3,41,42]. Glycolysis genes are overexpressed in various cancers^[35]. In addition to higher potentials for invasiveness and metastasis^[43], the glycolytic switch in cancer also increases cancer's sensitivity to external interference because of their higher dependence on aerobic glycolysis^[25-28].

Glucose deprivation, a method traditionally used to reduce glucose concentration in cultured cells for metabolic studies, has been used frequently in cancer research^[44-47]. Glucose deprivation limits glucose supply, forcing cancer cells to slow down proliferation or undergo apoptosis^[48-50]. Blocking glucose transport or glycolysis is similar to glucose deprivation, suggesting the possibility of restricting glucose supply with glucose transport or glycolysis inhibitors as an anticancer strategy.

Various inhibitors of glycolytic enzymes have shown significant anticancer efficacy. Most of the reported glycolysis inhibitors are summarized (Table 1 and Figure 1). The enzymes targeted include hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), and pyruvate dehydrogenase kinase (PDK). Related studies revealed that these inhibitors induced apoptosis in cancer cells^[51,52]. Moreover, inhibition of glycolysis has been shown to overcome drug resistance in multiple cancer cells associated with mitochondrial respiratory defect and hypoxia^[53]. Although numerous attempts to block glycolysis by using various inhibitors in cancer cells and in animal models have been

made, developing clinically effective and safe glucose metabolism-targeting therapeutics is still a challenging task.

Hexokinase (HK) as the first enzyme in glycolysis phosphorylates glucose to glucose-6-phosphate (G6P) irreversibly, which is a rate-limiting step. In cancer cells, type II HK (HK2) is bound to mitochondria, facilitating a high glycolytic flux rate and preventing cancer cell from apoptosis^[54]. HK2 is required for cancer initiation and maintenance and the systemic deletion of HK2 is therapeutic in mice bearing tumors^[55]. Thus, targeting HK2 may be an effective anti-cancer strategy.

2-deoxy-D-glucose (2-DG) is one of the most widely studied HK inhibitors. 2-DG is a glucose analog with a hydrogen group instead of a hydroxyl group in position 2 of glucose. Due to its structural similarity, 2-DG competes with glucose and inhibits HK with a K_i of 0.25 mmol/L^[56]. The product 2-deoxy-D-glucose-6-phosphate made from 2-DG cannot be processed in the following glycolytic steps and therefore blocks glycolysis, leading to ATP depletion, cell cycle arrest and cell death^[57,58]. Synergistic studies combining 2-DG and other anti-cancer drugs, such as adriamycin and paclitaxel, indicated that 2-DG is effective *in vivo* in combination with other drugs^[59]. 2-DG sensitizes glioblastoma cells to other anti-cancer treatments and radiation^[60-63]. Though effective, 2-DG is relatively toxic with side effects when administered to patients^[61,64]. This is at least in part because 2-DG has to be used at high concentrations, around and higher than 5 mmol/L, in order to compete with blood glucose^[65].

3-bromopyruvate (3-BP) is another HK inhibitor which has been shown to inhibit the progression of tumors *in vivo*^[66-68]. 3-BP also increases the total ROS in tumor cells^[69,70]. A recent study demonstrated that 3-BP inactivates ABC transporters, restoring drug sensitivity in cancer cells^[71]. 3-BP has also been studied in combination with various anti-cancer drugs for synergistic effects, and it has been found to be effective *in vitro*^[72] and *in vivo*^[73], although with some hepatotoxicity^[74]. However, 3-BP inhibits other enzymes, such as GAPDH, as well^[75]. Up to now, no clinical trials have been reported for 3-BP. This may be attributed to its low target specificity and relatively high toxicity.

Lonidamine specifically inhibits mitochondria-bound HK2, which is present mostly in cancer cells but not in normal cells^[76]. It effectively inhibits the cell growth, decreasing lactate and ATP generation, in cancer cells^[77,78]. Meanwhile, the combination of lonidamine with other anti-cancer agents reverts drug resistance and is effective in the treatment of various cancer cells in both pre-clinical and phase II / III studies^[78-80]. However, the combination of lonidamine and epirubicin resulted in no improvement in patients' survival^[81]. Though lonidamine has been widely studied, its hepatotoxicity resulted in the termination of several clinical trials^[82,83]. These studies of the HK2 inhibitors suggest that, although HK2 is a potential target, being the first and the rate-limiting step of glycolysis, inhibition of HK2 may result in severe side

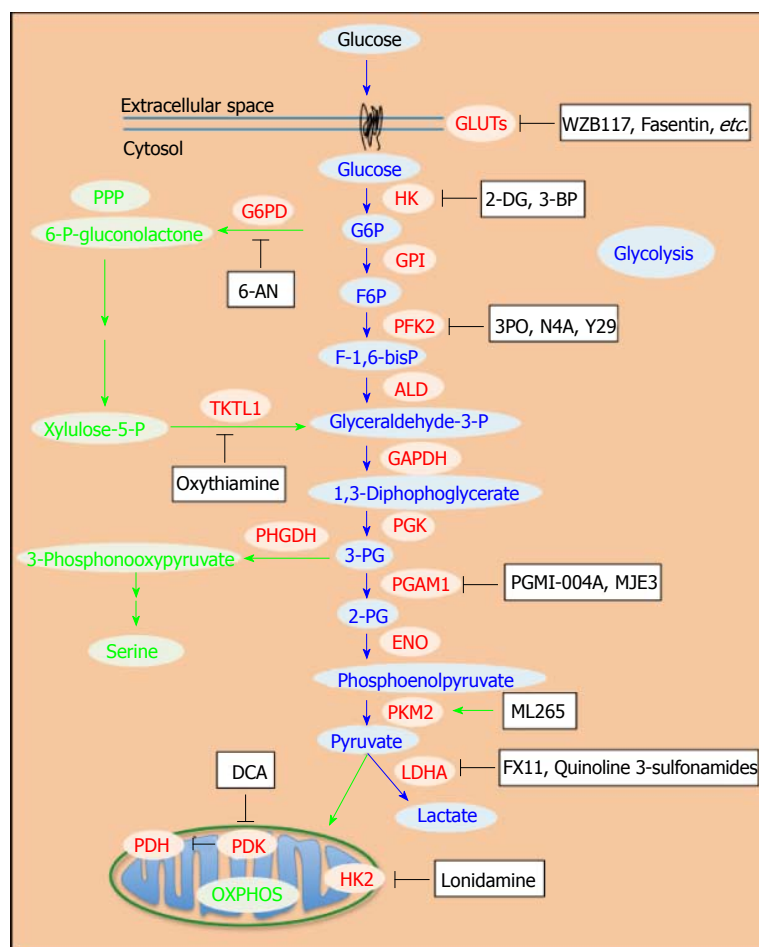


Figure 1 Glycolysis and inhibitors/activators of glycolysis as potential anti-cancer therapeutics. Glucose transporters and enzymes are shown in red and glycolytic intermediates are shown in blue. Inhibitors/activators are in black squares. PPP: Pentose phosphate pathway; OXPHOS: Oxidative phosphorylation; shown in green. GLUTs: Glucose transporters; HK: Hexokinase; GPI: Glucose-6-phosphate isomerase; PFK: Phosphofructokinase; ALD: Aldolase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PGK: Phosphoglycerate kinase; PGAM1: Phosphoglycerate mutase 1; ENO: Enolase; PKM2: Pyruvate kinase M2; LDH: Lactate dehydrogenase; PDK: Pyruvate dehydrogenase kinase; PDH: Pyruvate dehydrogenase; G6PD: Glucose-6-phosphate dehydrogenase; TKTL1: Transketolase-like enzyme 1; PHGDH: Phosphoglycerate dehydrogenase; G6P: Glucose 6-phosphate; F6P: Fructose 6-phosphate; F-1,6-bisP: Fructose 1,6-bisphosphate; 3-PG: 3-phosphoglycerate; 2-PG: 2-phosphoglycerate; 6-P-gluconolactone: 6-phosphogluconolactone; Xylulose-5-P: D-xylulose-5-phosphate; 2-DG: 2-deoxyglucose; 3-BP: 3-bromopyruvate; 3PO: 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; DCA: Dichloroacetate; 6-AN: 6-aminonicotinamide.

effects. However, the combination of HK2 inhibitors and other anti-cancer drugs may still be an alternative approach for HK2-overexpressing tumors.

Phosphofructokinase (PFK) has two isoforms. PFK1 promotes the chemical reaction of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F-1,6-bisP), while PFK2 catalyzes the synthesis of fructose-2,6-bisphosphate (F-2,6-biP) and reverses it back to F6P^[84]. In tumor cells, PFK2 is ubiquitously and constitutively active to produce F-2,6-biP^[85-87]. PFK2 is also inducible by hypoxia *in vivo*^[86,88], which is known as a microenvironment for tumor cell^[89]. Thus, targeting PFK may be a good anti-cancer strategy.

3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) is the most specific known PFK2 inhibitor with a K_i of 25 $\mu\text{mol/L}$ ^[82,90]. 3PO suppresses glucose uptake and glycolytic flux in multiple cancer cell lines, with IC_{50} values ranging from 1.4 to 24 $\mu\text{mol/L}$ ^[90]. Animal studies show that 3PO inhibits tumor growth *in vivo*^[90]. In addition, a chromene derivative, N4A, mimics F6P and is a competitive inhibitor of PFK2, with a K_i of 1.29 $\mu\text{mol/L}$ ^[91]. Its derivative, YZ9, has a K_i as low as 0.094 $\mu\text{mol/L}$ ^[91]. These inhibitors were shown to inhibit the proliferation of HeLa cells (human cervical cancer cells) and T47D cells (human adenocarcinoma cells) *in vitro*^[91]. Using high-throughput screening and structure activity relationship (SAR) studies, Brooke *et al.*^[92] identified derivatives of

5-triazolo-2-arylpyridazinone as a novel group of inhibitors of PFK2, with the lowest IC_{50} of 2.6 $\mu\text{mol/L}$. Although these inhibitors with extremely low IC_{50} s are potent and promising *in vitro*, *in vivo* studies are required to assess their toxicity in animals.

3-phosphoglycerate dehydrogenase (PHGDH) catalyzes the first step of the serine biosynthesis pathway (Figure 1). The increased serine synthesis flux attributed to PHGDH is essential to the viability of a subset of cancer cells in which the enzyme is overexpressed^[19,93,94]. Through negative-selection RNAi screening using a human breast cancer xenograft model, Possemato *et al.*^[93] showed that PHGDH is required for tumorigenesis *in vivo*. Meanwhile, using a metabolomics approach with isotope labeling, Locasale *et al.*^[19] showed that glycolytic flux is diverted into amino acid (serine and glycine) metabolism in cancer cells. This suggests that cancer cells use this specific pathway to promote oncogenesis. The PHGDH gene was found to be amplified recurrently in both breast cancers and melanoma^[19,93,95]. In addition, the protein levels of PHGDH are upregulated in 70% of estrogen receptor (ER)-negative breast cancers^[93]. Suppression of PHGDH in cancer cell lines with overexpressed PHGDH, but not in these without, causes a reduction in serine synthesis as well as cell proliferation^[19,93]. So far, no PHGDH inhibitors have been reported, although it appears to be a good target.

Table 1 Glycolytic inhibitors and modulators

| Compound name | Target protein | Status | Ref. |
|--------------------------|----------------------------|---|-------------|
| 2-DG | Inhibits HK | Phase I -completed (Jul 2008) | NCT00096707 |
| 3-BP | Inhibits HK | Phase I / II -terminated (Mar 2011) | NCT00633087 |
| Lonidamine | Inhibits mitochondrial HK2 | Pre-clinical | [66-74] |
| | | Phase II / III -terminated (Aug/Dec 2006) | NCT00237536 |
| | | | NCT00435448 |
| 3PO | Inhibits PFK2 | Pre-clinical | [90] |
| N4A, YZ9 | Inhibits PFK2 | Pre-clinical | [91] |
| PGMI-004A | Inhibits PGAM1 | Pre-clinical | [96] |
| MJE3 | Inhibits PGAM1 | Pre-clinical | [98] |
| TT-232 | Inhibits PKM2 | Phase II -completed (Mar 2008) | NCT00422786 |
| | | Phase II -terminated (Oct 2010) | NCT00735332 |
| Shikonin/alkannin | Inhibits PKM2 | Pre-clinical | [108] |
| ML265 (TEPP-46) | Activates PKM2 | Pre-clinical | [116,117] |
| FX11 | Inhibits LDHA | Pre-clinical | [126] |
| Quinoline 3-sulfonamides | Inhibit LDHA | Pre-clinical | [141] |
| DCA | Inhibits PDK | Phase I -ongoing | NCT00566410 |
| | | Phase I -ongoing | NCT01111097 |
| | | Phase II -completed (Aug 2009) | NCT00540176 |
| 6-AN | Inhibits G6PD | Pre-clinical | [159-161] |
| Oxythiamine | Inhibits TKTL1 | Pre-clinical | [170-173] |

2-DG: 2-deoxyglucose; 3-BP: 3-bromopyruvate; DCA: Dichloroacetate; 6-AN: 6-aminonicotinamide; HK: Hexokinase; PFK: Phosphofructokinase; PGAM: Phosphoglycerate mutase; PKM2: Pyruvate kinase M2; LDH: Lactate dehydrogenase; PDK: Pyruvate dehydrogenase kinase; G6PD: Glucose-6-phosphate dehydrogenase; TKTL1: Transketolase-like enzyme 1.

Phosphoglycerate mutase 1 (PGAM1) catalyzes 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG). In human cancer cells, loss of TP53 leads to upregulation of PGAM1^[96]. In addition, Tyr26 phosphorylation of PGAM1 stabilizes the active conformation of the enzyme^[97]. These regulations of PGAM1 contribute to the increased glycolysis and the rapid biosynthesis in cancer cells^[96,97].

Inhibition of PGAM1 by shRNA increased 3-PG and decreased 2-PG levels and inhibited the proliferation of cancer cells^[96]. Through *in situ* proteome reactivity profiling, PGAM1 inhibitor MJE3 was identified^[98]. MJE3 inhibits PGAM1 activity with an IC₅₀ of 33 μ mol/L and reduces the proliferation of breast cancer cells *in vitro*^[98]. PGMI-004A, an alizarin derivative, is another inhibitor of PGAM1 with an IC₅₀ of 13 μ mol/L, and it leads to significantly decreased glycolysis, pentose phosphate pathway (PPP) flux and biosynthesis, resulting in attenuated cancer cell proliferation and tumor growth *in vivo*^[96].

Pyruvate kinase (PK) irreversibly catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate coupled with the generation of ATP. PKM2 is the isoform highly expressed in embryonic cells and cancer cells during fast proliferation^[99]. The switch of PKM2 to PKM1 was able to inhibit tumor growth *in vivo*^[36]. PKM2 is inactive as a dimer and highly active as a tetramer. Regulation of the transition between the dimer and the tetramer forms depends on the F-1,6 bisP level^[100] or the phosphorylation of tyrosine residue 105 of PKM2, which is induced by oncogenic signals in cancer cells^[38]. Meanwhile, PKM2 activity is further influenced by serine and succinylaminoimidazolecarboxamide ribose-5'-phosphate (SAICAR), which adds additional complexity to the regulation of PKM2 in cells and suggests that the modulation of

PKM2 activity enables cancer cells to adapt their unique metabolic patterns to their specific pathological conditions^[38,101].

In tumor cells, the lower activity of PKM2 results in accumulation of upstream glycolytic metabolites for biosynthesis through PPP^[37,102]. In addition, the presence of histidine-phosphorylated PGAM1 has been found to correlate with the expression of PKM2 in both cancer cell lines and tumors^[103]. In fact, cancer cells with low PKM2 activity allow PEP to transfer its phosphate group to the histidine of PGAM1 and generate pyruvate. This alternate glycolytic pathway bypasses the activity of PKM2 and decouples ATP production from pyruvate generation, facilitating the high rate of glycolysis to support the biosynthesis observed in many proliferating cancer cells^[103]. This decoupled ATP production also suggests that ATP may not be the limiting factor for fast proliferation in cancer cells because cancer cells have access to increased interstitial ATP^[104-106].

Recently, Israelsen *et al.*^[107] demonstrated that PKM2 is not necessary for the proliferation of tumor cells and variable PKM2 expression was found in human tumors. These results suggest that varied PKM2 activity supports the different metabolic requirements of various cancer cells, each with unique metabolic conditions^[107]. Though the role of varied expression of the PKM2 isoform in cancer cells is still controversial, ongoing studies focus on both inhibitors and activators of PKM2 to inhibit cancer cell growth both *in vitro* and *in vivo*.

Shikonin and alkannin are potent PKM2 inhibitors. Both compounds lower PKM2 activity and decrease glycolysis in MCF-7 human breast cancer cells and A549 human lung cancer cells^[108]. TT-232, a synthetic heptapeptide, interferes with the cellular location of PKM2 in

tumor cells and induces apoptosis^[109]. However, the selectivity of these inhibitors is not very high for PKM2 and side effects were observed^[110,111].

In fact, PKM2 was found to be less active than PKM1^[36], indicating that cancer cells prefer to use a less active PK to regulate glycolysis and balance their metabolic needs. Thus, in order to inhibit cancer cell growth more effectively, activators, not inhibitors of PKM2, should be used.

Activators of PKM2, such as N, N'-diarylsulfonamides, thieno-pyrrole-pyridazinones and tetrahydroquinoline-6-sulfonamides, have been identified and studied through high throughput screening and SAR exploration^[112-114]. These compounds showed potent PKM2 activation activity with a highest AC₅₀ of 38 nmol/L^[112]. Kung *et al.*^[115] reported a series of quinolone sulfonamides with a unique allosteric binding mode, which activate PKM2 in A549 lung carcinoma cells. The activation of PKM2 reduces carbon flow to serine biosynthesis, which has been known to promote oncogenesis^[19,115]. This study suggests that targeting PKM2 confers metabolic stress to cancer cells and attenuates the unique metabolic pattern of cancer cells. Among these compounds, ML265 (or TEPP-46), a potent activator of PKM2 with an AC₅₀ of 92 nmol/L, was found to activate PKM2 by inducing the tetramerization of PKM2^[116,117]. ML265 has been shown to reduce tumor size, weight, and occurrence in animal models^[116,117]. Recently, Xu *et al.*^[118] described a structurally novel series of small molecule 3-(trifluoromethyl)-1H-pyrazole-5-carboxamides as potent PKM2 activators *in vitro*. Moreover, Guo *et al.*^[119] identified 2-((1H-benzo[d]imidazol-1-yl)methyl)-4H-pyrido (1,2-a) pyrimidin-4-ones as novel activators of PKM2 with a unique binding mode. However, their results also suggested that activation of PKM2 alone was insufficient to significantly alter the cancer metabolism^[119]. Although the complex roles of PKM2 in tumorigenesis remain to be elucidated, potent and selective activators of PKM2 may be valuable tools for solving the puzzle of PKM2 and combating cancer.

Lactate dehydrogenase (LDH) catalyzes the chemical conversions of pyruvate to lactate and NADH to NAD⁺ simultaneously. Upregulation of LDHA under c-Myc control promotes aerobic glycolysis and the growth of tumor cells^[120]. Increased expression of LDHA was identified in clinical samples of multiple tumor types^[121,122]. Inhibition of LDHA expression in fumarate hydratase deficient cells by RNA interference inhibited cell proliferation and tumorigenesis *in vivo*^[42,123]. Thus, LDHA is a potential anti-cancer target with multiple inhibitors already developed^[124].

Oxamate competes with pyruvate for LDHA binding with a K_i of 136 μ mol/L^[125]. However, oxamate also works as an inhibitor of aspartate aminotransferase with an even lower K_i of 28 μ mol/L^[125]. Thus, oxamate is a non-specific inhibitor of LDHA. FX-11,3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid, competing with NADH as a selective inhibitor of LDHA,

inhibited the growth of xenograft tumors^[126].

Galloflavin, a new LDHA inhibitor, reduced ATP generation, lactate production, and inhibited growth of human breast cancer cells. However, other mechanisms in addition to inhibition of LDHA were involved in cell death induced by galloflavin^[127]. Moorhouse *et al.*^[128] used a fragment-based click-chemistry-supported approach to synthesize a series of bifunctional inhibitors of LDHA. In this approach, the structures of both natural substrates pyruvate and NADH were mimicked and linked together in a bifunctional inhibitor. The lead compound has an IC₅₀ of 14.8 μ mol/L. ARIAD Pharmaceuticals and Genentech recently have identified numerous LDHA inhibitors^[129-132], and Ward *et al.*^[133] have identified plant-derived human LDHA inhibitors through high-throughput screening. However, these inhibitors need to be tested *in vitro* and *in vivo* in due course. Ward *et al.*^[134] used fragment-based lead generation as well as X-ray crystallography to develop very potent inhibitors of LDHA. The lead compound has a remarkable IC₅₀ of 0.27 μ mol/L. However, these potent LDHA inhibitors still need to be tested both *in vitro* and *in vivo* to demonstrate their potentials as anti-cancer therapeutics.

Granchi *et al.*^[135] designed and synthesized a series of N-hydroxyindole (NHI)-based compounds as competitive human LDHA inhibitors. Some representative compounds were tested and shown to possess anti-proliferation activity in multiple human cancer cell lines^[136-138]. NHI-1, one of these inhibitors, working with gemcitabine is active against pancreatic cancer cells synergistically^[139]. Interestingly, glycosylation of these NHI-based LDHA inhibitors increased potencies and improved cell permeability in cancer cells^[140]. Linking the glucose and the LDHA inhibitor facilitates the dual-targeting strategy.

Recently, Billiard *et al.*^[141] showed that quinoline 3-sulfonamides inhibit LDHA and reverse the Warburg effect (aerobic glycolysis) in multiple cancer cell lines. Interestingly, compound 1, an LDHA inhibitor in this study, also activates PKM2, if not directly, then at least in part due to the accumulation of F-1,6-bisP caused by LDHA inhibition. Unfortunately, because of low *in vivo* clearance rates and low oral bioavailability, the quinolone 3-sulfonamides are unsuitable for *in vivo* use^[141]. In sum, though several LDHA inhibitors have been identified, further efforts are needed to test their anti-cancer effects *in vivo* as well as in clinical trials.

Pyruvate dehydrogenase kinase (PDK) favors glycolysis over mitochondrial oxidative phosphorylation (OXPHOS) by blocking the activity of pyruvate dehydrogenase (PDH) by phosphorylating it^[142]. Under normal oxygen pressures, pyruvate goes to mitochondria and is converted to acetyl-CoA in a step catalyzed by PDH. Acetyl-CoA is an important metabolite involved in the citric acid cycle and OXPHOS. In studies in cancer cells, PDK1 expression was induced by HIF-1 in hypoxic conditions and shown to lead to increased glycolysis and suppressed OXPHOS^[143,144]. The expression of PDK1 is associated with poor prognosis in head-and-neck squa-

mous cancer^[145]. Also, the upregulation of PDK in cancer was associated with a more aggressive phenotype^[146]. For these reasons, PDK has been considered an attractive and promising anti-cancer target.

Dichloroacetate (DCA), an analog of pyruvate, has been identified as a PDK inhibitor and widely studied for its ability to inhibit lactate production and cancer growth^[147-151]. DCA decreases lactate production by shifting the pyruvate metabolism from glycolytic fermentation towards mitochondrial OXPHOS, and restores mitochondrial function, thus potentially restoring apoptosis-induction, allowing cancer cells to undergo programmed cell death and shrink the tumor^[53]. DCA's research and clinical trials were based on the belief that cancer cells' mitochondrial function is abnormal and therefore cancer cell growth will be reduced by upregulating and normalizing their OXPHOS. DCA was shown to be effective in suppressing the growth of cancer cells both *in vitro* and *in vivo*^[152-155]. Several human clinical trials of DCA started after the successful cell and animal studies and still ongoing. A phase II clinical trial for malignant glioblastoma has been completed and shows that DCA can be used safely in patients with glioblastoma, suggesting that DCA is a promising anti-cancer agent and inhibiting glycolysis is a potent and effective anti-cancer strategy^[156] (Table 1). In addition, several clinical trials combining DCA and other anti-cancer drugs or therapies are in progress. On the other hand, human studies indicate that DCA's anti-cancer effects, if any, may be cancer type-related. More basic biomedical studies need to be conducted on the compound before DCA's anticancer activity can be better evaluated.

Pentose phosphate pathway (PPP), a metabolic pathway branched off from glycolysis, provides metabolic intermediates for biosynthesis and NADPH for clearing ROS in cells. At the first step of PPP, glucose-6-phosphate dehydrogenase (G6PD) catalyzes the conversion of G6P to 6-phosphogluconolactone, coupled with generation of NADPH. G6PD has been shown to be overexpressed in cancer cells^[157,158]. Therefore, inhibition of G6PD is an attractive strategy to alter cancer metabolism and attenuate cancer growth. 6-aminonicotinamide (6-AN) is an inhibitor of G6PD that induces oxidative stress and sensitizes cancer cells to drugs^[159-161]. Recently, Preuss *et al.*^[162] used high-throughput screening to identify several hit compounds as novel inhibitors of G6PD with IC₅₀s of < 4 $\mu\text{mol/L}$. These G6PD inhibitors reduced the viability of MCF10-AT1 mammary carcinoma cells with an IC₅₀ of approximately 25 $\mu\text{mol/L}$ compared to approximately 50 $\mu\text{mol/L}$ for MCF10-A non-carcinoma cells^[162]. However, its *in vivo* efficacy remains to be investigated.

The enzyme transketolase (TKTL) is critical for both PPP and glycolysis^[157,163]. Transketolase-like enzyme 1 (TKTL1) has been shown to be increased in tumor cells^[164-166]. Down-regulation of TKTL1 inhibited cancer cell proliferation, tumor growth and metastasis^[167-169]. Thus, inhibiting TKTL1 is a potential anti-cancer strate-

gy. Oxythiamine inhibits TKTL and the growth of cancer cells both *in vitro* and *in vivo*^[170,171]. Also, oxythiamine interrupted signaling dynamics in pancreatic cancer cells^[172], and attenuated tumor cell metastasis^[173]. Further studies on oxythiamine are of interest.

GLUCOSE TRANSPORTERS AND UPREGULATION OF GLUCOSE TRANSPORTERS IN CANCER

Up to 90% of cancers demonstrate a phenotype of increased glucose uptake, as revealed by PET scan and other detection methods^[21,23,174,175]. Cancer cells also show an increased dependence on glucose as a source of energy and biosynthesis precursor for cell growth, while normal cells utilize lipids, amino acids and glucose in a more balanced fashion^[25,43]. Increased glucose uptake in cancer is achieved primarily by upregulation of glucose transporters (GLUTs)^[176-179] although the recent finding that animal cells transformed with a mutated (oncogenic) KRas gene exhibit macropinocytosis^[105] raises the possibility that macropinocytosis and other endocytosis may contribute significantly to glucose uptake in cancer cells. Current research finds that upregulation of GLUTs can be attributed to oncogenic alterations in cancer cells^[180].

GLUTs (SLC2A) are plasma membrane-associated transporters that facilitate glucose transport across the cell membrane down the glucose concentration gradients^[181]. Up to now, at least 14 different isoforms of GLUTs have been identified in human cells (Table 2)^[176]. All GLUTs share a common and highly conserved (97%) transmembrane domain composed of twelve membrane-spanning helices with less conserved and asymmetric extracellular and cytoplasmic domains^[183-185]. Different isoforms of GLUTs are structurally and functionally related proteins and divided into 3 classes according to the similarity of their amino acid sequences^[182]. They are expressed in various cell types based on cells' unique physiological requirements for glucose (Table 2)^[176]. This differential need and thus transport of glucose is achieved by varied affinities of the GLUTs for glucose^[176,186].

GLUTs that are most relevant to cancer are GLUT1 and GLUT3^[176,187,188]. GLUT1 is a basal glucose transporter expressed in almost all cell types^[189] and is up-regulated in almost all cancer types examined^[176-179]. PET scans and other analytical methods have revealed membranous overexpression of GLUT1 and increase in glucose uptake by cancer cells^[175]. GLUT1 expression level is correlated with the grade, proliferative activity, differentiation, and known prognostic markers in various cancers^[175,190-192]. Clinical studies also have shown that high levels of GLUT1 expression correlates with poor prognosis and survival^[192-195]. Normally, GLUT3 is expressed primarily in the tissues with high energy demand to supplement GLUT1^[176,196]. GLUT3 is over-expressed in various cancers compared with their non-cancerous tissues^[176,187,188,197]. GLUT2 is expressed in the liver, pan-

Table 2 Expression of glucose transporters and their major characteristics

| Protein | Class | Expression | Affinity to glucose | Major features | Expression in cancer |
|---------|-------|--|-------------------------------|---|-------------------------------------|
| GLUT1 | I | Ubiquitous (abundant in brain and erythrocytes) ^[207] | High ^[201,208,211] | Constitutive basal glucose uptake ^[207] | Over-expressed ^[176,203] |
| GLUT2 | I | Liver, retina, pancreatic islet cells ^[176,198] | Low ^[201,211] | Glucose sensing, fructose transport ^[176,200] | Abnormal ^[176,202-204] |
| GLUT3 | I | Brain ^[196] | High ^[201,211] | Supplements GLUT1 in brain ^[176,196] | Over-expressed ^[176,205] |
| GLUT4 | I | Muscle, fat, heart ^[210] | High ^[208,209,211] | Insulin responsive ^[210] | Abnormal ^[188] |
| GLUT5 | II | Intestine, testis, kidney, erythrocytes ^[213,214] | Very low ^[212] | Fructose transport ^[212] | Abnormal ^[176,203] |
| GLUT6 | III | Spleen, leukocytes, brain ^[215] | Low ^[215] | Sub-cellular redistribution ^[216] | UD ^[203] |
| GLUT7 | II | Liver, intestine, colon, testis, prostate ^[216,217] | High ^[217] | Glucose and fructose transport ^[217] | ND |
| GLUT8 | III | Testis, brain ^[219] | High ^[219] | Sub-cellular redistribution, multisubstrates ^[216] | Over-expressed ^[218] |
| GLUT9 | II | Liver, kidney, pancreatic cells ^[220,222] | High ^[221] | Multisubstrates ^[216] | UD ^[203] |
| GLUT10 | III | Liver, pancreas ^[223] | High ^[224] | Glucose transport ^[224] | ND |
| GLUT11 | II | Heart, muscle ^[225] | Low ^[225] | Inhibited by fructose ^[225] | ND |
| GLUT12 | III | Heart, prostate, muscle, fat, intestine ^[226] | High ^[227] | Insulin-reponsive ^[226] | Abnormal ^[206] |
| HMIT | III | Brain ^[228] | No | H ⁺ /myo-inositol transport ^[228] | ND |
| GLUT14 | I | Testis ^[229] | ND | ND | ND |

GLUTs: Glucose transporters; HMIT: H⁺/myo-inositol transporter; ND: Not determined; UD: Undetectable.

creatic islet cells, and retina cells^[176,198]. GLUT2 has low affinity and high capacity for glucose^[199,200]. GLUT2 also has high affinity for fructose^[201]. Abnormal levels of GLUT2 expression were detected in gastric, breast, and pancreatic cancers^[202-204]. In addition, GLUT4, GLUT5 and GLUT12 have been found to be abnormally expressed in various cancers^[187,188,203,205,206].

Transport of glucose from the extracellular space into the cytoplasm is the first rate-limiting step for glycolysis. Glucose metabolism is drastically upregulated in cancer. Thus, inhibition of aerobic glycolysis by blocking glucose uptake may be more efficient than inhibiting glycolytic enzymes in cells. Therefore, GLUTs are potential targets for anti-cancer therapies. All known glucose transporters and their major characteristics are summarized in Table 2.

ANTICANCER THERAPEUTICS TARGETING GLUCOSE TRANSPORTERS

The rapid growth and proliferation of cancer cells require a large amount of fuel, primarily and preferentially glucose. Numerous clinical and basic science studies have shown that glucose transport is upregulated in various cancers, by overexpressing GLUTs^[193,203,230-233]. Studies have identified GLUT1 and GLUT2 as the main glucose transporters in hundreds of tumors^[203]. GLUT1 expression was the most widely distributed, while GLUT2 was mainly expressed in breast, colon, and liver carcinomas^[203]. Upregulated GLUT3 protein expression was also detected in endometrial, breast and thyroid cancers^[233,234]. Recently, constitutive cell membrane localization of GLUT4 was found in myeloma cells^[235,236]. Because GLUTs increase glucose transport and enhance cancer cell growth, survival and drug resistance, they are good targets for cancer therapeutic intervention.

GLUT1 INHIBITORS

GLUT1 is the most widely expressed glucose transporter in different types of cancers^[189,194,237,238]. However,

GLUT1 was not targeted therapeutically until recently. This is not because GLUT1 is not a good target but because of the lack of specific and potent inhibitors. Anti-GLUT1 antibody was shown to be effective in reducing cancer cell growth *in vitro*, and the antibody treatment also resulted in cell cycle arrest of the cancer cells^[239]. Before and after the report of the GLUT1 antibody, several small molecule GLUT1 inhibitors have been reported. They will be described individually below.

WZB117

Liu *et al.*^[177] recently reported the identification of a group of novel small compounds that inhibit basal glucose transport by cancer. WZB117 is one of the small molecules that best inhibited GLUT1 and cancer cell growth *in vitro* and *in vivo*. Its anticancer efficacy and safety was demonstrated in a tumor model of human A549 lung cancer cells in nude mice^[28]. Daily intraperitoneal injection of WZB117 at 10 mg/kg reduced tumor size by more than 70%. Mechanism studies showed that WZB117 inhibited glucose transport in human red blood cells (RBC), in which GLUT1 is the only glucose transporter expressed^[28]. This conclusively shows that WZB117 inhibits GLUT1. However, it is presently unclear if WZB117 also inhibits other GLUTs. Computer docking studies show that WZB117 binds directly to GLUT1 using three hydrogen bonds with amino acid residues Asn34, Arg126, and Trp412 of the protein^[28]. Treatment with WZB117 resulted in changes in levels of GLUT1 protein, intracellular ATP, and related metabolic enzymes such as AMPK in cancer cells, leading to cell-cycle arrest, senescence, and necrosis in red blood cells and tumor cells (IC₅₀ = 10 μmol/L). Synergistic effect with cisplatin and paclitaxel was also demonstrated^[28]. A new generation of GLUT1 inhibitors based on the structure of WZB117 but with higher potency and stability are being synthesized and tested.

STF-31

A small molecule named STF-31 that selectively targets von Hippel-Lindau (VHL) -deficient renal cell carcinoma

(RCC) cells was reported by Chan *et al.*^[240]. They demonstrated that STF-31 inhibits VHL-deficient cancer cells by inhibiting GLUT1. It was shown that daily intraperitoneal injection of a soluble analogue of STF-31 effectively reduced the growth of tumors of VHL-deficient RCC cells in nude mice^[240]. STF-31 specifically targets RCCs because aberrant HIF stabilization regulated by VHL leads to diminished mitochondrial activity in these cells, causing them to become highly dependent on glucose uptake for glycolysis and ATP production. By directly binding GLUT1 and inhibiting glucose uptake, STF-31 targets an RCC-specific vulnerability with limited toxicity to normal kidney cells, which are strictly dependent on neither glycolysis nor GLUT1^[240]. Nevertheless, the target spectrum of STF-31 appears to be relatively narrow. The successful animal studies using WZB-117 and STF-31 show *in vivo* potential of GLUT1-targeting.

Fasentin

Fasentin was first identified as a compound that enhances the death receptor stimuli FAS-mediated cell death in FAS-resistant cancer cells in 2006^[241]. Its mechanism of action was further delineated when altered expression of genes associated with nutrient and glucose deprivation were detected^[242]. Culturing cells in low-glucose medium led to similar effects of fasentin and sensitized cells to FAS, supporting the conjecture that fasentin inhibits glucose uptake^[242]. Computer docking studies suggest fasentin interacts with a unique site on the intracellular domain of GLUT1^[242]. The role of fasentin as a chemical sensitizer through glucose transport inhibition was further supported by additional chemical studies^[242]. However, no *in vivo* study has been reported for fasentin.

Apigenin

Apigenin is a natural flavonoid compound existing abundantly in common fruits and vegetables^[243]. Previous studies have demonstrated apigenin's anti-mutagenic, anti-oxidant, anti-cancer, and anti-inflammatory activities^[244-247]. In a mechanism study, apigenin was shown to inhibit glucose uptake in a dose-dependent manner (in the 10-100 $\mu\text{mol/L}$ range) in CD18 and S2-013 human pancreatic cancer cell lines^[248]. Apigenin was determined to achieve this effect by inhibiting GLUT1 at both mRNA and protein levels^[248]. This was further investigated with PI3K inhibitors whose inhibitory effects on GLUT1 mRNA and protein expression are similar to apigenin's, suggesting that apigenin targets GLUT1 through a PI3K/Akt related pathway^[248]. Thus, apigenin inhibits GLUT1 indirectly.

Genistein

Genistein, an isoflavone, is a natural product present in plants such as soybeans^[249,250]. It is a known tyrosine kinase inhibitor and has been shown to exhibit therapeutic effects against a variety of health disorders such as obesity, diabetes and cancer, making it a promising agent for the treatment of metabolic diseases^[251,252]. Genistein is

also reported to be a potent inhibitor of GLUT1^[253,254]. It inhibits the transport of hexose and dehydroascorbic acid through GLUT1 in human HL-60 cells in a dose-dependent fashion^[253]. Further investigation demonstrated that genistein binds to the external surface of GLUT1, altering the binding of glucose to the external surface site of GLUT1^[254]. However, genistein does not appear to be specific for GLUT1.

Oxime-based GLUT1 inhibitors

Recently, a group of oxime-based GLUT1 inhibitors have been reported^[255]. These compounds possess a basic chemical structure different from either phloretin, WZB-117 or other reported GLUT1 inhibitors, and thus represent a novel group of GLUT1 inhibitory compounds. Some of these compounds are as potent as WZB117 in inhibiting glucose transport and cell proliferation in cancer cells^[255]. A detailed computer simulation study revealed the potential binding site for these compounds on GLUT1, which appears to be consistent with that reported for 17 β -estradiol and genistein^[256]. The simulation result and basic structure of these compounds provide bases for designing next generation GLUT1 inhibitors.

Pyrrolidinone-derived GLUT1 inhibitors

Using high-throughput screening coupled with ATP, cell cycle arrest, and lactate assays, two potent GLUT1 inhibitory compounds were identified^[257]. These compounds inhibit glucose transport mediated by erythrocyte membrane-derived vesicles with K_i values of 1.2 and 0.8 $\mu\text{mol/L}$, respectively^[257]. These compounds are GLUT1 inhibitors because only GLUT1 is expressed on erythrocytes. However, no *in vivo* study has been reported for these intriguing compounds.

GLUT2 INHIBITORS

Phloretin

Phloretin, a natural compound found in fruits such as apples and pears, is reported to be a GLUT2 inhibitor^[258-260]. Phloretin has been shown to retard tumor growth both *in vitro* and *in vivo* and induce apoptosis in leukemia, melanoma, and colon cancer cells^[261-263]. Results from human hepatocellular carcinoma HepG2 cells, which express high levels of GLUT2, suggest that phloretin-induced apoptosis involves inhibition of GLUT2-mediated glucose transport^[258]. Additional studies showed that the inhibitory properties of phloretin on GLUT2 sensitize cancer cells to paclitaxel, illustrating the potential use of phloretin in cancer therapy^[264].

Quercetin

Quercetin is a flavonoid compound in fruits, vegetables and grains. It was found to be an effective non-competitive GLUT2 inhibitor in *Xenopus* oocytes with a K_i of 22.8 $\mu\text{mol/L}$ ^[265]. In rats administered glucose, quercetin inhibits glucose absorption through GLUT2^[265]. Querce-

tin was also suggested to reduce the risk of lung cancer and other types of cancer^[266-268]. Quercetin aglycone was shown to affect some receptors associated with cancer development and modulate some signaling pathways involved in inflammation and carcinogenesis^[266], although no direct evidence links between inhibition of GLUT2 and cancer prevention. More studies are needed to explore the connection. Quercetin is likely to be a non-specific GLUT2 inhibitor since its anticancer activity cannot be completely explained by its GLUT2 inhibitory activity.

GLUT3 INHIBITORS

DNA-damaging anticancer agents

Some DNA-damaging anticancer agents including adriamycin, camptothecin and etoposide were reported to induce cancer cell death by reducing GLUT3 expression in HeLa cells^[269]. Real-time PCR results in HeLa cells and a tumorigenic HeLa cell hybrid showed that only the expression of GLUT3, rather than GLUT1, was suppressed by these medicines^[269]. Mechanism studies suggested that the suppression of GLUT3 expression induced by DNA-damaging agents was through the MEK-ERK pathway in a p53-independent manner^[269].

GSK-3 inhibitors

Recently, certain glycogen synthase kinase-3 (GSK-3) inhibitors were identified as inhibitors of GLUT3 expression in GLUT3-overexpressing tumorigenic HeLa hybrid cells as compared with non-tumorigenic counterparts that express GLUT1 alone^[270]. These inhibitors decreased GLUT3 expression at the transcriptional level through NF- κ B signaling in a p53-independent fashion, leading to apoptotic cell death^[270]. Thus, GSK-3 inhibitors do not interact with GLUT3 protein directly but reduce GLUT3 expression levels. No small molecule inhibitors of GLUT3 protein have been reported.

GLUT4 INHIBITORS

Ritonavir

Several HIV protease inhibitors were reported to exhibit inhibitory effects on GLUT4: the most potent is ritonavir^[235,271,272]. The effects of ritonavir against myeloma cells were investigated *in vitro*^[235]. It was demonstrated that the inhibitory effects of ritonavir were achieved by suppressing the glucose consumption mediated by GLUT4 in myeloma cells, which overexpress GLUT4, as well as localize it to the basal cell surface^[235]. The specificity of ritonavir for GLUT4 was confirmed by artificially introducing GLUT1-mediated glucose uptake, which resulted in resistance to prolonged ritonavir treatment^[235]. Half of the cell death induced by ritonavir was seen at a concentration of 20 μ mol/L^[235]. These and other study results highlight the therapeutic potential of ritonavir in mediating GLUT4 inhibition in myeloma treatment^[235,272]. Ritonavir has also been investigated for treatment of other types of cancer^[273-275] and undergone clinical trials (Clini-

calTrials.gov Identifier: NCT01009437, NCT01095094).

Silibinin

Silibinin, also known as silybin, is a natural flavonoid recently shown to be a GLUT4 inhibitor^[276,277]. Kinetic analysis revealed that silybin is a competitive inhibitor of GLUT4, modulating glucose transport in CHO cells with a K_i of 60 μ mol/L^[276]. Inhibitory effects of silibinin on cancer growth have been demonstrated in preclinical models^[278,279] and tested in clinical Phase I^[280,281] and Phase II trials (ClinicalTrials.gov Identifier: NCT00487721) for prostate cancer, indicating the relative safety of this anticancer agent. Because of its relatively weak GLUT4 inhibitory activity, silibinin's anticancer effects are likely to be elicited from multiple mechanisms.

From the studies cited above, it can be concluded that GLUTs are rate-limiting for glycolysis in specific tumor contexts. The identification and targeting of upregulated GLUTs in different tumors provide a promising approach to block glucose-regulated cancer metabolism and thus inhibit cancer growth. Key information for all the GLUT inhibitors described above is summarized in Table 3.

FUTURE DIRECTIONS AND CHALLENGES

From numerous examples cited in this review, it can be concluded that targeting glucose transport and metabolism offers several advantages: (1) It targets a protein, enzyme or process that is significantly altered or upregulated in cancer compared to those in normal cells. The differences between cancer and normal cells potentially provides a therapeutic window by which cancer cells can be effectively inhibited without harming patients' normal cells; (2) Targeting GLUTs is equivalent to inhibiting the entire process of glycolysis, leaving cancer cells fewer options for production of sufficient amount of ATP, NADPH, serine, *etc.* It may also be harder for cancer cells to bypass GLUT inhibition, leading to stronger and longer-lasting inhibition. To compensate for the shortage of glucose, cancer cells will have to use either other glucose transport mechanisms or other energy molecules, such as glutamine for biosynthesis and energy. Although this is possible, it is more difficult than merely bypassing the inhibition of a single enzyme in the middle of a signaling pathway; and (3) Cancer cells are addicted to glucose^[25,27], and thus more sensitive to glucose concentration changes triggered by GLUT inhibition than are normal cells. Cancer cells more readily enter cell cycle arrest or apoptose from glucose shortage^[28].

However, there are also some weaknesses associated with the strategy of glucose transport inhibition. These include: (1) GLUTs are expressed by both cancer and normal cells. Inhibiting cancer cells' GLUTs inevitably inhibits normal cells that also use GLUTs for their functions. The identification of a therapeutic window is absolutely essential for the success of this anticancer strategy. Fortunately, key organs in the body such as the

Table 3 Inhibitors of glucose transporters 1, glucose transporters 2, glucose transporters 3 and glucose transporters 4

| Inhibitor | Target GLUT | Status | Ref. |
|--|-------------|-----------------|---|
| WZB117 | GLUT1 | Animal study | Liu <i>et al</i> ^[28] , 2012 |
| STF-31 | GLUT1 | Animal study | Chan <i>et al</i> ^[240] , 2011 |
| Fasentin | GLUT1 | <i>In vitro</i> | Wood <i>et al</i> ^[242] , 2008 |
| Apigenin | GLUT1 | Phase II | NCT00609310 |
| Genistein | GLUT1 | Phase II / III | NCT00118040; NCT00584532 |
| Oxime-based GLUT1 inhibitors | GLUT1 | Animal study | Tuccinardi <i>et al</i> ^[255] , 2013 |
| Pyrrolidinone derived GLUT1 inhibitors | GLUT1 | <i>In vitro</i> | Ulanovskaya <i>et al</i> ^[257] , 2011 |
| Phloretin | GLUT2 | Animal study | Wu <i>et al</i> ^[258] , 2009 |
| Quercetin | GLUT2 | Phase I | NCT01912820 |
| DNA-damaging anticancer agents | GLUT3 | <i>In vitro</i> | Watanabe <i>et al</i> ^[269] , 2010 |
| GSK-3 inhibitors | GLUT3 | <i>In vitro</i> | Watanabe <i>et al</i> ^[270] , 2012 |
| Ritonavir | GLUT4 | Phase I / II | NCT01009437; NCT01095094 |
| Silibinin | GLUT4 | Phase I / II | Flaig <i>et al</i> ^[280] , 2007; NCT00487721 |

GLUTs: Glucose transporters.

brain and heart can use ketone bodies as a substitute for glucose^[282,283]. Therefore, GLUT inhibition should not result in significant energy shortage for these vital organs; and (2) Cancer cells' reliance on glucose is not absolute. Some cancer cells use glutamine^[284,285] and others can shift from glucose metabolism to glutamine metabolism^[286,287], bypassing glucose transport inhibition. Drugs targeting other metabolic pathways such as glutamine transport/metabolism or targeting cancer cell growth signaling may be used together with GLUT inhibitors to shut down cancer cells' energy metabolism and cell growth more effectively, leading to cancer cell death. These approaches need to be tested in cancer cells first and then in animal tumor models.

Recently, we have observed that our GLUT1 inhibitor WZB-117^[28] more effectively inhibits cancer cell lines that express the wild type KRas gene (KRas^{wt} cells) than KRas^{mut} cancer cell lines (unpublished observations). Although the reason for the difference is unclear, we speculate this may be associated with the "leakiness" of cancer cells to extracellular glucose and ATP. We base this on a recent finding published in a 2013 Nature paper that KRas^{mut} genotype is associated with a phenotype of macropinocytosis^[105], a type of endocytosis that non-specifically takes up extracellular molecules as large as proteins^[288]. In theory, KRas^{mut}-induced macropinocytosis should be able to take up glucose or ATP as well. Thus, to further enhance cancer treatment efficacy by GLUT inhibitors, it is imperative to ascertain not only which GLUT is upregulated in the targeted cancer, but also the genotype (such as KRas status) of the cancer. We also observed that WZB-117 was less effective in cancer cell lines with higher glycogen content (unpublished observation). It is possible that higher intracellular glycogen content confers some degree of resistance to glucose transport inhibitors. In theory, a longer duration of GLUT inhibition should be able to exhaust intracellular glycogen storage and change GLUT1 inhibitor-insensitive cells into sensitive ones. These new findings may enhance GLUT inhibitors' success in treating specific cancer types.

In summary, glucose transport and glycolysis inhibi-

tors have been shown to be promising anti-cancer agents that warrant further basic science and clinical investigation. Improvement in inhibitor's efficacy (IC₅₀), selectivity of the target, and identification of therapeutic windows while taking cancers' specific genotype and phenotype into account, are needed for such inhibitors to become effective anti-cancer therapeutics.

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Liver transplantation with grafts obtained after cardiac death-current advances in mastering the challenge

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models, namely, hypothermic machine perfusion and normothermic machine perfusion; we compare both methods, and delineate their major differences.

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Key words: Transplant hepatology; Liver transplantation; Organ donation; Extended criteria donors; Donation after cardiac death; *Ex-vivo* liver perfusion; Normothermic machine perfusion; Hypothermic machine perfusion

Core tip: There exists an increased need for liver grafts that currently exceed the availability of organs by a large margin. It is estimated that a third of the patients awaiting for transplantation will perish or become too ill due to the scarcity of grafts. This has led to a renewed interest in marginal organs as a potential pool. Most notably, donation after cardiac death livers has been targeted, and new strategies emerge to ameliorate their quality. *Ex-vivo* liver perfusion techniques could drastically change the paradigm of organ preservation, conditioning, and amelioration.

Abstract

The scarcity of donor livers has increased the interest in donation after cardiac death (DCD) as an additional pool to expand the availability of organs. However, the initial results of liver transplantation with DCD grafts have been suboptimal due to an increased rate of complications, as well as decreased graft survival. These challenges have led to many developments in DCD donation outcome, as well as basic and translational research. In this article we review the unique characteristics of DCD donors, nuances of DCD organ procurement, the effect of prolonged warm and cold ischemia times, and discuss major studies that compared DCD to donation after brain death liver transplantation, in terms of outcomes and complications. We also review the different methods of donor treatment that has been applied to ameliorate DCD organ outcome, and we discuss the role of machine perfusion techniques in organ reconditioning. We discuss the two major perfusion

Bazerbachi F, Selzner N, Seal JB, Selzner M. Liver transplantation with grafts obtained after cardiac death-current advances in mastering the challenge. *World J Transl Med* 2014; 3(2): 58-68 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v3/i2/58.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v3.i2.58>

INTRODUCTION

Donation after cardiac death (DCD) was the only mode of organ retrieval in the beginning of organ transplantation era. It was largely abandoned after the establishment of brain death criteria in favour of heart beating organ retrieval to minimize ischemic injury. However, the increasing organ shortage has resulted in a new interest to

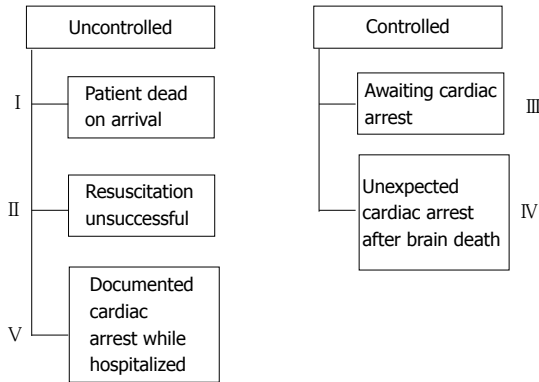


Figure 1 The maastricht classification of donation after cardiac death donors.

extend the donor pool for liver transplantation. Excellent outcomes have been reported for kidney transplantation with DCD organs, which triggered new interest for DCD liver transplantation in the 90's. One study concluded that DCD liver grafts can be used to dramatically reduce wait list time with outcomes comparable to those of standard criteria and donation after brain death (DBD)^[1]. Another study reported that a 5% increase in DCD donors will lead to a 27% relative reduction in the wait list volume^[2]. The proportion of DCD organs has increased compared with the past decades, and DCD liver transplantation remains at approximately 6% in the United States^[3]. However, this group of marginal organs is characterized by increased sensitivity to preservation injury, and the ischemia-reperfusion injury (IRI) pathway is exacerbated by the combination of warm and cold ischemia resulting in cellular injury and energy depletion.

CHARACTERISTICS OF DCD DONORS

In contrast to the irreversible coma state that defines brain death^[4], cardiac death is the irreversible desist of respiratory and circulatory functions. DCD donors [also known as non-heart-beating donors (NHBD)] are divided according to the modified Maastricht classification into 5 categories, which can further be reduced to two main groups (Figure 1): (1) Controlled DCD (categories III and IV), wherein circulatory and respiratory organ support is voluntarily withdrawn by the medical provider, in the setting of a dismal prognosis that renders cardio-respiratory support no longer in the patient's best interest and survival is deemed futile; and (2) Uncontrolled DCD (categories I, II, and V), in which cardiac death occurs suddenly, and resuscitation is unsuccessful or absent^[5].

Debate is ongoing regarding the exact definition of cardiac death, and whether loss of cardiac electricity should be established *vs* solely relying on the absence of heart sounds, pulse, and blood pressure^[5-7]. Today, the irreversible absence of pulse is accepted as the moment of death.

DCD ORGAN PROCUREMENT

After death is announced, organ procurement starts fol-

lowing a mandatory interlude designated to monitor for spontaneous return of cardiopulmonary function. The American Society of Transplant Surgeons (ASTS) recommends 2 min wait time, and the Society of Critical Care Medicine (SCCM) and Institute of Medicine (IOM) recommend 5 min of sustained death prior to the commencement of procurement^[8,9] (Figure 2).

The first described technique for DCD graft retrieval was the so-called "super-rapid technique" (SRT) presented by Starzl *et al*^[10], and involved en-bloc resection of the abdominal viscera, with subsequent separation of individual organs on the back-table while immersed in ice^[10,11]. This technique has been further refined, and the modified technique entails a fast thoraco-laparotomy, hypothermic perfusion of the abdominal aorta, venous exsanguination, cross-clamping of the supradiaphragmatic aorta, and may include portal venous hypothermic perfusion^[8]. The rate-limiting step in terminating warm ischemia time (WIT) is the cannulation of the aorta to allow hypothermic perfusion. In experienced centers, this essential step could be done within 1-2 min after declaration of death^[12].

In-situ cooling of the liver before and during procurement is imperative. Some have advocated for precannulation of the femoral arteries prior to withdrawing life support to further shorten the time until initiation of the cold flush. A double-balloon, triple-lumen (DBTL) catheter can be used to shunt the cold perfusate solely to abdominal viscera^[13]. Although cooling of abdominal organs is facilitated with this technique, ethical concerns have impeded its utilization^[14-16].

The role of the initial flushing solution has been controversially debated. DCD rat livers that were flushed with low viscosity solutions showed lower vascular resistance than those flushed with cold Belzer solution [University of Wisconsin solution (UW)] and led to better survival^[17]. In contrast, analysis of the UNOS database showed a decrease in graft survival when Histidine-Tryptophan-Ketoglutarate (HTK) *vs* UW solution was used as a preservative solution in DCD organs^[18].

THE CONCEPT OF WARM ISCHEMIA TIME

Warm ischemia time (WIT) refers to cellular ischemia under normothermic conditions, and entails two physiological periods^[19,20]: (1) Ischemia after withdrawal of life support until cold perfusion is commenced; and (2) Ischemia during implantation, after removal of the organ from ice until reperfusion.

The beginning of asystole is difficult to predict following the withdrawal of life support therapies (WLST). Should asystole not happen within 120 min of WLST, current guidelines recommend ending the attempted DCD organ retrieval and continuing ICU therapy. In about 30% of all attempted DCD organ retrievals death does not occur within the 120 min recommended waiting time. Different time points from the beginning of warm ischemia have been used. Some groups propose the use of total WIT

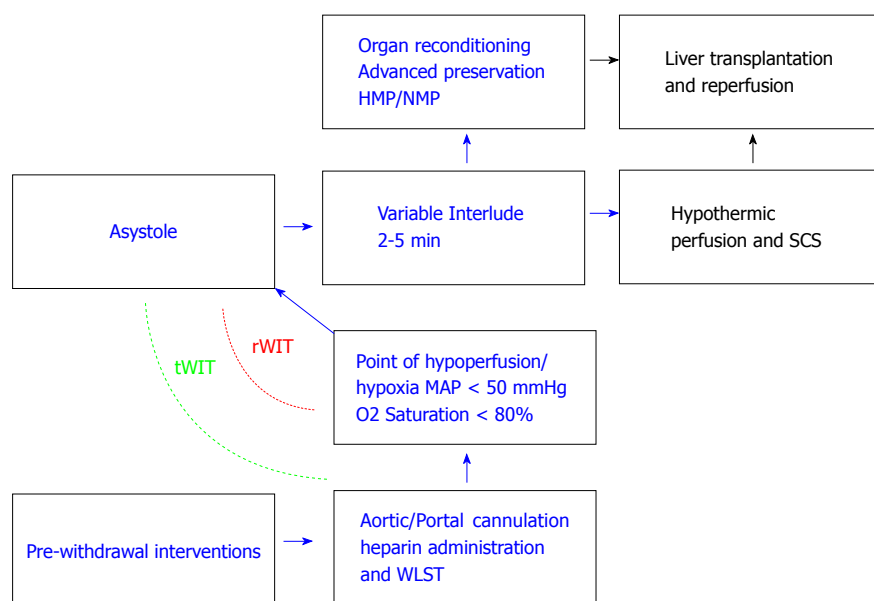


Figure 2 Donation after cardiac death organ procurement algorithm. Modified from Abradelo De Usera *et al.*^[24]. HMP: Hypothermic machine perfusion; NMP: Normothermic perfusion; SCS: Static cold storage; WLST: Withdrawal of life support therapies.

(tWIT): entailing the period from WLST until the start of preservation (whether it is sought through static cold storage (SCS), or through extracorporeal perfusion, organ preservation starts either as a total body cooling approach, or as in-situ cooling approach using the DBTL catheter^[21-24]). In contrast, others have proposed to consider the beginning of warm ischemia only if the arterial saturation of oxygen falls under 70%-80% and/or arterial hypotension occurs (MAP < 60-50 mmHg), also dubbed real warm ischemia time (rWIT). It ends once preservation commences^[24,25]. According to the ASTS, the maximal acceptable WIT for safe liver transplant is 30 min or less^[8,26].

Interestingly, single center studies produced conflicting reports regarding the association between WIT and graft survival^[27-29]. Similarly, the association between WIT and the rate of biliary complications has not been consistently established in all available studies^[28,30-32]. However, despite the inability to detect these associations, Ho *et al.*^[29] have shown that longer rWIT may predict poor survival after liver transplantation. Another recent study by Abt *et al.*^[33] demonstrated in a multivariate regression analysis, an association between graft survival and the slope of the systolic blood pressure using values during the first 10 min after donor extubation (SBP10). The authors propose to select donors with a favorable trajectory of blood pressure during the agonal phase^[33].

Warm ischemia injury is mediated by several mechanisms, including Na⁺/K⁺-ATPase dysfunction, inhibition of nitric oxide synthase (NOS), vascular microthrombosis, changes in bile salts composition, overproduction of hypoxanthine and free radicals, as well as overproduction of vasoconstrictors during reperfusion^[34-39].

LIVER TRANSPLANTATION WITH DCD GRAFTS: OUTCOMES AND COMPLICATIONS

Reich *et al.*^[40] reported the first single-center experience

with comparable outcome of DCD *vs* DBD liver transplantation. However, major studies that followed reported conflicting results (Table 1).

The initial outcomes of liver transplantation with DCD grafts have been suboptimal due to a high rate (20%-40%) of ischemic-type biliary strictures (ITBS)^[36,41-43], higher graft failure rate, as well as increased medical and surgical complications following the procedure.

According to one study by Jay *et al.*^[44], post transplantation costs were significantly higher in DCD versus DBD transplant recipients who experienced ITBS or re-transplantation. In their study, DCD costs continued to be higher when the analysis was censored for re-transplanted patients; this may suggest that morbidity is increased and may account for this increase in costs^[44]. It follows that an examination of the most common complications of DCD liver transplants may be necessary, if the full scope of economic burden is to be understood^[45].

Interestingly, in a study comparing 24 DCD recipients *vs* 16 DBD recipients, Yamamoto *et al.*^[45] showed that, despite an increased rate of hepatic artery thrombosis (HAT) and biliary complications, graft and patient survival did not differ between the groups. Their study suggested that improved surgical and medical management has led to amelioration of transplantation outcomes.

One of the immediate complications is primary graft failure (PGF) following ischemic insult resulting in re-transplantation or patient death. PGF after DCD liver transplantation has decreased in frequency over time, and is reported to be approximately 5% in the most recent studies^[31,43,46]. This improvement may be ascribed to improved surgical techniques and amelioration in organ preservation and extraction.

One of the later complications is ITBS [also dubbed as nonanastomotic biliary stricture (NABS), or ischemic cholangiopathy]. ITBS presents as non-anastomotic intrahepatic or extrahepatic biliary strictures (in the absence of arterial thrombosis), which occur within the first 3 mo

Table 1 Major studies that compared donation after cardiac death vs donation after brain death liver transplantation outcomes

| Ref. | Year | DCD transplants number | Recipient survival rate (%) at 1 yr, 3 yr, and 5 yr post-transplant | | | Graft survival rate (%) at 1 yr, 3 yr, and 5 yr post-transplant | | | ITBS rate | Retransplants rate |
|---|------|-------------------------------------|---|----|----|---|----|----|-----------|--------------------|
| Croome <i>et al</i> ^[98] | 2013 | HCC DCD = 242 Non-HCC DCD = 2117 | | | | 76 | 64 | 56 | | |
| Abt <i>et al</i> ^[33] | 2013 | 110 | | | | 86 | 77 | 71 | | 14% |
| Callaghan <i>et al</i> ^[99] | 2013 | 352 | | 81 | | | 73 | | | |
| Vanatta <i>et al</i> ^[100] | 2013 | 38 | 92 | 80 | | 92 | 74 | | 7% | 2% |
| Elaffandi <i>et al</i> ^[101] | 2012 | 108 | 84 | | | | | | | 2% |
| Taner <i>et al</i> ^[28] | 2012 | 200 | 93 | 85 | 81 | 81 | 73 | 69 | 12% | 5% |
| Meurisse <i>et al</i> ^[52] | 2012 | 30 | 93 | 85 | 85 | 90 | 82 | 82 | | 3% |
| DeOliveira <i>et al</i> ^[30] | 2011 | 167 | 87 | 85 | 81 | 85 | 83 | 78 | 2% | |
| Hong <i>et al</i> ^[102] | 2011 | 81 | | | | 78 | 62 | 53 | 10% | 12% |
| Mathur <i>et al</i> ^[53] | 2011 | 1567 | | | | | 65 | | | 13% |
| Dubbed <i>et al</i> ^[46] | 2010 | 55 | 85 | 80 | | 74 | 68 | | 14% | 18% |
| Yamamoto <i>et al</i> ^[45] | 2010 | 24 | 62 | 43 | 43 | 54 | 37 | 38 | | |
| Detry <i>et al</i> ^[103] | 2010 | 58 | 83 | 67 | | 72 | | | 38% | |
| de Vera <i>et al</i> ^[27] | 2009 | 141 | 79 | | 70 | 69 | | 56 | 16% | 18% |
| Grewal <i>et al</i> ^[43] | 2009 | 108 | 92 | 88 | 88 | 79 | 74 | 71 | 8% | 15% |
| Jiménez-Galanes <i>et al</i> ^[104] | 2009 | 20 | 86 | | | 80 | | | 5% | |
| Pine <i>et al</i> ^[105] | 2009 | 39 | 82 | 68 | | 80 | 64 | | 20% | |
| Nguyen <i>et al</i> ^[42] | 2009 | 19 | 90 | | 90 | 74 | | 63 | 10% | 16% |
| Fujita <i>et al</i> ^[106] | 2007 | 24 | 87 | 82 | | 69 | 56 | | | 21% |

DCD: Donation after cardiac death.

after transplantation. One hypothesis of ITBS etiology is the arterial supply theory. Since most of the blood that supplies the biliary system emanates from the hepatic arteries, severe decrease in hepatic artery supply may result in biliary necrosis and subsequent stenosis^[47,48]. The occurrence of this complication has been estimated to fall between 20% and 40% in DCD recipients, compared to 5% in DBD recipients. However, recent studies reported a decreased frequency of this complication, and estimated its incidence to be around 10%^[30,31,49].

ITBS appears to be particularly associated with DCD organs, with a 10 fold increase in incidence compared with DBD livers. Other risk factors are increased donor age, increased donor weight, and increased cold ischemia time (CIT) and/or WIT (especially WIT > 30 min)^[27,28,32,50-54].

Attempts have been made to reduce ITBS in orthotopic liver transplantation. Moench *et al*^[55] established the utility of arterial back-table pressure perfusion of the hepatic artery prior to transplantation in heart beating donor grafts, and showed an association with decreased ITBS rate in a multivariate analysis. Hashimoto *et al*^[36] investigated the use of tissue plasminogen activator (tPA) administration in 22 patients during DCD liver transplantation. In the implantation phase, tPA was injected in the hepatic artery prior to making the anastomosis. The authors found that this strategy decreased the incidence of ITBS to 9% in DCD liver grafts^[36].

WHO SHOULD RECEIVE DCD LIVERS?

As a result of these complications, strict acceptance criteria have been applied for DCD liver transplantation and only a small percentage of DCD livers are currently accepted for transplantation. Harring *et al*^[56] proposed

criteria for DCD transplant optimization that focused on strict selection for donors and recipients (donor age < 50 years and WIT < 20 min), however, current ASTS recommendations state that DCD liver grafts should be ideally used in younger recipients with age < 60 years and WIT < 30 min.

Some studies cautioned about using DCD grafts in HCV(+) recipients as they have found that HCV recurrence was more aggressive and advanced more rapidly in this cohort of patients, compared to DBD grafts^[57,58], although a recent registry analysis failed to detect this difference^[59]. Moreover, a recent match-controlled, retrospective analysis demonstrated that DCD liver grafts did not promote disease progression or negatively affect patient and graft survival in comparison with DBD liver grafts in HCV(+) patients^[60].

DONOR TREATMENT

Animal models were designed using several strategies to optimize DCD grafts, including administration of different pharmacologic agents^[61,62]. Administration of heparin and phentolamine prior to asystole resulted in an increase of acinar perfusion and sinusoidal density in rat livers^[61]. Experimental data have shown that tacrolimus may incur protection against hepatic IRI when administered intravenously or as a hepatic rinse^[63]. Recently, a study protocol has been published for a European randomized multicenter trial comparing *ex vivo* tacrolimus perfusion of marginal liver grafts *vs* placebo^[64]. Milrinone, a phosphodiesterase 3 inhibitor, exerts positive inotropic and vasodilatory effects, and has been reported to attenuate the graft injury caused by CIT, WIT, and subsequent IRI *via* an increase in intracellular cAMP levels^[65]. Pentoxifyl-

line is a methylxanthine compound and a phosphodiesterase inhibitor with hemorheological, as well as anti-inflammatory properties has also been shown to decrease IRI in animal models^[66,67].

MACHINE PERFUSION TECHNIQUES

As more programmes now accept increasing numbers of DCD livers in which organ function status is uncertain, the need for further evaluation and even reconditioning of the organ is emphasized.

Although the prevailing goal of organ preservation in the past has been to slow the metabolic rate by SCS, this strategy may not be optimal for livers from marginal donors.

Initially, SCS emerged as a method to optimally store organs and thus improve graft survival. However, this simple technique does not allow for adequate evaluation of the organ, as reduction of metabolism to about 5% by cold storage hinders the possibility of meaningful liver evaluation. Moreover, while hypothermia slows down metabolism, it does not prevent continuation of anaerobic glycolysis and does not stop the production of harmful by-products.

Therefore, several groups proposed the use of extracorporeal perfusion systems to reduce IRI, and ameliorate graft outcomes.

Originally suggested by Carrel and Lindbergh in the late 1930s for organs in general, *ex vivo* liver perfusion emerged as a potential protective strategy^[68-72]. The purpose of extracorporeal perfusion is to continuously support the preserved organ with nutrients and oxygen, and to eliminate toxic products from the cellular milieu. Newer studies evaluated these experimental techniques and their effect on late biliary injury^[73].

Ex vivo perfusion systems could be classified according to the perfusate temperature, and it includes: normothermia (35 °C-37 °C), mild hypothermia/subnormothermia (32 °C-35 °C), moderate hypothermia (28 °C-32 °C), severe hypothermia (20 °C-28 °C), and profound hypothermia (< 20 °C)^[74] (Table 2).

Henry *et al*^[75] and Guarrera *et al*^[76-78] performed hypothermic (4 °C) machine perfusion (HMP) of the hepatic artery and portal vein without oxygenation. The authors used sub-physiologic perfusion pressures, and no benefits of hypoxic HMP were observed in an animal model. However, in a case control study with 20 human liver transplants using low risk donors, the same group observed a decrease of serum AST/ALT after transplantation when HMP was compared with SCS.

In another porcine DCD model, de Rougemont *et al*^[71] studied the effects of oxygenated HMP prior to transplantation. Livers were exposed to 1 h WIT followed by 7 h of SCS preservation or 1 h of WIT plus 6 h of SCS and 1 h of oxygenated HMP. After liver transplantation, AST levels were similar in both groups. Median recipient survival after transplant was slightly increased by oxygenated HMP from 5 to 8 h.

Despite these results, early experiments that examined machine perfusion of animal liver grafts showed that a hypothermic perfusate is a risk factor for post-transplant HAT. Ikeda and colleagues demonstrated that, compared to normothermic perfusion (NMP), HMP was associated with increased hepatic artery resistance and decreased bile flow^[79]. More recently, Tolboom *et al*^[80] showed that bile production increased concordantly with increased perfusate temperature, and was the highest at a degree of 37 °C^[80]. Consequently, there existed an increased interest in normothermic techniques^[81].

In 2001, Schön *et al*^[82] were the first to successfully describe NMP in porcine livers. The Oxford group headed by Dr. Peter Friend showed conserved hepatic function with NMP up to 72 h^[83-85]. SCS cannot be completely avoided, even in NMP, due to the complexity of the procurement process, as well as the logistics of the apparatus. Although NMP could not salvage porcine livers that received 4 h of SCS prior to perfusion, it was able to assess liver function, and maintain cellular replenishment when used throughout the preservation period^[83,85-87]. Brockmann and colleagues showed that NMP was advantageous to DBD and DCD livers that endured a prolonged period of preservation (approximately 20 h)^[72].

Our group in Toronto^[70] was the first to examine bile duct injury using NMP in a DCD porcine model while simulating transplantation. Our study was designed to simulate a clinical scenario in which organs are retrieved at a remote donor hospital and transported with SCS to the transplant center to commence NMP, and our machine perfusion model utilized Steen solution^[88] for preservation rather than cellular products. Livers managed with SCS alone had significantly higher ALT levels, decreased oxygen extraction, and increased hepatic necrosis. Levels of bilirubin, phospholipids and bile salts in the bile fluid were fivefold decreased, while LDH was sixfold higher in the SCS *vs* NMP group. Hepatic artery perfusion was decreased and bile duct necrosis was increased as well, favoring NMP. The protective mechanisms of machine perfusion remain under investigation^[89].

Despite these advances, the majority of the studies that examined machine perfusion, focused on early liver graft injury and acute survival. However, in humans, the majority of biliary lesions occur within the first year after transplantation^[90].

OUTLOOK FOR FUTURE RESEARCH

Regional perfusion (RP) of the liver is used in-vivo, prior to organ retrieval, and act as a bridge between asystole and retrieval, thus limiting WIT, and mitigating ischemia. Moreover, it prevents the depletion of mitochondrial ATP stores, favoring aerobic metabolism, and acting as an ischemia pre-conditioning period^[91-93].

Future research is needed to focus on synergistic liver perfusion modalities such as RP extracorporeal oxygenation, followed by NMP.

The NMP system could also benefit from optimiza-

Table 2 Hypothermic vs normothermic machine perfusion of liver grafts

| Hypothermic machine perfusion HMP | Normothermic machine perfusion NMP |
|--|--|
| Temperature 0 °C-4 °C Logistically easier Modest resumption of energy production with low perfusion rate Improves the state of mitochondria during preservation Performed at sub-physiologic pressures ^[107] Requires low perfusion rates ^[108] No requirement for a specific oxygen carrier in the perfusate as demand for O ₂ is low ^[108] Less occurrence of graft infection considering the hypothermic state More tendency for endothelial cell, kupffer cell, and macrophage cell damage due to shear stress and hypothermic activation ^[110-113] When compared to SCS it decreases inflammatory cytokines but no difference in graft or patient survival was found ^[77,114] May help protect marginal livers by converting PNF into allograft dysfunction ^[71] | Temperature 37 °C Logistically demanding Recreates the physiological milieu by maintenance of normal temperature Performed at physiological pressures ^[70,82] Requires high perfusion rates ^[108] Oxygen is provided by using blood, modified hemoglobin, or using a high oxygen tension in special preservation solutions ^[70,82,84,88,109] Reduces IRI Provides nutrients (glucose, amino acids, <i>etc.</i>), medications to prevent micro-circulatory failure (<i>e.g.</i> , prostacyclin, heparin, antibiotics), and oxygen Allows the assessment of organ viability (<i>e.g.</i> , Galactose elimination, factor V production, bile flow) May allow the use of gene therapy prior to transplantation, to reduce the risk of rejection, or decrease the ischemia-reperfusion injury ^[115-117] |

HMP: Hypothermic machine perfusion; SCS: Static cold storage; NMP: Normothermic perfusion.

tion in terms of portability. In its current form, NMP-dependent techniques cannot avoid a period of SCS prior to perfusion. Experimental data suggest that prolonged cold ischemia of the organ before attachment to the *ex-vivo* perfusion system could impair the protective effects^[86,94].

Another area to explore is liver assessment methods during *ex-vivo* perfusion to predict function and viability of DCD liver grafts. If clinical validation of such parameters could be established, the procurement team would be able to determine suboptimal grafts without putting the recipient at risk with the liver transplant procedure. Alternatively, optimal DCD grafts would avoid unjustified rejection, therefore adding more livers to the donor pool^[87].

NMP may also provide ground for pre-transplantation gene therapy of donor grafts. Cypel *et al*^[95] investigated this method with an adenoviral vector encoding human interleukin-10 (AdhIL-10) to repair injured donor lungs *ex-vivo* (through NMP) before transplantation. In their study, AdhIL-10-treated lungs showed significant improvement in function when compared to controls, a favorable shift from proinflammatory to anti-inflammatory cytokine expression, and recovery of alveolar-blood barrier integrity^[95]. The range of potential targets for gene therapy prior to transplantation includes recruitment of heat shock proteins (some of which have been shown to protect against IRI^[96]), modulation of co-stimulatory and apoptosis pathways, amelioration of immunologic profile to prevent rejection, and manipulation of leukocyte recruitment^[87,97].

CONCLUSION

The scarcity of donor livers has resulted in an increased interest in extended criteria donors (ECD), and more specifically DCD grafts, as a potential source to signifi-

cantly expand the donor pool. Initially, this pool provided disappointing results as it was associated with high incidence of ITBS, HAT, and PGF, however, outcome has improved with better donor selection and pre-transplant treatment. Future application of machine perfusion modalities might allow graft assessment and repair, resulting in more extensive use of DCD liver grafts and provide ground for pre-transplantation conditioning of organs.

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Assessment of platelet function: Laboratory and point-of-care methods

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platelet-rich plasma are traditionally utilized to aid in the diagnosis and management of patients with platelet and hemostatic disorders and used as diagnostic tools both in bleeding and thrombotic diathesis in specialized laboratories. Now, new and renewed automated systems have been introduced to provide a simple, rapid assessment of platelet function including point of care methods. These new methodologies are also suitable for being used in non-specialized laboratories and in critical area for assessing platelet function in whole blood without the requirement of sample processing. Some of these methods are also beginning to be incorporated into routine clinical use and can be utilized as not only as first panel for the diagnosis of platelet dysfunction, but also for monitoring anti-platelet therapy and to potentially assess risk of both bleeding and/or thrombosis.

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Key words: Platelets; Method; Test; Point of care testing; Laboratory assessment; Bleeding; Thrombosis; Platelet function

Abstract

In the event of blood vessel damage, human platelets are promptly recruited on the site of injury and, after their adhesion, activation and aggregation, prevent blood loss with the formation of a clot. The consequence of abnormal regulation can be either hemorrhage or the development of thrombosis. Qualitative and/or quantitative defects in platelets promote bleeding, whereas the residual reactivity of platelets, despite antiplatelet therapies, play an important role in promoting arterial thrombotic complications. Platelet function is traditionally assessed to investigate the origin of a bleeding syndrome, to predict the risk of bleeding prior surgery or during pregnancy or to monitor the efficacy of antiplatelet therapy in thrombotic syndromes that, now, can be considered a new discipline. "Old" platelet function laboratory tests such as the evaluation of bleeding time and the platelet aggregation analysis in

Core tip: This review discussed the scenario of available platelet function laboratory and point-of-care methods suitable in different clinical setting. As this matter has become of crucial importance in the bleeding management and for monitoring antiplatelet therapies, improved ability to assess platelet function in a timely and efficient manner is essential. Traditional platelet function methods, requiring a fair degree of expertise, have been limited to specialized laboratory. Many efforts have been carried out for improving platelet function assays for centralized laboratory, such as different point-of-care testing methodologies have been developed. Moreover, different guidelines and recommendations for their method standardization are growing.

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INTRODUCTION

Platelets are multifunctional cells that play a role in many pathophysiological processes including haemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defense and tumor growth/metastasis^[1,2]. Bizzozzero in the late 1800s first described platelets, identifying them as distinct cells and observing that aggregated platelets form thrombi into damaged parts of vessel^[3].

Notwithstanding the multiple roles of platelets, the available platelet tests investigate those functions directly involved in haemostasis^[4,5]. The fine relationships between platelets and the vessel wall, *i.e.*, the primary haemostasis, is the first phase of haemostatic process. At the injury of a vessel wall platelets are involved in sequential functional responses including adhesion, spreading, shape change, aggregation, release reaction, exposure of a procoagulant surface and clot retraction. The progression of these different steps conveys the activated platelets rapidly to form a hemostatic plug that occludes the site of lesion to prevent blood loss^[6]. If one of these functions and/or platelet number are defective, then hemostasis is impaired and an associated increased risk of bleeding could be also present. On the other hand, an increase in platelet count or reactivity may lead to unsuitable thrombus formation. Upon and within atherosclerotic lesions platelets adhere, aggregate with the development of arterial thrombi that may result in stroke and myocardial infarction, two of the major causes of morbidity and mortality in the western world^[7]. The prevention of arterial thrombotic complications, the antiplatelet therapy and its monitoring, can be beneficial, but their management should be carefully conducted without increasing the risk of bleeding^[1,8-11].

To date, platelet function testing has been used to identify the possible causes of bleeding^[12] to monitor pro-haemostatic therapy in patients at high risk of bleeding and to verify normal platelet function prior and during surgery^[13,14]. Recently, different methodologies have progressively developed for monitoring the response to antiplatelet therapy and for the identification of patients with residual platelet reactivity at risk of thrombotic complications^[15-18].

Development of platelet function testing

At the beginning of 1900s the bleeding time (BT) by Duke procedure^[19], was the first test for evaluating the capacity of platelets to form a plug. For long time, this test has been considered a useful screening test to identify both congenital or acquired platelet disorders^[20].

The cornerstone for the diagnosis of platelet function

was the platelet aggregation in platelet-rich plasma (PRP) according to Born's studies^[21]. This method measures the capacity of platelet to aggregate to each other in response to external aggregating agents-agonists, *i.e.*, adenosine-diphosphate (ADP), arachidonic acid (AA), collagen, epinephrine (EPI) and others^[22]. Since the late 1980s new laboratory tests of platelet function have become available, such as flow cytometry as well as the evaluation of platelet nucleotides^[23].

Because platelet dysfunction may be due to a wide multiplicity of defects, to diagnose an affected platelet function is difficult and there are no pivotal screening tests. The current laboratory assessment of platelet defects usually investigates platelet adhesion/aggregation and/or measurement of granule content/release. However, these tests are labor intensive, costly, time consuming and require a fair degree of expertise and experience. These problems have mainly limited their extensive clinic use. Actually, these methodologies are available only in specialized clinical laboratories dedicated to the studies of pathophysiological processes including haemostasis and thrombosis. As the evaluation of platelet function has become of crucial importance in the management of severe bleeding, improved ability to assess platelet function in a timely and efficient manner is essential. During the last two decades, different point of care testing (POCT) instruments for the assessment of platelet function at the bedside of patients at high risk of bleeding or thrombotic complications have been developed. Now, simple platelet function tests on whole blood (WB), that may be employed as POCT at bedside or within non-specialized laboratories, have been proposed^[24-28]. In Table 1 the different laboratory and point-of-care assays for the evaluation of platelet function are reported; in Table 2 advantages and disadvantages of these methodologies are indicated and in Table 3 the clinical value of the principal platelet tests is specified.

This report attempts to focus on the scenario of available platelet function POCT with the pertinent instrumentation more suitable for the use in different clinical setting of critical area such for the diagnosis of inherited and acquired bleeding disorders or for monitoring residual platelet reactivity of patients on antiplatelet treatment.

PLATELET FUNCTION LABORATORY TESTING

Bleeding time

The skin Bleeding time (BT) is the oldest test for assessing *in vivo* primary haemostasis^[20]. BT assesses the capacity of platelets to form a haemostatic plug. The time, that the platelets employ to occlude an *in vivo* skin wound, is recorded by evaluating the ability of platelets to stop the bleeding^[29]. BT still remains a useful test to identify both congenital and acquired disorders of primary haemostasis in those laboratories that don't perform other platelet function tests. The technique is easy and quick to per-

Table 1 Laboratory and point-of-care assays for evaluation of platelet function

| Platelet function tests | Principle of method | Application of the methods |
|---|---|---|
| Platelet adhesion studies Bleeding time | <i>In vivo</i> stopping of blood flow | Screening test of platelet function on defects of primary hemostasis |
| Platelet Function Analyzer - PFA-100/InnovancePFA-200 | <i>In vitro</i> stopping of high shear blood flow by platelet plug in whole blood | Assessment of bleeding risk, thrombotic risk, drug effects Sensitive to severe platelet dysfunctions Detection of VWD |
| Impact Cone and Plate(let) analyzer | Shear-induced platelet adhesion/aggregation onto surface in whole blood | Screening of congenital primary hemostasis abnormalities Evaluation of platelet response to aspirin and clopidogrel (scarce data). |
| Platelet-To-Platelet Aggregation Studies Light transmission platelet aggregation | Low shear platelet-to-platelet aggregation in response to agonists in platelet-rich-plasma | Screening test for bleeding behavior Diagnostic for platelet surface glycoprotein defects Monitoring of the platelet response to antiplatelet agents |
| Impedance platelet aggregation | Low shear platelet-to-platelet aggregation in response to agonists in whole blood | Screening test for bleeding behavior Diagnostic for platelet surface glycoprotein defects Monitoring of the platelet response to antiplatelet agents |
| VerifyNow system | Fibrinogen-platelet agglutination in response to agonist in whole blood | Monitoring of the platelet response to antiplatelet agents |
| Plateletworks | Platelet counting pre- and post-activation in whole blood | Monitoring of the platelet response to antiplatelet agents |
| Analysis of Clot Formation Thromboelastography/ Thromboelastometry | Monitoring of rate and quality of clot formation in whole blood based on viscoelastic blood changes | Assessment of global haemostasis Possible definition of different platelet and clotting abnormalities Diagnosis and treatment of bleeding after cardiac surgery, liver transplantation, trauma and PPH |
| Platelet function tests to investigate platelet activation Flow cytometry | Cell counting, cell sorting, biomarker detection and protein engineering laser-based detection of suspending fluorescent label platelets in a stream of fluid | Expression of platelet specific surface and/or cytoplasmatic markers; VASP phosphorylation state ¹ (Monitoring of CD41/61, CD42, CD62P, etc. Activation markers directly dependent on thienopyridine target) |
| Radio- or Enzyme Linked-Immune Assays: Soluble markers determination ¹ | Ligand binding assays | Measurement of Beta-thromboglobulin, PF4, GPV, Soluble P-Selectin, Thromboxanes |

¹Not planned in this report. GP: Glycoprotein; PPH: Post-partum hemorrhage; VASP: Vasodilator-stimulated phosphoprotein; VWD: Von Willebrand Disease.

form without any WB processing; but it can be affected by an inaccurate operator managing and by skin thickness and temperature. Notwithstanding, BT was fulfilled by the use of an available device to standardize the size and the depth of cut, a lack of precision and uncertain correlation with clinical patient state remain. No study has clearly established the ability of BT evaluation to predict the risk of bleeding in patients^[30] and only a study reported that BT could predict clinical bleeding in patients with acute myocardial infarction undergoing thrombolytic therapy^[31]. Moreover, this test is not used routinely to monitor the effect of antiplatelet therapy^[32].

Platelet aggregation on platelet-rich plasma

The Light Transmission Aggregometry (LTA), method performed on PRP and developed in the 1960s^[21,33], is still considered as the gold standard test for investigat-

ing platelet functions. This analysis measures *in vitro* the platelet-to-platelet aggregation in a glycoprotein(GP) II b III a-dependent manner, the most important function of platelets. PRP and platelet poor plasma (PPP), obtained after opportune centrifugation of citrated blood samples, are used to perform LTA. The addition of an agonist to optically dense PRP, promotes platelet aggregation resulting in an increase of brightness of plasma sample. The aggregometer records the rate and extent percentage of increase in light transmission from 0% (maximal optical density of PRP) to 100% (no optical density of autologous PPP) by a photometer. Multi-channel easy to use aggregometers are available to achieve platelet aggregation tests including automatic setting of 100% (PPP) and 0% (PRP) baselines of light transmission, computer aid and storage of results and disposable stirring bar-preloaded cuvettes. Different agonists can be added to PRP sample

Table 2 Advantages and disadvantages of different platelet function methodologies

| Platelet tests | Advantages | Disadvantages |
|--|---|--|
| Bleeding time | Physiological <i>In vivo</i> test Easy, quick No WB processing | Operator dependent Invasive Poorly standardized Dependent on different variables (skin thickness, t°C) |
| Light transmission platelet aggregation in PRP | Historical gold standard Flexible Diagnostic method Different agonists available Sensitive for anti-plt therapy | Pre- and analytic variables Time-consuming High sample volume Sample preparation |
| WB Impedance Platelet Aggregometry | No sample preparation Flexible Diagnostic method Different agonists available Sensitive for anti-plt therapy | Limited HCT and platelet count range |
| Flow Cytometry | Close to POCT (Multiple system) Small blood volumes Diagnosis <i>ex vivo</i> of platelet activation Evaluation of efficacy of thienopyridyne therapy | Expensive Specialized equipment Experienced operator Careful sample processing Probable, possible artifacts Nonflexible Platelet count- HCT-dependent Not sensitivity for platelet secretion defects. |
| Platelet Function Analyzer -PFA-100 /Innovance PFA-200 | <i>In vitro</i> standardized BT POCT Easy, quick Sensitive to severe platelet dysfunctions | |
| VerifyNow system | POCT WB assay Easy, quick No WB processing | Expensive Nonflexible Monitoring antiplatelet therapy only Limited HCT and platelet count |
| Impact Cone and Plate(let) analyzer | WB assay Global platelet function Small sample volume | Expensive Experienced staff Lacking of clinical studies Not widely available |
| Plateletworks | POC WB system Minimal sample preparation Easy, rapid screening test | Indirect assay Required platelet count method Not so well studied |
| Viscoelastic methods | POCT Global hemostasis test Anticoagulation monitoring Predicts bleeding Reduces blood transfusions Improve clinical outcome | Measure clot properties Depend on: platelet function, coagulation and fibrinolysis factors More studies are needed |

HCT: Hematocrit; plt: Platelet; POCT: Point-of-Care Testing; PRP: Platelet-rich-plasma; WB: Whole blood.

in order to obtain information about many different aspects of platelet function. Different parameters can be obtained from the evaluation of the aggregation trace: lag phase, shape change, primary and secondary aggregation, slope, and the maximal aggregation (%) at a fixed time.

However, despite the widespread use of LTA test, it is poorly standardized and variation between laboratory practice has been evidenced^[34,35]. Because LTA is recognized to be the most important and common assay that clinical laboratories can perform to diagnose platelet function disorders, its procedure is constantly substantiated by an ongoing standardization process. Recently, specific guidelines for LTA that want to stabilize/normalize the correct procedure, have been published^[36-39].

Concisely, these guidelines discuss the possible problematic pre-analytical, analytical and post-examination aspects of LTA, in order to guide toward an accepted, agreed and standardized procedure. Regarding some

principal pre-analytic aspects, a complete record of medication taken by patients should be done prior the blood sampling. Blood withdrawal should be atraumatically performed with the use of 19 and 21 gauge needles. Evacuated tube systems are accepted and the anticoagulant recommended is the buffered trisodium citrate at the concentration of 109 mmol/L (described as 3.2%). Also the Anticoagulant-Citrate-Dextrose solution (formula) A (ACD-A), that maintains the pH at 7.2 may be used. The citrated blood specimens must be gently mixed, maintained at room temperature (RT) and softly, but rapidly, transferred to the laboratory. Samples should be tested no more than 4 h from withdrawals. Regarding some principal analytic aspects, the PRP should be obtained by centrifugation at RT at 170-200 g for 10 min, whereas the autologous PPP may be prepared by centrifugation (after removal of PRP or using whole samples) at 1500 g for at least 15 min at RT. The adjustment of platelet

Table 3 Major platelet function tests: Clinical value

| Platelet tests | Clinical value | Ref. |
|---|---|--|
| Light transmission platelet aggregation | Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (3) detection of VWD (RIPA test); (4) diagnostic for platelet surface glycoprotein defects. | Moffat <i>et al</i> ^[34] Hayward <i>et al</i> ^[41] Gadisseur <i>et al</i> ^[43] Breet <i>et al</i> ^[44] Buonamici <i>et al</i> ^[45] Panicia <i>et al</i> ^[51,65] Gum <i>et al</i> ^[63] Rechner ^[107] |
| Whole blood platelet aggregation | Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (3) acquired bleeding risk: antiplatelet therapy, surgical coagulopathy; (4) detection of VWD (RIPA test); (5) diagnostic for HIT. | Panicia <i>et al</i> ^[72] Panicia <i>et al</i> ^[73] Sibbing <i>et al</i> ^[74] Sibbing <i>et al</i> ^[75] Würtz <i>et al</i> ^[77] Bolliger <i>et al</i> ^[78] Morel-Kopp <i>et al</i> ^[79] Ranucci <i>et al</i> ^[81] Görlinger <i>et al</i> ^[84] Hayward <i>et al</i> ^[25] |
| PFA-100 Innovance PFA-200 | Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) detection of VWD; (3) acquired bleeding risk: anti-plt therapy, surgical coagulopathy; (4) thrombotic risk also in relation to potential failure of anti-plt therapy; (5) platelet function in pregnancy, kidney or liver disease. | Favaloro ^[94] Koessler <i>et al</i> ^[92] Marcucci <i>et al</i> ^[103] Reny <i>et al</i> ^[104] Crescente <i>et al</i> ^[105] Raman <i>et al</i> ^[108] Cammerer <i>et al</i> ^[109] Chauleur <i>et al</i> ^[113] |
| VerifyNow system | Assessment of: (1) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (2) low platelet reactivity of patients on antiplatelet treatment to stratify risk of bleeding events (scarce clinical data). | Breet <i>et al</i> ^[44] Panicia <i>et al</i> ^[51,65] Tantry <i>et al</i> ^[116] Marcucci <i>et al</i> ^[119] Price <i>et al</i> ^[120] Angiolillo <i>et al</i> ^[121] |

HIT: Heparin-Induced Thrombocytopenia; plt: Platelet; RIPA: Ristocetin-Induced Platelet Aggregation; VWD: Von Willebrand Disease.

count of PRP is still matter of debate. The need of adjustment of PRP with autologous PPP occurs in general for standardizing the platelet count between 200 and 300×10^9 platelets/L and in particular for lowering the platelet count for matching it with that of a thrombocytopenic patient^[36]. Previous in house reference intervals (RI) for the % maximal aggregation response specific for each concentration of agonist used must be established on healthy adult volunteers (these RI can be applied to children older than neonates). LTA tracings should be studied and the final interpretative comment shall be organized by a laboratory physician. The principal agonists are commonly used at the following recommended final concentrations: ADP, 2.0-10 $\mu\text{mol/L}$; arachidonic acid, 0.5-1.64 mmol/L (usually 1.0 mmol/L); collagen, 1-5 $\mu\text{g/mL}$ (typically 2 $\mu\text{g/mL}$); epinephrine, 5-10 $\mu\text{mol/L}$ (typically 5.0 $\mu\text{mol/L}$); ristocetin, 0.5-0.6 mg/mL at low concentration and 1.2-1.5 mg/mL at high concentration.

To date, platelet aggregometry is still the most widely used method for identifying and diagnosing platelet function disorders or for monitoring antiplatelet therapies. Actually, this analysis is considered the first panel test to study hemorrhagic patient with inherited or acquired platelet dysfunctions^[37,40-43]. When congenital/acquired bleeding disorders are suspected, apart from the most

commonly agonists ADP, AA and collagen - used principally for monitoring antiplatelet therapies - other agonists should be also used: ristocetin, epinephrine, thrombin receptor activating peptide (TRAP), thromboxane A2 mimetic U46619, calcium ionophore A23187.

Monitoring antiplatelet therapies by using LTA allows to predict major adverse cardiovascular events (MACE) in cardiovascular patients at high risk. The rate of residual platelet reactivity defined by ADP-, AA-LTA or both has been associated with the development of ischemic events both in ACS patients and in those with stable coronary artery disease^[44-49].

ADP agonist is generally used to investigate congenital/acquired bleeding disorders by LTA^[22]. In the presence of different platelet alteration, ADP induced platelet aggregation may result reduced (P2Y12 defects, storage pool deficiency of α and δ granules, and defects of α granules) or severely impaired (Glanzmann's thrombasthenia)^[42,50]. ADP at high concentrations (*i.e.*, $\geq 10 \mu\text{mol/L}$) is used to monitor thienopyridines effect: ticlopidine, clopidogrel, prasugrel and ticagrelor act through the P2Y12 ADP receptor causing selective inhibition of responses to ADP^[44,45,51-55]. For the classification of patients responsive or not to clopidogrel therapy, a collectively shared cut-off value of 70% for 10 $\mu\text{mol/L}$ ADP-in-

duced maximal extent aggregation was found^[45,51,56,57]. AA is the agonist of choice to investigate the efficacy of the ASA antiplatelet therapy^[58-60]. ASA is able to inhibit platelet aggregation by irreversible inactivation of the COX-1 enzyme resulting in an inhibition of the TXA2 production^[61]. The concentrations of 1 and 1.3 mmol/L AA are usually used to monitor antiplatelet therapy and the cut-off value of 20% is used to identify patients responsive or not to ASA treatment^[62-67]. Platelet aggregation profile induced by collagen (1-5 µg/mL) is characterized by a lag phase before aggregation arises. Collagen binds to the GPVI and GP I a/ II a platelet receptors inducing granule release and TXA2 generation. Recently, RPR identified by collagen aggregation in ACS patients on ASA has been reported associated with cardiovascular events^[67,68] and with the polymorphism C807T predisposing to MACE^[69]. Collagen induced platelet aggregation can be impaired in different condition of platelet function disorders, such as: Glanzmann's thrombastenia, abnormalities of the signal-transduction pathways caused by COX-1 deficiency (aspirin like defect) or defects of platelet granules (α and/or δ storage pool deficiency)^[42]. Epinephrine (5-10 µmol/L) is a weak agonist that binds to the α_2 -adrenergic receptor on the surface of platelets leading to inhibition of adenylyclase and the release of calcium ions. Platelet aggregation induced by epinephrine is similar to that obtained with ADP and characterized by an initial primary wave of aggregation, the release of stored ADP from the platelet dense bodies and second wave sustained aggregation^[38]. ASA inhibits aggregation to any concentration of epinephrine^[67]. Impaired response to epinephrine can be present in some congenital platelet disorders such as the Wiskott-Aldrich syndrome or the Quebec platelet syndrome. Ristocetin (1.2-1.5 mg/mL) causes platelet agglutination through the Von Willebrand Factor (VWF) and GPIb-IX-V complex. In the presence of Bernard-Soulier syndrome a severely impaired platelet agglutination induced by ristocetin is present. Moreover, LTA test performed by using different concentrations of ristocetin (0.6-1.2-1.5 mg/mL), exerts an important role to analyze possible VW Disease (VWD) and to differentiate the VWD variants^[42,43].

In summary, LTA test is considered the first diagnostic step in the evaluation of platelet disorders. Since these platelet alterations are complex, in order to perform a diagnostic hypothesis, LTA results should be supported by further and more specific tests. Lumiaggregometry method for the identification of impaired platelet secretion (*i.e.*, the measurement of the platelet content of adenosine nucleotide and serotonin), flow cytometry analysis or western blotting test for the identification of expression of specific platelet component and the evaluation of deficiency of α and δ granules by electron microscopy can specifically confirm the LTA results and should be performed as second diagnostic step^[42].

Platelet aggregation on WB

Platelet aggregation on WB is achieved by impedance

platelet aggregometry, based on the principle that activated platelets expose their surface receptors which allow them to bind to artificial surfaces^[70,71]. This test measures the change in electrical resistance or impedance between two electrodes set at a fixed distance within WB sample. The platelet adhesion to electrodes and the response to classical agonists get other platelets aggregate to those stacked to the electrodes, increasing the impedance. The extent of the increase in impedance is normally recorded in Ohm. The use of WB allow to assess platelet function under more physiological conditions taking into account that also the contributions of other blood elements that may affect platelet function. In addition, another important aspect is that WB aggregometry takes place on surfaces. Platelet aggregation on WB has many advantages as well as the use of small sample volume, the immediate analysis without no sample manipulation, loss of time or possible failure of subpopulation of platelets.

Recently, a new multiple electrode aggregometry (MEA) by using a five channel computerized WB aggregometer (Multiple Platelet Function Analyzer - Dynabyte - Roche Diagnostics, Germany) equipped by disposable cuvettes ready to use with two independent sensor units and an automated pipetting has become available. The increase of impedance is detected for each sensor unit separately and calculated automatically as area under curve (AUC). By using this device with these advantages, MEA has acquired the high valence for being considered a POCT. Because MEA may use different agonists (similarly to LTA), it is suitable for diagnosis of bleeding and also for monitoring antiplatelet therapy^[72-77]. Indeed, MEA has been used to investigate the presence of VWD in patient with severe aortic stenosis^[78] and on the other hand the high thrombotic risk due to heparin induced thrombocytopenia (HIT)^[79].

In particular, MEA, beyond the identification of cardiovascular patients at risk of MACE^[72-75], is able to discriminate those patients that have a too much high inhibition of platelet function and at risk of bleeding^[80]. In the same manner, Ranucci *et al.*^[81] reported that the use of MEA before cardiac surgery allowed to identify those patient at risk of bleeding. Different reports elucidated that MEA might be able to identify preoperatively those patients at risk of blood loss after cardiac surgery^[82,83] and it is de facto entered as rapid and useful tool for the management of postoperative severe bleeding^[84]. More recently, Malek *et al.*^[85] reported that low extent of TRAP-induced platelet aggregation by using this method was a factor independently associated with intramyocardial hemorrhage of patients with myocardial infarction.

Flow cytometry platelet analysis

Platelet analysis by using flow cytometry (FC) may offer information on the functional status *in vivo* of platelets^[23]. This technique allows the evaluation of the physical and antigenic properties of platelets, *i.e.*, surface expression of receptors, bound ligands, secretion, presence of platelet aggregates and leukocyte-platelet aggregates. FC is

able to measure cell size and granularity of a large population of cells, not only the platelets and to quantify the fluorescence emitted by fluorochrome-labeled antibodies and ligands bound to the cells evaluated.

FC can be a useful tool for the diagnosis of inherited or acquired platelet dysfunctions (*i.e.*, Bernard-Soulier Syndrome or HIT, respectively). In addition, FC is able to recognize the pathological activation state of platelets (*i.e.*, in the setting of acute coronary syndromes or cardiopulmonary bypass); the efficacy of antiplatelet drugs^[86] and, finally, the state of stored platelet for the evaluation of efficacy of platelet transfusion^[87,88].

A panel of antibodies may be used to study in detail the membrane glycoprotein receptors of platelets. To count binding, antibodies may be directly conjugated with different fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE). But, also a species-specific secondary antibody coupled to a fluorochrome can be used to recognize a primary antibody linked to surface antigens^[23]. For FC both PRP and WB can be used. Prior fixation of platelets with paraformaldehyde stabilizes surface antigens and consents transport of reagent components. In the WB, the use of a double labeling binding allows the identification of platelets or mixed cell aggregates^[89,90]. The results of FC are represented in the form of histograms with mean fluorescence intensity (MFI) plotted against cell number.

PLATELET FUNCTION POINT-OF-CARE TESTING

The Platelet Function Analyser - PFA-100/ Innovance PFA-200

This POC method PFA-100/Innovance PFA-200 (Siemens, Munich, Germany) assesses platelet function in WB and has been considered the standardization of BT^[24,25,91]. The PFA-100 and the updated system Innovance PFA-200^[92] by using apposite cartridges simulates primary haemostasis under shear stress conditions. Citrated WB is drew at high shear stress rate through a defined microscopic aperture (147 μm) into a collagen-coated membrane (C) filled with either epinephrine (EPI), CEPI cartridge, or ADP, CADP cartridge. In response to shear stress and agonists platelets undergo adhesion and aggregation upon the membrane forming a platelet clot which occludes the aperture. The time taken to occlude the hole is the closure time (CT), a measure of overall platelet-related haemostasis and this interval will be prolonged depending on the platelet activity. The use of two different cartridges with distinct agonists allows to distinguish the platelet function alterations due to intrinsic defects (principally by using CADP cartridge) or to antiplatelet therapy with ASA (CEPI cartridge)^[93-97], whereas the new Innovance cartridge is affected by thienopyridine therapy^[98]. In comparison to BT test, this method is revealed more sensitive^[27,46] especially for diagnosis of VWD and platelet function defects^[25].

The PFA-100 is sensitive to many variables that in-

fluence platelet function as well as low platelet count and haematocrit. Thus, to exclude thrombocytopenia or anemia, a WB count should always be performed prior test. In addition, it has been demonstrated that different determinants such as high levels of VWF, fibrinogen or erythrocytes tend to shorten CEPI CT^[99,100]. Moreover, the PFA CT by CEPI cartridge could reveal high residual platelet reactivity despite aspirin therapy, and consequently predict the risk of ischemic events^[101-103]. In ACS patients on ASA treatment, a high concordance between LTA and PFA-100 CEPI test results and a significant negative predictive value for the PFA system have been reported^[65]. In addition, PFA CEPI shortened CT was demonstrated to be significant and independent predictor of MACEs in patients with AMI undergoing primary PCI^[103-105].

Assessment of platelet dysfunction with PFA-100 in different clinical setting or in patients undergoing different kinds of elective surgeries, may provide useful information for postoperative blood transfusion management^[106,107]. Especially in cardiac surgery PFA methodology showed a high predictive value of platelet function for management of intra- and postoperative blood loss^[108-111]. In patients with biventricular assist device implantation on treatment with clopidogrel, the strict monitoring of impaired platelet function with this method (by using CADP cartridge) allowed them to go under successful transplantation with no major blood loss^[112]. Prolonged CTs by CADP assay were found to be independent risk factors for post-partum hemorrhage (PPH) severity^[113] and prolonged CTs by CADP cartridge have been consistently described to be correlated in women with menorrhagia^[114]. The pre-surgical correction of the prolonged PFA-100 CT with DDAVP treatment, allowed to maintain the number of postoperative blood transfusions not significantly different from that of patients with normal presurgical PFA CT^[106].

It has been suggested that PFA system could be used as a screening tool that could be integrated into a panel of existing tests^[38,42]. In particular, it is reported that this test presents a high negative predictive value^[63,96]: so, in the presence of normal CT in a suspected platelet defect, further detailed analysis, *e.g.*, platelet aggregation, might be eliminated from the investigation^[5].

VerifyNow system

The VerifyNow system (ITC, Edison, NJ, United States) is a POC turbidimetric-based optical detection device that measures platelet aggregation in a system cartridge containing fibrinogen-coated beads and specific agonist^[115]. The instrument measures changes in light transmission and thus records the rate of aggregation in WB. This methodology originally was developed for monitoring antiplatelet therapy with to GP II b/IIIa antagonists. Now, the system provides other 2 different assays each sensitive to targeted drugs: Aspirin Assay with AA as agonist (sensitive to ASA) and P2Y12 Assay with ADP as agonist and PGE₁ as suppressor of intracellular free calcium lev-

els to reduce the non-specific contribution of the ADP-binding to P2Y1 receptors (sensitive to thienopyridines). The VerifyNow system allows a rapid assessment of the platelet function also without the requirement of a specialized laboratory. Since the VerifyNow is a cartridge-based WB assay, it is not necessary to perform tests with any blood manipulation and instrument handling. Actually, this methodology is so waived that it is largely used to monitor antiplatelet therapies^[116].

For Aspirin Assay, results are expressed as Aspirin Reaction Units (ARU) and for the identification of responsiveness to ASA treatment a specific cut-off value of 550 ARU is recommended by manufacturers^[117]. In stroke patients on low dose of ASA^[96] and in coronary artery disease patients on dual antiplatelet therapy^[65,46,118] a moderate agreement between VerifyNow system and LTA results was observed. For the VerifyNow P2Y12 assay results are expressed as P2Y12 Reaction Units (PRU). Different laboratory and clinical studies relative to patient on different thienopyridines have tried to choose a cut-off value for discriminating patients not responsive to drug^[44,51,119-122].

The potential role of this system for prediction of postoperative bleeding in surgical practice remains placed for the evaluation of the extent of inhibition of platelet function in response to antiplatelet medication^[123]. Actually, by using this system antiplatelet therapy for outpatients or patients immediately after surgery could be tailored to the individual depending on the results.

Plateletworks

Plateletworks system is a POC assay based on platelet aggregation on WB. This system consists of the Plateletworks aggregation kits and the Ichor blood counter (Helena Laboratories, Beaumont, TX, United States). The Plateletworks procedure compares the platelet count measured in the control sample (EDTA tube) with those obtained after aggregation in citrate blood with either collagen, ADP or AA (citrate tube plus agonist). Platelet aggregation is measured as the decrease of platelet count. Results are available in minutes and without any manipulation of blood sample^[124]. This method has showed a relationship with LTA, VerifyNow system and Thromboelastography^[125,126] and may be used to monitor antiplatelet therapy^[127]. Plateletworks gives information about both platelet count and function within an acute care situation. However, it is still under consideration and has not reported to predict clinical outcomes.

IMPACT Cone And Plate(Let) Analyzer

IMPACT (Image Analysis Monitoring Platelet Adhesion Cone and Plate Technology) Cone and Plate(let) Analyzer (CPA) (DiaMed, Cressier, Switzerland) is a new POC completely automated system that evaluates platelet function simulating *in vitro* primary haemostasis^[4,27,28,128]. Citrated WB is exposed to shear stress by the spinning of a cone in a standardized polystyrene plate. After automated staining, the percentage of the well surface covered by

platelet aggregates -representing platelet adhesion - and the average size of the aggregates (per μm^2) - representing platelet aggregation - are measured by image analysis software.

This system is highly dependent on plasma VWF, fibrinogen binding the platelet glycoproteins GPIb and GP II b/IIIa to the plastic surface. Therefore, this instrument methodology should be a reliable device for the diagnosis of platelet defects. Moreover, the addition of the agonists AA and ADP in the system allows to monitor dual antiplatelet therapy^[128-130]. This system still needs an experienced use and additional studies must be conducted for assessing its possible role for monitoring inherited or acquired platelet dysfunctions.

Viscoelastic methods

These methods are global tests for the assessment of haemostatic process, based on the measurements of changes in viscoelastic forces in WB. These analyses are able to assess the extent of platelet count and function, clotting and fibrinolytic activation^[131,132]. To date, three principal systems are available: Thromboelastography, performed on "old" renewed devices (TEG, Haemoscope, Niles, IL, United States), Thromboelastometry, formerly called Rotational Thromboelastography, performed on a new device (ROTEM, TEM Int., Munich, Germany) and Sonoclot analysis performed on a new device (Sonoclot Signature, Sienco, Boulder, CO, United States). All these systems providing a graphic representation of clot formation and lysis, are now used as a bedside monitor in different clinical setting such as cardiac surgery, liver transplantation and trauma center^[133,134]. For TEG and ROTEM, in a rotating system consisting of a pin suspended by a torsion wire in a cup the WB clot entraps the pin promoting a motion that increases as the clot strengthens and decreases when the clot lyses. In Sonoclot device in the cup the pin is moved up and down at ultrasonic rate.

Different studies^[135-142] reported these systems to be predictive of risk of increased postoperative bleeding. Other reports have stated the use of different parameters provided by these tests are predictors of both postoperative bleeding and blood product use^[143-145].

Thrombelastograph Platelet Mapping System has been developed to monitor antiplatelet therapy^[146-149]. A weak clot is formed by the addition of reptilase and factor XIII, by adding AA or ADP the clot strength is increased allowing this assay to be sensitive to dual antiplatelet therapy.

However, further large prospective studies should be performed in order to define the possible role of these devices in monitoring antiplatelet therapy.

FUTURE PERSPECTIVES

As reported in this review, several *in vitro* tests for the assessment of platelet (dys)function in order to screen different idiopathic or acquired pathological conditions

-hemorrhagic and/or prothrombotic status - have been developed. Now, platelet testing is mostly used thanks also to the recent and constant standardization effort. These available tests allow to study global platelet function including the different steps of platelet activation. For example, the POC platelet tests simultaneously evaluate *in vitro* platelet adhesion and aggregation; platelet aggregometry in PRP and in WB (by using the new Multiplate system) is a comprehensive examination of platelet secretion and aggregation phenomena, also considering the role of other blood cells (platelet aggregation in WB); viscoelastic methods analyze the global hemostasis with the regard of clot retraction (Tables 2 and 3).

To date, platelet function tests are available to address the different phases of platelet activation. Platelet assays, evaluating platelet adhesion under static or flow conditions and platelet spreading have been developed^[107,150]. Platelet adhesion tests in static condition, using a large number of different surfaces - glass beads, cultured vascular cells, purified matrix proteins or complete subendothelial extracellular matrix from cultured endothelial cells - that goes to the detriment of univocal results and standardized procedure, might be achieved^[151]. Under flow conditions, platelets adhesion is affected by rheological conditions such as shear rate, presence of red blood cells, red blood cell deformability, and viscosity of the medium. In this multitude of conditions, platelet adhesion can be evaluated by using microfluidic devices for example biochip containing several different adhesion molecules^[152,153]. Platelet spreading tests, using fluorescence microscopy or scanning electron microscopy are frequently employed^[154]. Platelet secretion may be evaluated measuring the concentration of several compound released - nucleotides (ATP, ADP), serotonin (5-HT), Platelet Factor 4 (PF4), beta-thromboglobulin, thrombospondin-1) by using different methodologies such as: ELISA, HPLC, fluorescence microscopy or flow cytometry^[42,155,156]. The assessment of these distinct steps - platelet adhesion, secretion and interactions with circulating cells - might be helpful to better define pathological conditions related to different platelet dysfunction. However, most of these assays, prevalently aimed for research studies show different clinical impact and methodological challenges. Main limitations of the application of these assays in clinical practice are the scarcity of clinical and laboratory data, often divergent each other, and the lack of clear indications or guidelines for a correct use of such tests. In the future, specific, standardized, more rapid and easy tests - whose clinical value has been well defined - for the study of single steps of platelet function or for the definition of clinical value of new platelet biomarkers by using new tests showing high sensitivity and specificity, are desirable for routinely laboratory analysis.

New potential biomarkers of platelet activation

Recent studies have shown that the interaction of activated platelets with CD34+ cells might potentially contribute in the differentiation of CD34+ cells to endothelial

progenitor cells (EPCs)^[157] and mature endothelial cells (EC)^[158]. The identification of cellular mediators, tissue specific chemokines, factors and molecular determinants involved in this interactions could be useful to identify new strategies for the vascular repair and tissue regeneration in ischemic organs^[159,160].

In this contest, the chemokine CXCL12 (stromal cell-derived factor-1 α , SDF-1 α), principally produced by platelet and stored in α granules, but also released from endothelial cells, is directly involved^[161]. The principal role of CXCL12 is related to the platelet activation accompanied with P-selectin expression and release of different platelet chemokines^[162,163]. In the site of vascular injury, CXCL12 stimulates the differentiation of CD34+ cells into EPCs and ECs, so exerting an important role in neointima formation^[157,162]. Actually, the measurement of CXCL12 and/or the rapid identification of platelet-CD34+ cell complexes in the future might be used, on hand, as assessment of a predictive biomarker of ischemic events in combination with other vascular parameters and, other hand, to early detect CD34+ cells as biomarkers for cardiovascular diseases or for tissue renewal and/or repair.

CONCLUSION

New guidelines for platelet function testing have been written in the 2011^[38] and, recently, new procedures for improving the ongoing standardization of LTA have been reported in the 2013^[39]. From the late 1980's to nowadays, the study effort, in the field of application of platelet function methods as diagnostic tool for evaluating bleeding disorders and monitoring the efficacy of antiplatelet therapies, is at this time again in progress. However, the increasing number of new POC methods for the assessment of platelet function is making possible the introduction of these tests into the routine laboratory and opening the door for the their application in different clinical settings such as inherited bleeding disorders, cardiovascular intensive care, trauma coagulopathy, liver transplantation and obstetric care for the prediction of bleeding.

To date, the improvement of reliable, advanced and innovative, but simple to use WB methodologies, that simulate primary hemostasis, is allowing to screen rapidly patients and to guide the clinicians for an appropriate diagnosis of bleeding risk or for tailoring correctly the antiplatelet therapy. Surely, the general consensus is that the *in vivo* BT should be replaced. On the other hand, the use of platelet aggregometry in PRP or WB at the light of new instruments should be implemented into routine laboratories. Similarly, some POC platelet function tests could also be, actually, used as instruments for evaluating bleeding risk, thrombotic risk and monitoring antiplatelet therapy not only at the bedside, but also in centralized or in satellite laboratories. Conversely, platelet function testing is become increasingly used in critical area outside of the specialized laboratory. Although the presence of

these new methodologies represents an important improvement, a validation procedure, the study of reliability and quality control testing of these point of care tests is becoming an increasingly important issue^[38].

In conclusion, old and new platelet function tests are now available. Many tests are beginning to prove to be useful supplements to the existing set of platelet function tests, but large prospective well designed clinical trials are necessary for defining the true applications of these tests. In the future, the developments in platelet genome and proteome may lead advances in the field of platelet function testing which may have a significant impact upon the diagnosis and management of patient affected by hemorrhagic or thrombotic defects.

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Nanotechnological approaches in diabetes treatment: A new horizon

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Abstract

Diabetes is a chronic metabolic disorder that affects millions of people worldwide and takes a heavy toll on human life. Treatment of diabetics often poses a problem in selection of the proper drug, its dose and unwanted side effects. Therefore, newer drugs with the least side effects but with highest efficiency are being relentlessly searched for. In recent years, nanotechnology has given new hope for the formulation of various drugs against a myriad of diseases, including diabetes. This review tries to give an overview of the advantages of various new drugs being used, including a wide range of nanoformulations of orthodox as well complementary and alternative medicines. Several studies and research reports based on nanotechnological approaches in the formulation of anti-diabetic drugs have pointed out the fact that research in the formulation of nanodrugs improved strategies for combating diabetes based on the plausible molecular mechanism of action of the drugs. Furthermore, attempts have also been made to delineate the optimum drug concentration and time of exposure in order to recommend a scientifically validated drug dose response in developing different therapeutic strategies. Thus, to a considerable extent, recent studies have contributed towards improving the

life expectancy and quality of life of diabetics, through both targeted orthodox medicine and complementary medicine, particularly those obtained from natural resources.

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Key words: Diabetes; Complementary and orthodox medicine(s); Orthodox anti-diabetic medicines; Nanotechnology and nanomedicine; Nanoformulation

Core tip: This review on diabetes aims to provide information available on research carried out on both traditional and modern medicine practices, highlighting some recent ones including use of nanomedicines that would hopefully be able to give patients a better quality of longer life. This review also focuses on some unresolved issues and concerns about the benefits of using plant products and nanoformulations in reducing side effects and provides convincing evidence of their ameliorative properties.

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INTRODUCTION

The onset of diabetes mellitus (DM) is marked initially by an impaired glucose tolerance that sometimes can produce severe symptoms needing immediate medical attention. Diabetes is mainly caused by dysfunction of the β cells of the pancreas. This in turn leads to decreased production of the hormone insulin and/or increased resistance to the action of insulin in the peripheral tissues^[1].

Diabetes can be categorized into two types: type 1 and

type 2. Type 1 diabetes, or juvenile-onset diabetes, develops when the body's errant immune system attacks itself and damages and destroys the pancreatic β cells that produce the blood glucose regulating hormone insulin. To survive, people with type 1 diabetes must have an exogenous delivery of insulin hormone. This form of diabetes usually strikes children and young adults, although the onset of the disease may occur at any age^[2]. In adults, type 1 diabetes accounts for about 5% of all diagnosed cases of diabetes. Risk factors for type 1 diabetes may be autoimmune, genetic or environmental. No known way to prevent type 1 diabetes exists. Several clinical trials for preventing type 1 diabetes are currently in progress or being planned^[3].

Type 2 diabetes (non-insulin-dependent diabetes mellitus; NIDDM) or adult-onset diabetes usually begins as an initial phase of progressive insulin resistance/insensitiveness, with an ensuing reduction in the ability of the pancreatic hormone to promote peripheral glucose disposal and to suppress hepatic glucose output^[4]. To compensate, the pancreas pumps out increasing amounts of insulin to normalize blood glucose levels. Over time, as long as a decade, this ever increasing production becomes unsustainable and the pancreas' ability to produce insulin declines. As a result, the blood glucose level rises and, because it is unable to enter the body's cells, it begins to appear in the urine and causes increased urination. Established risk factors for type 2 diabetes include older age, obesity, physical inactivity, stress, family history and genetic polymorphism^[5]. Type 2 DM patients need to follow a diet and exercise program to control their blood glucose levels. If this first line treatment does not control blood sugar levels effectively, an oral medication can be added to the treatment plan. In certain circumstances, patients with type 2 diabetes may also need insulin injections. Many patients also need to control their blood pressure and cholesterol levels. Type 2 diabetes accounts for about 90% to 95% of all diagnosed cases of diabetes. African Americans, Hispanic/Latino Americans, American Indians and some Asian Americans are at particularly high risk for type 2 diabetes, along with its complications, and are also being diagnosed, although still rare, as children and adolescents^[3].

Gestational diabetes is a form of glucose intolerance diagnosed during pregnancy, is more common among obese women and women with a family history of diabetes, and requires treatment to optimize maternal blood glucose levels to lessen the risk of complications in the infant^[6,7]. Other types of diabetes result from specific genetic conditions, such as maturity-onset diabetes of youth, surgery, medications, infections, pancreatic disease and other illnesses. Such types of diabetes account for 1% to 5% of all diagnosed cases.

The present review is based on several studies and research reports on possible side effects emanating from the use of orthodox medicines that justifies the search for measures to eliminate these unwanted toxic effects. These efforts have culminated in the experimental approach

towards formulation and use of nanotechnologically synthesized anti-diabetic drugs and evaluation of their adaptability and acceptability in the medical fraternity. To achieve this goal, a primary extensive literature search was made and all information related to this research area was procured through search engines like SCOPUS, PUBMED, MEDLINE, GOOGLE, *etc.*, with proper key words. Relevant information from the year 1973 through 2013 in the area of nanotechnology based design on anti-diabetic drugs were covered and incorporated briefly in this review.

Tables are provided which include some prominent study reports highlighting the bioactive constituents/major bioactive compounds found in potent anti-diabetic phytomedicines (CAMs) which could potentially be used for nanoformulations of anti-diabetic drugs in future. As this is a review work, raw data could not be provided from the original papers as such, but the source from where original data can be procured from the actual papers has been cited. This review has been divided into several subheadings for discussion of certain important aspects for ease of the readers.

ORTHODOX MEDICINAL REGIMEN

The first line treatment for diabetes is usually diet and exercise and sometimes these measures alone are sufficient to bring blood glucose levels back to the normal range. If these measures do not effectively control blood glucose levels, one or a combination of medications may be necessary to control hyperglycemia. The medications for diabetes are from various classes; each class contains one or more specific drugs. Some of these drugs are taken orally and others must be injected. Various diabetes drugs work in different ways to lower blood sugar. A drug may work by: (1) stimulating the pancreas to produce and release more insulin; (2) inhibiting the production and release of glucose from the liver; or (3) blocking the action of gastric enzymes for carbohydrate catabolism or making tissues more sensitive to insulin.

MOST FREQUENTLY USED ANTI-DIABETIC DRUGS

Several anti-diabetic drugs are being used but they often have side effects (Figure 1^[8-16]). The side effects of these drugs often preclude their use in many diabetic patients with an extremely high blood glucose level.

Insulin: The most prominent biological molecule associated with diabetes

Insulin is one such therapeutic agent that is extensively used for the treatment of both type 1 and type 2 diabetes patients.

Physiologically, insulin hormone is secreted by the islet β cells of the pancreas to lower blood glucose by stimulating the uptake of glucose into skeletal muscle and fat

| Orthodox medicine | Drawbacks | Ref. |
|--|--|--|
| <p>Sulfonylureas</p> <p><chem>Nc1ccc(cc1)S(=O)(=O)NC(=O)NCC</chem> Carbutamide</p> <p><chem>c1ccc(cc1)S(=O)(=O)NC(=O)Nc2ccccc2</chem> Glibenclamide ou Glyburide</p> | Hypoglycemia, weight gain | Kunte <i>et al.</i> ^[8] , Patel <i>et al.</i> ^[9] |
| <p>Biguanides (metformin)</p> <p><chem>CN(C)C(=N)NC(=N)N</chem></p> | Abdominal discomfort, diarrhea, nausea or vomiting, loss of appetite, metallic taste | McIntosh <i>et al.</i> ^[10] , Bolen <i>et al.</i> ^[11] |
| <p>Alpha-glucosidase inhibitors</p> <p><chem>OC[C@H]1O[C@H](O[C@@H]2[C@@H](O)[C@H](O)[C@@H](CO)O[C@H]2O)[C@H](O)[C@H](O)[C@H]1O</chem> Acarbose</p> | Abdominal pain, diarrhea and flatulence | Benalla <i>et al.</i> ^[12] , McCulloch ^[13] |
| <p>Amylin analogs</p> <p>Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Pro-Ile-Leu-Pro-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH₂</p> | Hypoglycemia, abdominal pain, headache, nausea, decrease in appetite, vomiting, diarrhea and flatulence | Jones ^[14] |
| <p>Prandin (repaglinide) stralix (nateglinide)</p> <p><chem>CC(C)[C@H]1CC[C@@H](C2=CC=CC=C2N1C(=O)CCc3ccc(OC(=O)O)cc3)C</chem> Prandin (repaglinide)</p> <p><chem>CC(C)C1CCC(CC1)C(=O)NC[C@H](C(=O)O)Cc2ccccc2</chem> stralix (nateglinide)</p> | Respiratory infections, flu-like symptoms, hypoglycemia, headache, dizziness, upset stomach, back or joint pain | Alison ^[15] |
| <p>Canagliflozin (Invokana) [inhibitor of subtype 2 sodium-glucose transport protein (SGLT2)]</p> <p><chem>OC[C@H](O)[C@H](O)[C@H](O)C(=O)c1ccc(cc1)Cc2cc3cc(ccc3s2)C4=CC=C(C=C4)F</chem> Canagliflozin</p> | Yeast infections, urinary tract infections, sudden drop in blood pressure, dizziness, fainting, heart risk in first 30 d | Chao ^[16] |

Figure 1 Frequently used anti-diabetic drugs and their side effects.

balance, thereby regulating the blood glucose level. Insulin resistance can result from mutations or post-translational modifications of the insulin receptor or insulin peptide itself or any of its downstream effector molecules^[17]. Preproinsulin, the initial precursor of insulin, at the time of its synthesis generally gets cleaved at the posttranslational process to form proinsulin which further cleaves to form insulin. Therefore, even a mild depletion in the level of secretion of insulin or mutation in the insulin

molecule or its receptor system leads to the initiation and progression of hyperglycemia. Exogenous supplemental insulin administration thus is one of the therapeutic remedies for diabetes. Insulin is classified according to how it works in the body: (1) depending on the time of onset, that is, how soon it starts working; (2) depending on the peak, that is, when it is working most effectively; and (3) depending on the duration, that is, how long it lasts in the body. For example, insulin glargine (<http://www.>

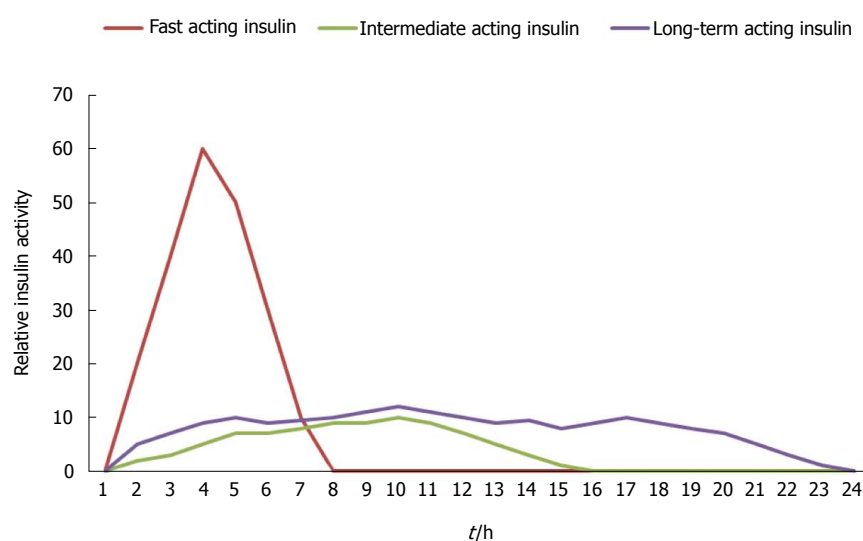


Figure 2 Diagrammatic representation of insulin activity profile.

ncbi.nlm.nih.gov/pubmedhealth/PMHT0010728/) and insulin detemir (<http://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0000962/>) are examples of long acting insulin that works slowly over a period of about 24 h. On the other hand, insulin lispro (<http://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0000957/>), marketed by Eli Lilly and Company as “Humalog”, is a fast acting insulin analogue. It was first approved for use in the United States in 1996, making it the first insulin analogue to enter the market. A literature survey also suggests that insulin aspart protamine and insulin aspart are combinations of fast-acting insulin and an intermediate-acting type of human insulin (http://en.wikipedia.org/wiki/Insulin_aspart). Diagrammatic representation of the insulin activity profile is shown in Figure 2.

Biosynthetic “human” insulin is now manufactured for widespread clinical use exploring genetic engineering techniques and using recombinant DNA technology, which the manufacturers claim reduces the presence of many impurities. Eli Lilly marketed the first such insulin, Humulin, in 1982. Humulin was the first medication produced using modern genetic engineering techniques in which actual human DNA is inserted into a host cell (*E. coli* in this case). The host cells are then allowed to grow and reproduce normally and, due to the inserted human DNA, they produce a synthetic version of human insulin.

Problems in using insulin

In spite of the extensive use of artificial insulin, several problems gradually develop with insulin as a long-term clinical treatment for diabetes. These may be the mode of administration, selecting the “right” dose and timing, selecting an appropriate insulin preparation (typically on “speed of onset and duration of action” grounds), adjusting dosage and timing to fit food intake, amounts and types, adjusting dosage and timing to fit exercise undertaken, for instance during the increased stress of illness,

variability in absorption into the bloodstream *via* subcutaneous delivery, *etc.* In fact, the dosage is non-physiological in that a subcutaneous bolus dose of insulin alone is administered instead of a combination of insulin and C-peptide being released gradually and directly into the portal vein. It is simply a nuisance for patients to inject whenever they eat carbohydrate or have a high blood glucose reading. Furthermore, it is dangerous in the case of a mistake, most especially injecting an increased dosage of insulin causing hypoglycemia, which causes a dangerous fall in blood glucose level, even threatening life.

The most common modes of application of exogenous insulin are subcutaneous injection and an insulin pump. More recently, various other modes of insulin administration include inhalation (Food and Drug Administration approved the use of Exubera, the first inhalable insulin), transdermal and intranasal (Nasulin). Oral insulin capsules have even been formulated in recent years by a biotechnology company (Oramed Pharmaceuticals Inc., Kafer Hi-Tech, based in Jerusalem, Israel), which is currently conducting Phase 2B clinical trials of its oral insulin capsule, ORMD-0801, on 30 patients diagnosed with type 2 diabetes. The technology is based on two components: (1) a chemical make-up that protects insulin during passage through the gastrointestinal tract; and (2) absorption enhancers so that insulin could be absorbed by the intestine.

A combination therapy of insulin and other anti-diabetic drugs appears to be most beneficial in diabetic patients who still have a residual insulin secretory capacity. A combination of insulin therapy and sulphonylurea is more effective than insulin alone in treating patients with type 2 diabetes after secondary failure of oral drugs, leading to better glucose profiles and/or decreased insulin needs. The mechanisms by which insulin-sulphonylurea therapy improves glycemic regulation and decreases insulin requirements involve an increase in endogenous insu-

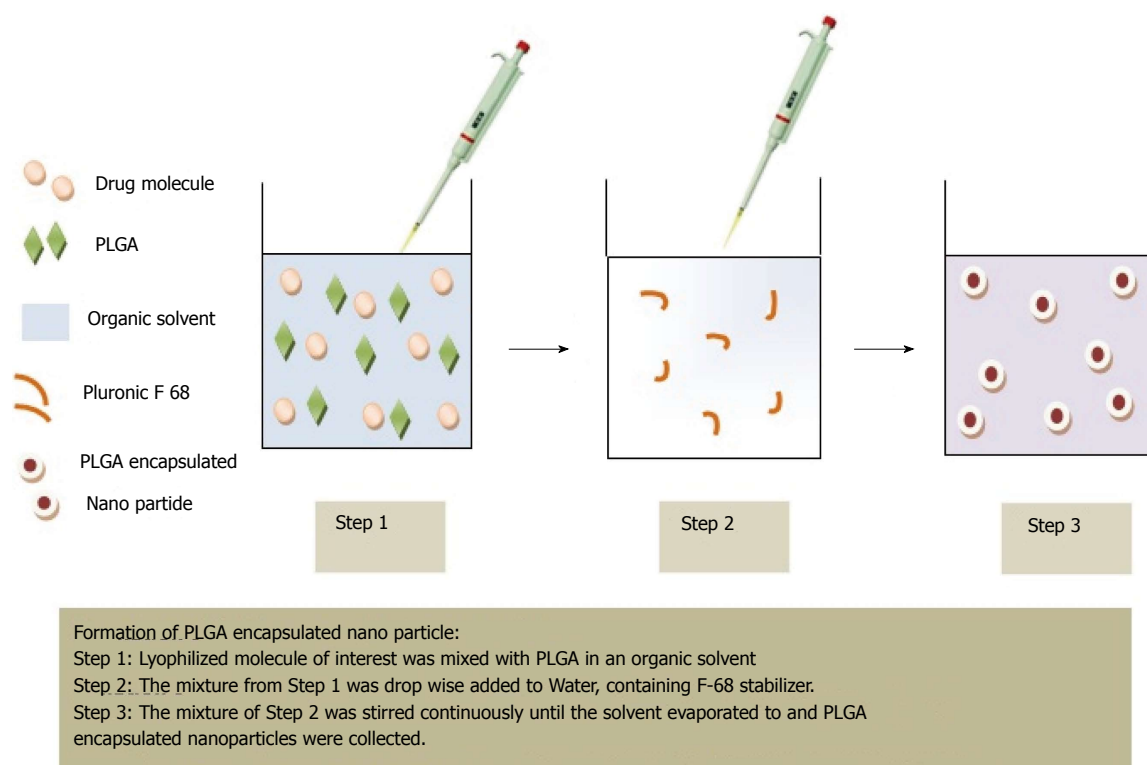


Figure 3 Steps involved during the formulation of [poly (lactide-co-glycolide)] polymers encapsulated nanoparticles. PLGA: [Poly (lactide-co-glycolide)] polymers.

lin secretion and possibly some extra-pancreatic actions of the sulfonylureas on muscle and liver^[18]. However, this combination is not widely used now for curing diabetic patients.

Use of complementary and alternative medicines in diabetes therapy

In view of undesirable side effects of orthodox therapies, a search is on to find ways to avoid some of these by using drugs that are equally effective but with no or little side effects. The results of the preclinical study could prove useful for phase 2 clinical trials in which the morbidity and mortality of DM complicated by the side effects of drug-induced hypoglycemia may be reduced by the practice of integrated medicine (CAM). Several compounds that constitute the major sources of chemical diversity, in a purified or structurally identified form with biological activities, are broadly defined as “natural products”. The products derived from these natural sources, *e.g.*, plants, animals and microorganisms, are often used in crude therapeutic formulations and serve the regimen of CAM^[19,20].

There is growing awareness of the role and practice of integrated medicine in the field of metabolic disorders, particularly in oncology^[21-25] and diabetes^[26-33], to give patients a better quality of life by alleviating some of their sufferings. This is based in part on a flood of reported scientific data about medicinal plants, including those with anti-diabetic potential, and partly on the support provided for its practice by governmental agencies and the WHO. Several ethanolic

plant extracts are now used in Ayurvedic or homeopathic formulations. The principal difference between these two modes of treatment lies primarily in the use of a lesser amount of drug in the case of homeopathy. The use of plant extract as an ingredient or component among a mixture of several other substances with medicinal properties is well accepted, even by the orthodox mode of treatment (allopathy) and also in other CAM modes. Several CAMs could interact with a wide variety of proteins and other biological targets for specific purposes, *i.e.*, they bind to a variety of protein domains and folding motifs that lead to modulating or inhibiting protein-protein interaction, thereby making these molecules behave as effective modulators of cellular processes such as immune responses, signal transduction, mitosis, apoptosis, inhibitors of apoptosis and potential anti-oxidants^[34,35]. Some of the phytochemicals have already been established as mainstream drug(s) with identified and characterized chemical ingredients for marked public use now to combat diabetes and its complications. They share high chemical diversity, biochemical specificity, molecular mass, number of chiral centers, molecular flexibility and distribution of heavy metals suitable for therapeutic applications^[36]. A list of such phytochemicals with identified biological active components is given in Table 1^[37-66]. In addition to several mechanisms to ameliorate diabetic complications, these phytochemicals also render potential anti-oxidant activities which impart an extra advantage to their diabetes attenuative properties, like increasing insulin secretion, insulin receptors in RBCs, repair and regeneration of pancreatic islets, increasing glycogen synthesis, regulating

Table 1 Bio-active constituents/ major bioactive compounds found in potent anti-diabetic phytomedicines

| CAMs | Bioactive ingredient(s) | Functions | Ref. |
|---|--|--|---|
| Ficus carica, Ficus religiosa | Ficain, Sitosterol-d-glucoside leucocyanidin 3-O-beta-d-galactosyl cellobioside, leucopelargonidin-3-O- alpha-L rhamnoside | Protease enzyme antioxidant | http://www.ficain.com/ Bnouham <i>et al</i> ^[37] , Ayodhya <i>et al</i> ^[38] |
| Nigella sativa | Thymoquinone | Reduces appetite, glucose absorption, hepatic gluconeogenesis, cholesterol, triglycerides, body weight, stimulates glucose induced secretion of insulin | Mathur <i>et al</i> ^[39] |
| Trigonella foenum-graecum | Fenugreekine, 4- hydroxyisoleucine, galactomannan | Liver detoxifier, increase insulin receptors in RBC, improve glucose utilization in peripheral tissues, stimulate insulin secretion | http://diabetes- drugsandcure.blogspot. in/2013/03/fenugreek-for- diabetes_13.html , Madar <i>et al</i> ^[40] |
| Cinnamomum cassia, Cinnamomum zeylanicum | Cinnamaldehyde, methylhydroxy chalcone polymer | Lower blood glucose, triglyceride, cholesterol, elevate plasma insulin | Jarvill-Taylor <i>et al</i> ^[41] |
| Euonymus alatus Kalanchoe pinnata, Eucommia utmoides | Quercetine | Stimulate insulin for glucose uptake, regeneration of pancreatic islet | Fang <i>et al</i> ^[42] |
| Gynura procumbens, Euonymus alatus | Kaemferol | Hypoglycemic effect | Fang <i>et al</i> ^[42] |
| Ecklonia cava | Dieckol | Inhibitor for α -glucosidase and α -amylase | Lee <i>et al</i> ^[43] |
| Tinospora cispa | Apigenin | Increase plasma insulin level | Noor ^[44] |
| Bumelia sartorum | Bassic acid | Increase insulin secretion and glycogen synthesis | Kerry <i>et al</i> ^[45] |
| Gymnema sylvestre | Gymnemic acid | Increase generation of β -cells | Ahmed <i>et al</i> ^[46] |
| Olea europaea | Hydroxytyrosol, oleuropein | Anti-oxidant, slow digestion and absorption | Al-Azzawie <i>et al</i> ^[47] , Jemai <i>et al</i> ^[48] |
| Momordica, Charantia | Momordins, oleanolic acid, glycosides | Prevent absorption of sugar | Mitra ^[49] |
| Panax ginseng | Ginsenoside 20(S)-Rg(3) | Anti-oxidant, lowers triglycerides and cholesterol | Kang <i>et al</i> ^[50] |
| Syzygium jambolanum | Morroniside | Anti-oxidant, regeneration of β cells, drug-DNA interaction, regulates signal proteins | Samadder <i>et al</i> ^[33] |
| Eugenia jambolana | 1-O-galloyl castalagin, casuarinin, alkaloid jambosine, glycoside jamboline, quercetin, betulinic acid, b- sitosterol, eugenin, ellagic, gallic acid, bergenin | Slow down diastatic conversion of starch into sugar, increase insulin secretion, inhibit insulin depletion | Ayyanar ^[51] , Morton ^[52] |
| Pterocarpus marsupium | (-)-Epicatechin | Enhance insulin release and conversion of proinsulin to insulin, strengthen and activate insulin signaling proteins, regulates glucose production through AKT and AMPK modulation | Ahmad <i>et al</i> ^[53] , Rizvi <i>et al</i> ^[54] , Cordero-Herrera <i>et al</i> ^[55] |
| Allium sativum | Allicin, n- acetylcysteine, Acetylcysteine | Antioxidant, enhance serum insulin by combining with cysteine and sparing it from SH group reactions | Mathew <i>et al</i> ^[56] , Jain <i>et al</i> ^[57] |
| Cassia fistule | Catechin | Glucose oxidizing and insulin mimetic activities | Daisy <i>et al</i> ^[58] , Kamiyama <i>et al</i> ^[59] |
| Curcuma longa | Curcumin | Prevention and treatment of diabetic encephalopathy | Kuhad <i>et al</i> ^[60] |
| Leandra lacunosa | Ursolic acid | Inhibit blood glucose level | Cunha <i>et al</i> ^[61] |
| Hemionitis arifolia | Coumarin | Stimulate β -cells to secrete insulin | Nair <i>et al</i> ^[62] , da Cunha <i>et al</i> ^[63] |
| Ajuga iva | Naringenin | Anti-oxidant, reduce lipid peroxidation | Taleb-Senouci <i>et al</i> ^[64] |
| Anoectochilus roxburghii | Kinsenoside | Repair β -cell in pancreatic islet injury | Li <i>et al</i> ^[65] |
| Coprinus comatus | Comatin | Maintain low level of glucose, improve glucose tolerance | Ding <i>et al</i> ^[66] |

CAMs: Compounds found in potent anti-diabetic phytomedicines.

action of insulin signaling proteins, *etc.*^[33,40,42,45,54,55,65]. The therapeutic potentials and efficiency of biologically active molecules are essentially dependent on the identification of their bio-target and active sites. Interestingly, some of the phytomedicines have been found to interact with double-stranded DNA, which provides us with useful information concerning the drug-nucleotide interaction; this would bear testimony to the fact that the DNA acts as the molecular target of those drugs^[33,67,68]. However, one important ques-

tion remains about the exactness of the dose of a few herbal formulations and the exact and optimum drug doses to be administered during different forms of diabetes which still need to be validated, calling for further research.

Nanotechnology: A new platform for formulating anti-diabetic drugs

Keeping pace with the discovery of modern nanosciences, where improved and advanced drugs are being

tested on biological systems, it has become necessary to seek an outlook at designing a more cell/tissue specific drug with better efficacy in a minimum dosage. Recently, considerable progress has been made in developing biodegradable nanoparticles as effective vehicles for the delivery of proteins and peptides^[69]. These polymer drug delivery systems offer many advantages as they can carry and deliver the drug to a target site, have the ability to deliver proteins, peptides and genes, increase the therapeutic benefits and minimize the side effects of the drug^[70,71]. The poly (lactide-co-glycolide) polymers (PLGA), being biocompatible, have been used as controlled release delivery systems for parenteral and implantable applications^[72]. A successful PLGA nanoparticulate system, as shown in Figure 3, has a high drug loading capacity as it allows a small quantity of the carrier during a single administration. This approach of PLGA encapsulation has been used to encapsulate a wide variety of hydrophobic drugs, including natural products curcumin^[73,74], coumarin^[75-77], plant extracts used as homeopathic mother tinctures^[25,78,79], coenzyme Q10^[80], estradiol^[81], protein^[82,83] and others. A brief step wise procedure of PLGA encapsulation of any drug is demonstrated in Figure 1. PLGA, a biodegradable polymer, is approved for human use by the United States Food and Drug Administration and the polymer readily decomposes without any induction of inflammation or immune reactions^[84]. Nanoparticles made of PLGA conjugated with glyco-heptapeptides was also shown to cross the blood brain barrier (BBB) after *in vivo* administration^[76,82,85].

Beneficial role of nano insulin in diabetes therapy

Insulin is the most effective drug in the treatment of advanced stage diabetes. Despite the significant advancement in the field of pharmaceutical research, development of a proper insulin delivery system remains a challenge^[86].

In this respect, biodegradable nanoparticulate delivery systems have been proposed for the safe and controlled parenteral administration of peptides^[87]. The biodegradable and biocompatible PLGA polymers possess various unique properties for the design of a sustained release drug delivery application^[88-90].

Formulations of PLGA encapsulated micro and/or nano insulin (Table 1) have been tested in recent years using various stabilizers *via* several administered routes of entry^[82,83]. Overall results of these studies open up the possibility of using nano insulin as an effective new anti-diabetic strategy that may target any of the several mechanisms that are involved in the development of diabetes; these may be done by adopting the proper correctional measures to bring the regulatory events back to the right track. The major targets of nano insulin are mainly focussed on the various glucose transporters (GLUTs) present in the pancreas, muscle, brain, *etc.*, which are primarily involved in the influx of glucose into several organs to maintain glucose homeostasis in the body. Samadder *et al.*^[82,83] observed that nano insulin could modulate

expression levels of several GLUTs better than that by unencapsulated insulin in a diabetic condition and could bring their expression level near to normal values. Even the mitochondrial signaling pathway that is normally affected in diabetic conditions could be favorably affected by the administration of nano insulin compared to that by the unencapsulated insulin. However, more work on other animal models is needed prior to conducting pre-clinical human trials for evaluating its actual efficacy and beneficial use in diabetic patients.

The need for an increasing dose of insulin administration is frequently observed with the lapse of time and progress of the disease for effective control. Increase in dose also increases the risk of developing hypoglycemia suddenly. Therefore, the dose of insulin is often a great concern in effective management of the disease. One of the primary goals of using nano insulin is to reduce the dose of insulin and help in suspended release of insulin from its nanocapsule to make the best use of its optimum efficacy. A suspended release of insulin was observed in diabetic mice when this nano insulin was subjected to *i.p* injection. Moreover, it was possible to obtain similar results at a dose of nano insulin several folds (10 fold or so) less than that of the unencapsulated form of insulin, as reported by Samadder *et al.*^[82,83] in experimental diabetic mice. Thus, in certain arsenic contaminated areas where diabetes predominantly occurs at a large scale, nano insulin may be found to be particularly helpful in reducing the cost and for better management of the disease. Furthermore, a non toxic PLGA coating more readily degrades and increases the bioavailability to a great extent and can prove to be an effective agent of targeted drug delivery with the desirable suspended release.

PLGA is composed of biodegradable, biocompatible and non-toxic polymers and has satisfactory nanoencapsulation potentials with smaller size and uniform spatial planar frequency, giving it the ability to enter cells and act faster, and provides an alternative approach for encapsulation of insulin for an optimized cost-effective use in the control of diabetes because the quantity of the drug entering the body is reduced^[82,83]. Nanoparticles possess the potential to modulate several biomarkers by different amounts, depending on their amount taken for the encapsulation (20 mg insulin in this case) and the final yield after formulations of the encapsulated form (approximately 200 mg nano insulin). Hence, the actual amount of the original drug substance in PLGA-encapsulated nano insulin is minimized by about 10-fold (approximately in this case) but nonetheless provides similar efficacy as that of their unencapsulated counterpart^[82,83].

Several other formulations of insulin nanoparticles were also administered to check if they can render protection to the drug carried through gastric acid and if they are able to get through the intestinal wall to enter into the liver and ultimately to the bloodstream^[91,92].

Transdermal delivery of nano insulin is also an attractive alternative therapy as it can control release of the drug and avoid possible drug degradation resulting from

gastrointestinal tract (GIT) of first-pass liver effects. Although the mechanisms of action of PLGA encapsulated nano insulin in several forms of diabetes are not known and still need proper investigation, some Indian researchers believe that people with diabetes may soon take a pill of insulin-loaded nanoparticles instead of having to give themselves painful injections^[92]. The pills, coated with tiny nanoparticles, protect insulin as it enters the stomach and keep blood sugar levels stable for 10 h. The minute nanoparticles are smaller than 100 nanometres across, attract water on the inside and are water-repelling on the outside. When they reach the bloodstream, they break down in response to the pH of blood and then release the insulin. The animal experiments demonstrated that the nanoparticles enter the bloodstream and end up in organs such as the liver and kidney. In diabetic pigs, the pill containing the nanoparticles led to control of blood glucose after eating^[67]. The size and distribution profile of the nanoparticles, smaller than 100 nanometers, has been characterized by the use of atomic force microscopy and dynamic light scattering. Some experts opine that while the research is promising, it will be some time before such a pill can be tried on humans (Available from: URL: <http://www.news-medical.net/news/2009/01/27/45264.aspx>).

The latest advance in this field of research is the nano insulin pump. A small capsule the size of a tiny silicon chip containing pancreatic cells has been created for this purpose. It has micro pores which allow the squamous red blood cells and other small molecules in and out of the capsule. It restricts larger cells such as phagocytes, antibodies and other immunoresponsive cells and proteins to enter; hence, it keeps the pancreatic cells inside safe from danger but also provides nutrients and allows them to release insulin according to the amount of glucose in the blood at that particular moment. The tiny pump needs to be mounted on a disposable skin patch or underneath the skin to provide continuous insulin infusion to diabetic patients. This new technology will help diabetics so that they can be completely free from dietary regulations and the restrictive systematic regime. They will no longer be dependent on insulin injections and their blood glucose levels will be adjusted according to their glucose level at that moment of time. This would enable them to lead a normal life. The benefits would be especially useful for the young who are always active, allowing them to feel like a person without diabetes. The unhappiness diabetics feel with fluctuating weight gain, especially during teenage years, would be diminished and help them feel more mentally secure and confident amongst their peers.

Use of nanoencapsulated CAM medicines in diabetes treatment

In addition to the wide therapeutic arsenals of modern medicine in combating diabetes, it is necessary to develop a traditionally adapted but more advanced complementary and alternative drug formulation to treat several symptoms of diabetes and its complications^[2] in order

to improve the validation and dose selection strategies of several phytochemicals which are already in use. There are several drugs of plant origin containing substantial amounts of alkaloids, glycosides and flavonoids with strong antioxidant properties for the treatment of diabetes which are described in ancient literature. However, these drugs prove to be mostly effective in long-term treatment and so often lose their importance when compared to the faster onset of action of orthodox medicines. Therefore, efforts are needed to enhance their action and increase their bioavailability to targeted organs/organ systems.

Among the wide range of alternative therapies that manifest potential anti-diabetic properties, PLGA nanoencapsulated forms of *Syzygium jambolanum*^[93] (SJ) and *Gymnema sylvestre*^[94] (GS) have been tested and shown to have relatively more anti-hyperglycemic effects than their unencapsulated counterparts in various experimental models.

Ravichandran^[94] reported that gymnemic acids, the main phytoconstituents of GS, possess potential natural pharmacological activities like suppression of taste sensitivity to sweetness, inhibition of intestinal glucose absorption and lowering plasma glucose levels. Nanonization of active drug components are shown to improve their physiological action. In this study, nanoparticulate formulations of gymnemic acids were studied for their pharmacokinetic and pharmacodynamic behaviors compared with that of some marketed products. The nanoformulation exhibited significantly enhanced anti-diabetic activity compared to marketed products^[94].

Although the study on nano-GS was only undertaken on a "glucose level" content parameter, another study conducted by our own group, Samadder *et al.*^[93], conducted both *in vitro* (in L6 cells) and *in vivo* (in mice) experiments to assess the relative efficacy of nano SJ against its unencapsulated counterpart. The physicochemical characterization of the formulated nano-SJ was undertaken by several standard protocols. Bio-markers and signal proteins associated with stress and hyperglycemia were also critically analyzed to determine the relative efficacy of nano-SJ against SJ. Nano-SJ was also found to have localized in the brain tissue of mice, suggesting that it could efficiently cross the blood brain barrier. Brain, specially the hypothalamic region, has been proposed to be the glucose sensor region which plays a critical role in initiating the counter regulatory response to glucose homeostasis. Transport of glucose across the brain capillary and into neurons in this region is mediated by a different glucose transporter (GLUT) gene family. BBB participates in brain sensing of blood glucose concentration. Under normal physiological conditions, glucose is the major metabolic fuel in the brain and therefore adequate insulin mediated glucose supply is essential for the maintenance of cerebral energy production^[95]. The ability of nano-SJ to cross the BBB therefore has great implications in terms of its potentiality to maintain insulin supply leading to optimum glucose homeostasis. Therefore, the overall results suggest that

nano-SJ had a greater potential than that of SJ, indicating the possibility of using NSJ in the future drug design and management of hyperglycemia and stress.

Can nanotechnology bring relief to diabetics?

Several instances of earlier studies reported many possibilities of nanotechnology to implement new ways of treating diabetes. As the world population increases, greater resources are needed to sustain society. An alternative to this issue is to be highly efficient and this could be achieved through nanotechnology. With this new technology, diabetics may become completely free from dietary regulations and the restrictive systematic regime. Some devices are so adjustable that diabetics will no longer be dependent on insulin injections and their blood glucose levels will be adjusted according to their glucose level at that moment in time. This would enable them to lead a normal life, especially the young who are always active. It helps the patient to feel more mentally secure/better and confident, as well as being cost-effective in other aspects as it requires fewer resources with a much more effective outcome. Efficiency is essential as the world population increases and therefore economical efficiency is the most stable way of supporting the billions of patients with diabetes.

Nanotechnology: Scope of future research

A survey of the literature suggests that a lot of work has been undertaken to establish the anti-diabetic potentials of several drugs, ranging from traditional (homeopathy and Ayurvedic) to formulated nanomedicines, but the field of nanotechnology or nanomedicine needs special attention. In this context, there is an open area of research to establish standard nanodrugs, explore more advanced insulin therapy, their nanoformulations, delivery and the pathway through which they act. Finding the truth of which drugs are really capable of bringing about corrective modulations of some parameters (maybe genes) and are scientifically acceptable with protocols/methodologies adopted that can be repeated by others is absolutely necessary. Exploring this area of research will not only bring a new dimension in the regimen of treatment to diabetic patients, but could also be a step forward towards building a platform for development of newer scientifically tested drugs by following an advanced procedure of drug designing.

The discovery and development of potent anti-diabetic drugs has been greatly hampered due to a lack of a suitable preclinical model with respect to the optimum dose of the drugs to check the efficacy of candidate agents. To bridge the gap, these drugs should include the realm of natural products, *i.e.*, CAM, but at the same time should be target-specific in action and utilize the different aspects of nanotechnology. The use of CAM drugs in nanoformulations would not only be biodegradable, biocompatible and non-toxic polymers in nature, but also would have a greater ability to enter cells and have a faster action, thereby providing an alternative approach for an

optimized cost-effective use of the original drug substances in much reduced (several fold) quantity and entering the body in nanoforms. If the testing in animal models of these drugs is successful, their true potential should then be explored in higher hierarchical animal orders. Finally, a better understanding of the plausible mechanism of the drugs and a proper scientific validation of the drug dose response should be engineered in the highest animal model, a human trial, while developing different therapeutic strategies. Hopefully, research in these directions can achieve the goal of improving the life expectancy and the quality of life of diabetics in the future.

CONCLUSION

In recent years, research on the formulation of advanced organ/tissue/cell-specific drugs that aim to enhance bioavailability of drugs to target organisms or organ systems with better efficacy at a minimum dosage is a top priority area. In this context, nanoencapsulated drugs appear to have greater advantages due to their: (1) small size; (2) more rapid entry into target cells; (3) biodegradable nature; (4) ability to render greater bioavailability of the drug; (5) lesser amount of drug requirement; and (6) ability to cross the BBB. Hopefully, further in-depth research in this direction can pave the way for the discovery of newer drugs that are more precise and organ/tissue-specific in nature from the plant kingdom by utilizing nanotechnology.

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Arsenic-induced abnormalities in glucose metabolism: Biochemical basis and potential therapeutic and nutritional interventions

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the behavior of arsenic as an antagonist or synergist on glucose homeostasis and insulin secretion is not yet fully understood. The present review delineates the relationship between arsenic and the biochemical basis of its relationship to glucose metabolism. This review also addresses potential therapeutic and nutritional interventions for attenuating arsenic toxicity. Several other potential nutritional supplements are highlighted in the review that could be used to combat arsenic toxicity.

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Key words: Arsenic toxicity; Glucose metabolism; Nutritional aspects

Core tip: This review illustrated the interference caused by arsenic in enzymes, genes and transcription factors involved in glucose metabolism and possible nutritional aspects for attenuating arsenic toxicity.

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Abstract

Health hazards due to the consumption of heavy metals such as arsenic have become a worldwide problem. Metabolism of arsenic produces various intermediates which are more toxic and cause toxicity. Arsenic exposure results in impairment of glucose metabolism, insulin secretion in pancreatic β -cells, altered gene expressions and signal transduction, and affects insulin-stimulated glucose uptake in adipocytes or skeletal muscle cells. Arsenic toxicity causes abnormalities in glucose metabolism through an increase in oxidative stress. Arsenic interferes with the sulfhydryl groups and phosphate groups present in various enzymes involved in glucose metabolism including pyruvate dehydrogenase and α -ketoglutarate dehydrogenase, and contributes to their impairment. Arsenic inhibits glucose transporters present in the cell membrane, alters expression of genes involved in glucose metabolism, transcription factors and inflammatory cytokines which stimulate oxidative stress. Some theories suggest that arsenic exposure under diabetic conditions inhibits hyperglycemia. However, the exact mechanism behind

INTRODUCTION

Arsenic is a toxic heavy metal and belongs to the 5th group in the periodic table. It is present in both inorganic and organic forms in different surroundings and its level is increased by anthropogenic contamination^[1]. It is a ubiquitous element and is found in four oxidation states -3, 0, +3, and +5. It is an environmental contaminant of worldwide concern due to its high toxicity and presence

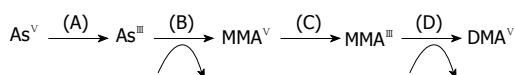


Figure 1 Arsenic methylation pathway in the human body^[25]. A: Arsenate reductase or purine nucleoside phosphorylase (PNP); B: Arsenite methyl transferase (As3MT); C: Glutathione S-transferase omega 1 or 2 (GSTO1, GSTO2); D: Arsenite methyl transferase (As3MT). SAHC: S-adenosylhomocysteine; SAM: S-adenosylmethionine; MMAV: Monomethylarsenic acid; MMAIII: Monomethylarsonous acid; DMAV: Dimethylarsenic acid; DMAIII: Dimethylarsinous acid.

in groundwater aquifers. Arsenic contamination in water has been found in countries such as Canada, India, Bangladesh, United States, China, Taiwan, Mexico, Poland, Japan, Nepal^[2] and Iran^[3]. Inorganic arsenic is believed to be the major form of arsenic in water, soil and various foods^[4] and is said to be a group I carcinogen based on clinical studies^[5].

Flora reported that the major exposure route of inorganic arsenic (iAs) is by contaminated drinking water in India, Bangladesh, China and American countries. Argentina (200 ppb), Mexico (400 ppb), Taiwan (50-1980 ppb), and the Indo-Bangladesh region (800 ppb) are countries where arsenic concentration in drinking water is reported to be beyond WHO guidelines maximum permissible value (10 ppb)^[6].

Epidemiological studies in various regions of the world with high levels of arsenic in groundwater have associated arsenic exposure with increased risks of different types of cancer (skin, liver, kidney and lung), arteriosclerosis and cardiovascular diseases, diabetes, hypertension and neurological diseases (Alzheimer and Parkinson)^[7-13]. Arsenic stimulates alterations in oxidative stress, cell calcium signaling, impairment of cell mitochondrial function and affects cell cycle progression^[14-17]. Some of these toxic effects at cellular and molecular levels ultimately lead to cancer^[18]. Although arsenic induces adverse health effects, all exposed humans do not develop arsenic symptoms related to exposure, suggesting that genetic susceptibility is also an important aspect involved in the human response to arsenic exposure.

Metabolism of arsenic in the human body

Metabolism of arsenic takes place in the liver where the first step is methylation. The presence of monomethylarsenic acid (MMA^V) and dimethylarsenic acid (DMA^V) indicates the methylation of arsenic in bile and urine. Monomethylarsenic acid is comparatively more toxic than dimethylarsenic acid^[19]. It was previously suggested that arsenic metabolism was a detoxification procedure, but now it is reported that intermediates of arsenic metabolism generate more toxicity. Absorbed arsenic undergoes biomethylation to form MMA^V and DMA^V (urinary excretion products) and are more toxic than iAs^[20]. Pentavalent arsenic (iAs^V) is quickly reduced to trivalent arsenic (iAs^{III}) and is then enzymatically methylated in humans and animals, which is then excreted *via* urine in the form of the dimethylated metabolite DMA^V^[21-24]. Methylation of arsenic requires S-adenosylmethionine as the methyl donor

and glutathione sulfhydryl as a vital co-factor^[25] (Figure 1).

Along with the major metabolite, DMA^V, dimethylmonothioarsenic acid (DMMTA^V), a thiolated metabolite, is also found in urine as a minor metabolite^[26-29]. In addition, DMMTA^V and dimethyldithioarsenic acid (DMDTA^V) are found in organs *in vivo* and *in vitro*^[30-32]. Moreover, iAs consumed by marine organisms is converted into arsenosugars and arsenobetaines and their thiolated metabolites are recognized as minor marine arsenic metabolites^[33-36]. Arsenic is ingested as arsenate or arsenite, is altered into the dimethylated form for excretion, and inorganic arsenicals and their metabolite viz., DMA. Among these arsenic metabolites, DMDTA^V and DMMTA^V are the current arsenic metabolites observed in urine and organs in man and animals^[26-29,31,32]. It has been suggested that DMMTA^V is simply absorbed by organs/tissues and is more toxic in nature^[37]. DMMTA^V is absorbed efficiently by organs in a different way to that of DMDTA^V, although DMMTA^V and DMDTA^V are both thioarsenicals. In addition, the distribution and metabolism of DMMTA^V are similar to DMA^{III} in hamsters, while the distribution and metabolism of DMDTA^V are similar to those of DMA^V^[38].

Oxidative stress

Arsenic causes toxicity *via* oxidative stress by affecting the antioxidant enzymes^[6,39]. It stimulates the production of reactive oxygen species (ROS) which results in the induction of adverse health effects^[20,40]. The mitochondrion is the chief site of ROS generation in cells and enhanced ROS formation is due to the abnormal function of electron transfer through the respiratory chain in mitochondria which in turn results in the production of hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radicals (OH[•])^[41]. Furthermore, in the electron transport chain, complexes I and III are the major leak sites for ROS formation, as some of the electrons passing through the mitochondrial respiratory chain leak out to molecular oxygen (O₂) to form superoxide radicals and then dismutate to H₂O₂. Increased ROS causes cellular and metabolic impairment through oxidative damage, which results in physiological abnormalities and deleterious chronic disorders. H₂O₂ is produced during the oxidation of As^(III) to As^(V) when intermediary arsine species are formed such as dimethylarsinic radicals [(CH₃)₂As[•]] and dimethylarsinic peroxy [(CH₃)₂AsOO[•]] involving O₂^{•-}^[42]. Arsenic leads to an increase in consumption of oxygen by cells, which results in ROS production and hence an increase in oxidative stress^[43]. Hepatic and renal heme oxygenase isoform-1 (HO-1) are also involved in the production of ROS by iAs which in turn results in extra free iron and biliverdin formation^[44]. This free iron participates in the Fenton reaction resulting in the formation of hydroxyl free radical (•OH) which attacks DNA^[45].

ROS produced intracellularly at the time of physiological processes, regulate cell functions, for instance endocytic pathways, autophagy, gene expression, intracellu-

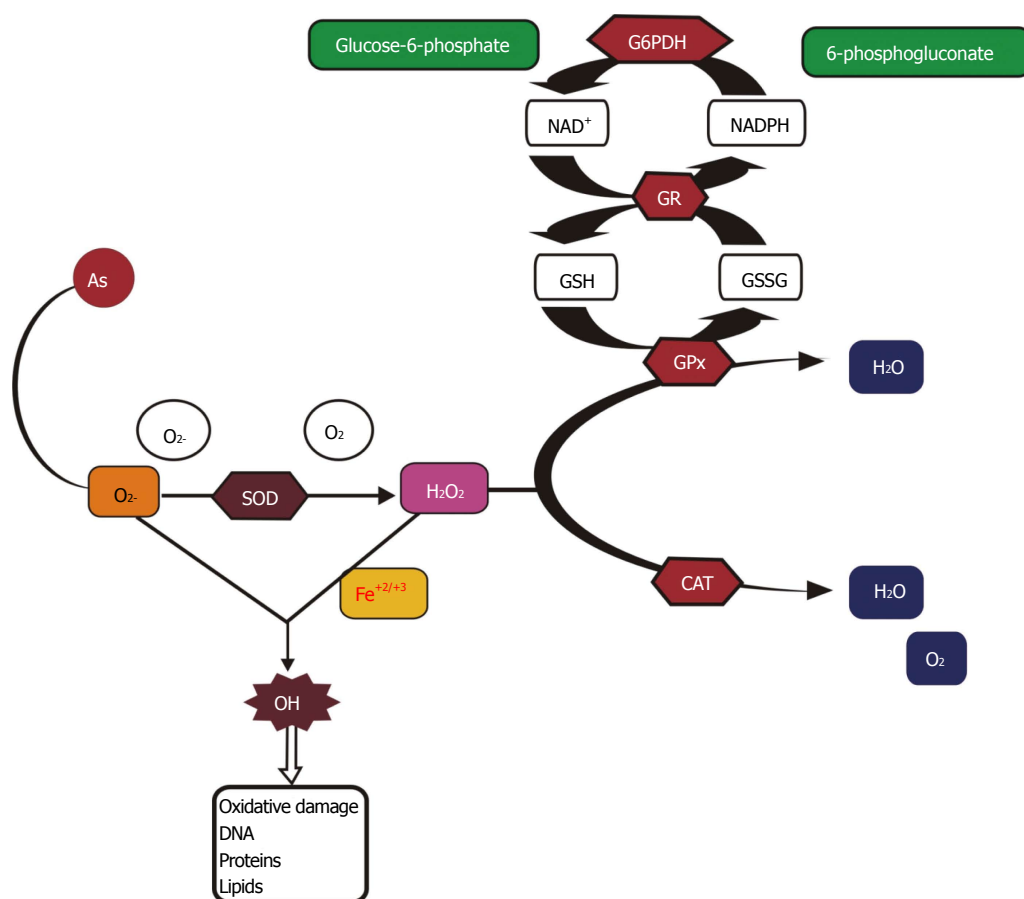


Figure 2 Mechanism of arsenic toxicity: Arsenic enhances the production of superoxide anion radical which results in a higher oxidant level than antioxidant enzymes involved in the detoxification of superoxide anion radical viz., superoxide dismutase, catalase, glutathione reductase and glucose-6-phosphate dehydrogenase. SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; G-6-PDH: Glucose-6-phosphate dehydrogenase; NAD⁺: Nicotinamide adenine dinucleotide; NADPH: Nicotinamide adenine dinucleotide phosphate reduced.

lar Ca^{2+} , glucose homeostasis, hypoxic and inflammatory responses^[46-49]. ROS function as second messengers due to stimulation/suppression of numerous signaling features by the oxidation of sulfhydryl groups and by changing the intracellular redox status, therefore inducing cell signaling pathways, downstream gene expression and cell reproduction or death^[13,20]. The signaling molecules affected include protein tyrosine kinases and phosphatases, protein serine/threonine kinases and phosphatases, small G proteins, lipid signaling, Ca^{2+} signaling and transcription factors^[50]. Biochemical reactions such as glycation results in the formation of advanced glycation end-products (AGEs) and protein oxidation causes alterations in cells which in turn results in the formation of disulfides between cysteine and methionine residues, cyclization of polyunsaturated fatty acid residues of phospholipids forming malondialdehyde (MDA), lipid peroxidation, 4-hydroxy-2-nonenal (HNE) and nucleic acid oxidation^[7,8,51,52]. Free radicals produced during iAs metabolism are the source of oxidative stress^[45]. Low concentrations of MMA^{III} and DMA^{III} are cytotoxic in human and rat skin, bladder, lung cells and human hepatocytes^[53-56]. Cellular offense in response to methylated metabolites is involved in genotoxicity with strong proof of oxidative stress as a causal factor. Genotoxicity of MMA^{III} and

DMA^{III} can be reversed by ROS inhibitors^[57]. Moreover, methylated metabolites mainly DMA^{III} and trimethylarsenic oxide (TMAO), also play a role in arsenic-induced genotoxicity^[58]. Cells having low methylation capabilities are more prone to cytotoxicity by arsenic specifying that other mechanisms are also employed in cytotoxicity induced by arsenic. An *in vitro* study on mammalian cell lines showed that there was no clear link between arsenic methylation capability by cells and resulting cytotoxicity induced by sodium arsenite^[59]. The possible mechanism of arsenic toxicity is depicted in Figure 2.

Arsenic and diabetes mellitus

Diabetes mellitus is one of the world's oldest known diseases. Type 2 diabetes mellitus (T2DM) is a widespread global metabolic disorder, distinguished by the unusual metabolism of carbohydrates and lipids, mainly resulting either from a fault in insulin secretion and/or insulin action, or adipocyte functioning^[60]. In T2DM, the entire body glucose homeostasis is disrupted due to insulin resistance and impaired glucose uptake by peripheral tissues, consisting of skeletal muscle and adipose tissue. In these tissues, glucose homeostasis is regulated by a mechanism involving insulin-dependent stimulation of glucose uptake.

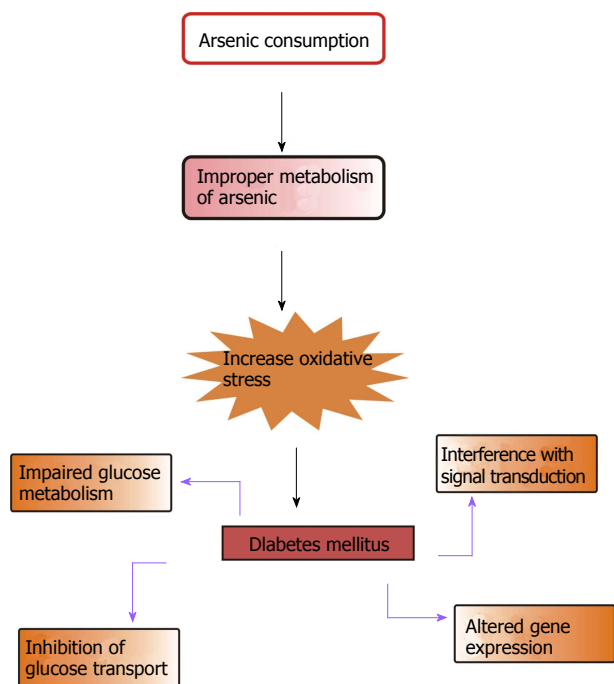


Figure 3 Possible biochemical approaches by which arsenic induces diabetes.

The worldwide incidence of diabetes among people aged 20-79 years was approximately 6.4% in 2010. This rate is supposed to rise to 70% in developing countries and 20% in developed countries from 2010 to 2030^[61]. Globally, more than 0.39 million people die every year from diabetes which is due to increase in the next decade^[62,63]. T2DM is more prevalent than type 1 diabetes mellitus. In India, the World Health Organization (WHO) reported that about 32 million people suffered from diabetes in 2000. According to the International Diabetes Federation (IDF), the total number of diabetic patients is nearly 40.9 million which is supposed to increase to 69.9 million in 2025^[64]. Environmental and lifestyle factors are the main causes of this remarkable increase in T2DM prevalence^[65,66].

Epidemiological studies suggest that T2DM is one of the most familiar non-cancerous metabolic disorders correlated with chronic exposure to iAs. Lai *et al.*^[67] in 1994 first established the link between diabetes and iAs. The correlation between arsenic toxicity and diabetes mellitus is a burning issue. Increased prevalence of T2DM is associated with the use of drinking water containing high levels of iAs and chronic occupational exposure to iAs^[68-75]. This is more prevalent in people consuming contaminated water in Bangladesh and Taiwan and in those working in copper smelters and the art glass industry in Sweden^[67,70-72,74-76]. According to the American Diabetes Association, diabetes due to arsenic toxicity or arsenic-induced diabetes may be classified under “the other specific types”^[77]. In epidemiologic studies, arsenic exposed subjects showed symptoms of diabetes mellitus similar to T2DM^[70]. As the symptoms were almost identical to those of T2DM, it is considered that the pathophysiol-

ogy of diabetes mellitus induced by arsenic is more likely to be similar to that of T2DM^[6]. According to Wang *et al.*^[78], there is a relationship between increased risk of metabolic syndrome, one of the most important cardiovascular disease risk factors and exposure to iAs in the general population^[78]. Figure 3 shows the possible way by which arsenic causes diabetes mellitus.

Arsenate replaces the phosphate group

Arsenate (As^{V}) replaces the phosphate group in various biochemical reactions owing to their similar structure and properties^[79]. Arsenate reacts *in vitro* with glucose and gluconate^[80,81] to form glucose-6-arsenate and 6-arsenogluconate, respectively, which are corresponding similar to glucose-6-phosphate and 6-phosphogluconate. Arsenate also replaces the phosphate group in the sodium pump and anion exchange transport system of human erythrocytes^[82]. Arsenate inhibits ATP formation during glycolysis by substituting arsenate for the phosphate anion in a process known as arsenolysis. In one of the steps of glycolysis, the phosphate group is enzymatically linked to D-glyceraldehyde-3-phosphate to form 1,3-diphospho-D-glycerate. In this reaction, phosphate is replaced by arsenate to form an unstable anhydride, 1-arsenato-3-phospho-D-glycerate, and hydrolyzes into arsenate and 3-phosphoglycerate. The instability of arsenic anhydride is due to the longer As-O bond length compared with the P-O bond length^[79]. ATP is not generated during glycolysis in the presence of arsenate^[83,84]. At the mitochondrial level, arsenolysis may occur during oxidative phosphorylation in the presence of succinate to form adenosine-5'-diphosphate (ADP) arsenate^[81]. ADP-phosphate formed during oxidative phosphorylation is difficult to hydrolyze in comparison to ADP-arsenate. During the process of cellular respiration, arsenolysis diminished ATP production by substituting phosphate with arsenate in respiratory pathways. An *in vitro* study suggested that arsenate exposure caused a reduction in ATP in rabbit and human erythrocytes^[85,86]. The activity of hexokinase is inhibited at higher concentrations of arsenate^[87]. In contrast, the two pentavalent forms of methylated metabolites, monomethylarsonate and dimethylarsonate do not disturb the metabolism of phosphate or bind to sulfhydryl groups^[88].

Affinity for sulfhydryl group

Arsenic affinity for thiols, especially the vicinal thiols of enzymes, is an accepted mechanism for arsenic toxicity, thereby inhibits catalytic activity of an enzyme by binding to a thiol-containing active site^[84]. Trivalent arsenicals easily react *in vitro* with molecules having a sulfhydryl group, for instance cysteine and reduced glutathione (GSH)^[85]. The complex linking vicinal thiols and arsenic is generally strong. Three main pathways by which arsenic decreases cellular GSH level have been suggested: (1) In the reduction of arsenates to arsenites, GSH functions as an electron donor; (2) Arsenite has a strong affinity for GSH; and (3) Arsenic-induced free radicals oxidize GSH.

As a consequence of obstruction of the Kreb's cycle

and disruption of oxidative phosphorylation by arsenic, a reduction in cellular ATP followed by cell death occur. Due to the interaction of arsenic with thiol groups, methylated trivalent arsenicals such as MMA^{III} inhibits GSH reductase and thioredoxin reductase^[89,90]. Cellular redox conditions are modified by the activities of methylated arsenicals, which in turn results in cytotoxicity. GSH protects cells from cytotoxins and is also involved in the metabolism of arsenic, through the formation of GSH conjugates. Numerous proteins with regulatory functions such as nuclear factor kappa B (NFκB) and adiponectin (AP)-1 are susceptible to cellular redox conditions. These proteins are regulated by GSH by altering the redox state of particular sulfhydryl groups of target proteins including stress kinases, transcription factors and caspases^[91].

Arsenic impairs pathways of glucose catabolism

Insulin-independent diabetes is the common form of diabetes mellitus among people chronically exposed to iAs^[71]. It has been suggested that at the cellular level, iAs or its metabolites disturb glucose metabolism and insulin signaling. Trivalent arsenicals are moderately effective inhibitors of numerous enzymes involved in glucose metabolism such as succinyl Co-A synthase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (PDH)^[92,93]. The PDH complex is the most studied enzyme and is considered most sensitive to inhibition by arsenite. During cell respiration, pyruvate is converted into acetyl-CoA in the presence of the pyruvate dehydrogenase enzyme complex (dihydrolipoyl transacetylase, dihydrolipoyl dehydrogenase, pyruvate decarboxylase, thiamine pyrophosphate, lipoic acid, CoASH, FAD, NAD⁺). Arsenite inhibits PDH by binding to the lipoic acid moiety^[94]. It has been reported that MMA^{III} is a stronger inhibitor of PDH than arsenite^[93]. The Krebs' cycle provides reducing power in the electron transport chain for ATP generation. Inhibition of PDH leads to decreased generation of ATP and energy resulting in cell damage and cell death.

In addition, an organic derivative of arsenite, phenylarsine oxide (PAO) inhibits basal or insulin stimulated glucose uptake by canine kidney cells, adipocytes and intact skeletal muscle^[95-100]. Arsenic interferes with sulfhydryl-containing enzymes such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, competes with the phosphate binding sites on glycolytic enzymes, uncouples oxidative phosphorylation and impairs glucose metabolism^[101,102]. Arsenic interferes with phosphate binding sites in ATP resulting in the formation of ADP-arsenate which inhibits metabolic pathways which require ATP. Glucose-6-phosphate is an essential mediator for glycolysis, glycogenesis, gluconeogenesis and glycogenolysis, and the pentose phosphate pathway (PPP). The PPP generates nicotinamide adenine dinucleotide phosphate (NADPH), an essential cofactor for glutathione reduction. Insufficient production of NADPH from the PPP further interrupts the cell's ability to deal with oxidative stress^[103]. Glucose-6-phosphate dehydrogenase (G6PDH) activity in mice exposed to arsenic, was significantly re-

duced in a time-related manner^[104]. G6PDH is an enzyme of the PPP, an alternate metabolic pathway for glucose. Reduced blood activity of G6PDH can lead to oxidative stress-induced diabetes and diminished nitric oxide generation^[105,106]. Exposure to arsenic also results in an increase in glycosylated hemoglobin level which indicates high blood glucose level and was reported in Danish people working in the wood industry^[73]. Thus, individuals exposed to iAs both from the environment and occupationally exposed are prone to diabetes mellitus.

Modulation of insulin signal transduction pathways

Signal transduction pathways activated by insulin which result in glucose uptake have been widely studied. This consists of binding of the insulin molecule to the α-subunit of the insulin receptor followed by activation of the tyrosine kinase moiety leading to autophosphorylation of the β-subunit of the insulin receptor, and consequent phosphorylation of insulin receptor substrate 1 or 2, phosphorylation and activation of phosphatidylinositol 3-kinase, and phosphorylation of phosphatidylinositol-4,5-bisphosphate at the cell membrane to phosphatidylinositol-3,4,5-triphosphate (PIP₃)^[107-109]. PIP₃ promotes phosphorylation of protein kinase B (PKB/AKT) and protein kinase C (PKC) enzymes that is, PKC λ and PKC ζ^[110,111]. The phosphorylation of PKB/AKT results in the transport of GLUT4 from the perinuclear space to the plasmalemma and the activation of glucose uptake^[112,113].

iAs^{III} and methylated arsenicals interfere with the main signal transduction pathways in human cells^[114]. Arsenic exposed cells show inhibition of the expression or activation of PKB/AKT, which is an essential component of the insulin stimulated signal transduction pathway. Thus, insulin-dependent signal transduction at the PKB/AKT level is inhibited, which is responsible for hyperglycemia in humans exposed to iAs. An *in vitro* study showed that iAs^{III} interrupts the expression and phosphorylation of PKB/AKT and inhibits insulin-stimulated glucose uptake and mobilization of GLUT4^[115]. Arsenic is also involved in the modulation of the mitogen-activated protein kinases (MAPK) pathway and related growth factors^[114,116]. The MAPK signaling pathway regulates stepwise phosphorylation of protein kinases and terminates the activation of transcription factors needed for cellular proliferation, differentiation or apoptosis.

Arsenic specifically inhibits glucose transport

Sulfhydryl groups play an essential role in insulin-dependent and insulin-independent mediated glucose transport (GLUT). The thiol component forms a structural bond linking the A and B polypeptide chains of insulin, the α and β subunits of the insulin receptor and the exofacial sulfhydryl moiety present on glucose transporters at the plasma membrane^[6]. PAO forms stable cyclic thio-arsenite complexes with vicinal or paired sulfhydryl groups of cellular proteins and inhibits glucose transport in adipocytes^[95,117]. Moreover, PAO prevents insulin-stimulated glucose transport without affecting insulin binding to its

receptor^[117]. PAO only affects insulin-dependent GLUT4 present in adipocytes and myocytes^[118].

However, the effect of arsenite on glucose transport is dose-dependent. Studies have shown that arsenite stimulates glucose uptake at higher concentrations, while at low level, glucose uptake decreases^[119]. Arsenite does not disturb regulation of GLUT4 gene expression, thus overall GLUT4 quantity does not alter. Walton *et al*^[115] examined the dose-dependent decrease in insulin-stimulated glucose uptake in 3T3-L1 adipocytes treated with iAs and its metabolites.

Effect on gene expression

There are many studies which support that diabetes is induced by arsenic *via* alteration in gene expression. When isolated rat pancreatic β -cells were exposed to 5 $\mu\text{mol/L}$ arsenite for 72 h, mRNA expression and insulin secretion decreased^[9]. An *in vitro* study showed that exposure to arsenite decreased the gene expression and activity of catalase, whereas the production of ROS increased^[120]. Peroxisome proliferative-activated receptor- γ (PPAR- γ) (a transcription factor) controls the main gene expression for insulin sensitivity. When mouse adipocytes from the C3H 10T1/2 cell line were exposed to 6 $\mu\text{mol/L}$ arsenite, alteration in the expression of PPAR- γ and AP-2 genes occurred which resulted in the inhibition of mRNA and reversal of adipocyte differentiation^[121]. The transcription of cytokines, namely tumor necrosis factor- α (TNF- α) and interleukins (IL), required in insulin resistance, are regulated by NF- κ B^[122]. When human bronchial epithelial cell lines were exposed to 18 $\mu\text{mol/L}$ arsenite for 12 h, NF- κ B dependent genes were activated. In contrast, exposure to 12.5 $\mu\text{mol/L}$ arsenic in TNF- α stimulated HeLa cells for 2 h resulted in inhibition of NF- κ B activation and I κ B degradation^[123,124]. When human GM847 fibroblast cells were exposed to 0.1 and 5 $\mu\text{mol/L}$ arsenite for 24 h, upregulation and expression of c-fos and c-jun genes and DNA binding activity of AP-1 takes place^[125].

Arsenic upregulates inflammatory cytokines from mononuclear cells. When human peripheral mononuclear cells were exposed to very low arsenite levels, TNF- α production increased 2-fold^[126]. Studies have shown that when blood arsenic level ranged from 0.128 to 0.62 $\mu\text{mol/L}$, the expression of IL-6 increased 3-fold^[127]. Expression and phosphorylation of AKT were suppressed when 3T3-L1 adipocytes were exposed to trivalent arsenicals^[115]. Activation of AKT by PDK-1 phosphorylation, is also inhibited by arsenite^[128]. In adipocyte cells, exposure to arsenite at high levels reduced the expression and phosphorylation of AKT genes, while at low levels expression was stimulated^[115,129]. In addition, the expression of phosphoenol pyruvate carboxykinase (PEPCK) increased in chick embryos after exposure to high dose arsenite, which may be due to the interaction of arsenic with glucocorticoid receptor complexes^[130,131].

EVIDENCE OF ALTERED ARSENIC METABOLISM IN DIABETES MELLITUS

T2DM is hypothetically related to variations in blood ar-

senic concentration and there is evidence to suggest that arsenic metabolism is modified in people with T2DM and these factors have precise roles in the pathogenesis and progression of this disorder^[132]. Many *in vivo* and *in vitro* studies have shown that iAs induces diabetes, but some experiments contradict these reports. There are convincing reports that diabetes alters the pharmacodynamics and pharmacokinetics of drugs/xenobiotics in humans and animals^[133,134]. Our previous studies showed that arsenic exposure causes the inhibition of hyperglycemia in diabetic rats and mice (Kulshrestha *et al*, Unpublished observation). Arsenic exposure in diabetic rat results in the promotion of insulin secretion and decrement of arsenic concentrations^[135,136].

The causal correlation between arsenic exposure and diabetes mellitus is still debated. Various epidemiological studies performed in arsenic-contaminated regions proved the relationship between chronic arsenic exposure and diabetes mellitus, however, the exact mechanism is not known^[137]. Various animal studies on the effects of exposure to arsenic on glucose metabolism and insulin secretion show inconsistent results due to variations in animal species, dose and time of exposure^[128,137]. The studies carried out by Wang *et al*^[138] in both humans and rats suggested that glucose metabolism is altered by arsenic.

ARSENIC AND NUTRITIONAL STATUS

Hsueh *et al*^[139,140] suggested that arsenic toxicity is associated with nutritional status in residents living in arsenic-contaminated areas such as Taiwan. As arsenic causes toxicity *via* oxidative stress, thereby decreasing antioxidant enzyme activity, it is possible that there is a link between arsenic-induced diabetes mellitus and antioxidant deficiency, and that the individual consuming less antioxidants has an increased risk of diabetes mellitus and cardiovascular disease^[141]. Thus, good nutritional status with sufficient antioxidant intake reduces the chance of arsenic-induced diseases. As arsenic interferes with GSH, people with diabetes have a lower level of GSH^[142]. It was found that selenium, which is required for GSH biosynthesis, is significantly lower in arsenic exposed subjects than in normal controls^[143,144]. GSH is required for the correct action of insulin and increased uptake of glucose, and is obligatory for the excretion of arsenic^[145]. Animal studies have shown that nutritional status modifies arsenic toxicity. A choline or methionine deficient diet (source of methyl donor group) results in a reduction in arsenic methylation, which leads to high retention of arsenic in the body and an increase in toxicity^[146-148]. Therefore, by consuming methionine rich diets, arsenic toxicity can be alleviated.

Preventive and therapeutic measures are available against arsenic toxicity. The roles of chelating agents, antioxidants, natural/herbal remedies as protective/therapeutic agents against arsenic toxicity are discussed below.

Chelating agents

The formation of a metal ion complex is known as che-

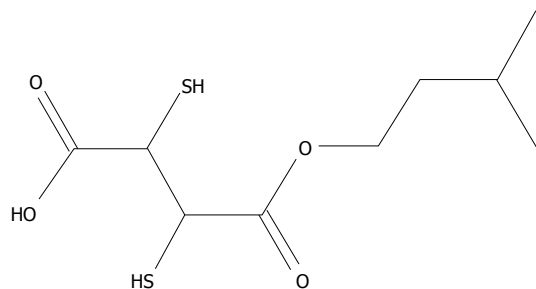


Figure 4 Mono isoamyl 2,3-dimercaptosuccinic acid.

lation in which two or more separate coordinate bonds are formed between monodentate or polydentate ligands and metal ions. These ligands are referred to as chelators or chelating agents and are organic compounds able to link together metal ions to form a complex structure called chelates. In chelation therapy, chelating agents are used to detoxify toxic heavy metals such as arsenic, and convert them to a chemically inert form with greater water solubility, which increases their excretion by the kidney without further interaction within the body. Various chelating agents are used to treat arsenic toxicity^[149]. The first chelating agent used was British Anti Lewisite (BAL) which was used during World War II. This is a dithiol compound used as a therapeutic agent against heavy metal toxicity. Despite its capacity to treat metal toxicity, its use is limited due to a low therapeutic index^[150]. Other metal chelators such as meso-2,3-dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propanesulphonic acid (DMPS) can be administered for a much longer time due to their very low toxicity^[151]. DMSA decreases the arsenic burden in cells by inhibiting the constant formation of ROS^[152]. Subsequently, numerous esters of DMSA have been produced to achieve more advantageous chelation. Flora *et al*^[153] found that administration of dimethyl DMSA (DMDMSA), diethyl DMSA (DEDMSA), diisoamyl DMSA and diisopropyl DMSA (DiPDMSA) led to a decrease in arsenic content in blood and soft tissues, but was less effective in recovering biochemical alterations following sub-chronic arsenic exposure in rats^[153]. Kreppel *et al*^[154] observed that administration of the monoesters, mono isoamyl DMSA (MiADMSA), mono n-amyl DMSA (MnDMSA), mono n-butyl DMSA (MnBDMSA) and mono i-butyl DMSA (MiBDMSA) were able to reduce the arsenic concentration in tissues, of which MiADMSA and MnADMSA were found to be most effective in mice^[154]. Administration of MiADMSA (Figure 4) and mono methyl DMSA (MmDMSA) (Figure 5) resulted in a reduction in arsenic concentration in blood and soft tissues in experimental animals^[155,156]. Despite the beneficial effects of chelating agents against arsenic toxicity, they have some drawbacks, such as non-specificity, low therapeutic index, failure to permeate the plasma membrane and metal redeployment, and induce side effects including headache, nausea, and vomiting, thus their use has been limited^[157]. Although these chelating agents enhance arsenic excretion, these agents have numerous

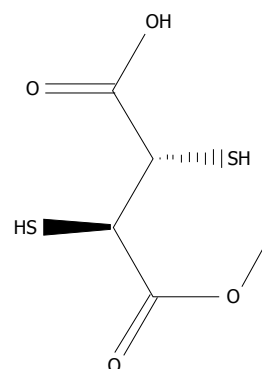


Figure 5 Mono methyl 2,3-dimercaptosuccinic acid.

drawbacks. Therefore, the identification of novel therapies without side-effects and complete medical recovery in terms of altered biochemical variables such as complete removal of metals are necessary.

Antioxidants

Arsenic exposure results in the production of ROS, thus cellular antioxidants are reduced. To prevent the increased production of ROS and their deleterious effects, the body's antioxidant system which consists of superoxide dismutase (SOD), glutathione reductase (GR), catalase, glutathione peroxidase (GPx), and reduced glutathione (GSH) scavenge ROS. In addition to this endogenous system, antioxidant status is improved by the administration of exogenous antioxidants such as vitamin C and E, quercetin, N-acetylcysteine (NAC), and α -lipoic acid.

N-acetylcysteine (NAC), the thiol-based antioxidant, is an originator of L-cysteine and GSH and stimulates glutathione synthesis (Figure 6). It protects cellular components against oxidative stress^[6,158]. It stimulates the production of GSH, hence retaining intracellular GSH level^[159]. NAC plays an essential role in the chelation of toxic metals^[160,161]. Co-administration of NAC and zinc alleviates arsenic-induced hepatic and renal toxicity^[162]. Flora *et al*^[163] developed a new treatment strategy consisting of combination therapy with DMSA and NAC, to achieve better results against arsenic toxicity in rats. NAC is effective against arsenic toxicity and recovered the level of hepatic malondialdehyde^[164]. The protective effect of NAC against arsenic toxicity in animals has been suggested by Hemalatha *et al*^[165] and Reddy *et al*^[158].

Quercetin (3,3',4',5,7-pentahydroxyflavon) is a bioflavonoid found in fruits, vegetables, seeds and flowers (Figure 7). It has very strong antioxidant properties and prevents cell apoptosis caused by oxidative stress^[166]. Quercetin scavenges superoxide radicals and protects against lipid peroxidation and chelates metal ions. An *in vitro* study showed that quercetin prevented cytotoxicity due to low-density lipoproteins^[167]. Quercetin co-administration with a thiol chelator was found to be more efficient in reducing body arsenic burden^[168].

α -lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid) is a dithiol antioxidant produced from octanoic acid in the

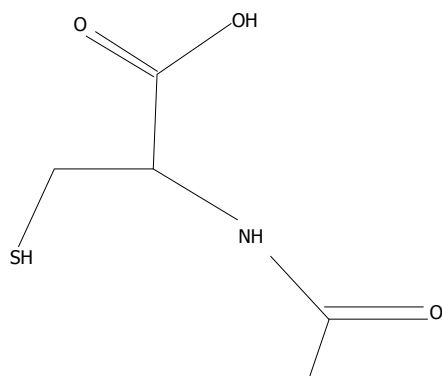


Figure 6 N-acetylcysteine.

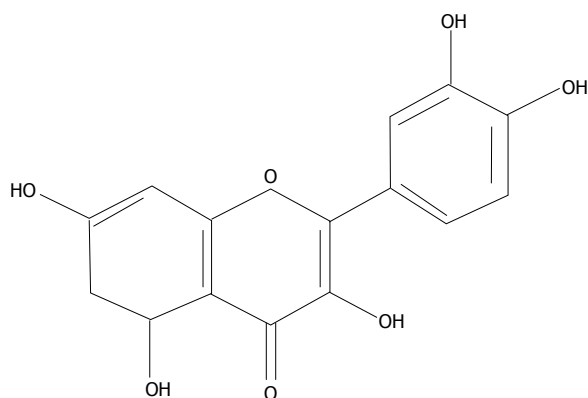


Figure 7 Quercetin.

mitochondria. LA is an essential cofactor for α -ketoacid dehydrogenase in mitochondria. In addition to production, LA is also consumed in the diet from wheat germ, beer, yeast, and red meat^[169]. After consumption, it is taken up into the circulatory system and traverses the blood-brain barrier, where it is reduced to dihydrolipoate^[170]. LA and dihydrolipoic acid (DHLA) are able to scavenge free radicals and chelate metals (Figure 8). LA treatment reduces arsenic-induced oxidative damage *in vivo* due to its chelation and free radical scavenging properties^[171,172].

A range of vitamins possess antioxidant properties against arsenic poisoning. The consumption of vitamins A, C and E plays a protective role against arsenic toxicity^[173]. Vitamin C is hydrophilic and is an intracellular and extracellular antioxidant capable of scavenging ROS *in vivo* and *in vitro* by electron transfer to inhibit lipid peroxidation. It traps free radicals and protects biomembranes from oxidative damage. Vitamin C alleviates arsenic-induced oxidative stress in mouse liver^[174]. Its advantageous effect is due to its capability to form a complex with arsenic^[6].

Vitamin E is a lipid soluble vitamin and its active form is α -tocopherol. It is assembled in lipophilic sites of the cell membrane and protects the membrane against oxidative damage. It donates an electron to the peroxy radical, which is produced during lipid peroxidation^[175]. Vitamin E has the ability to scavenge free radicals, hence

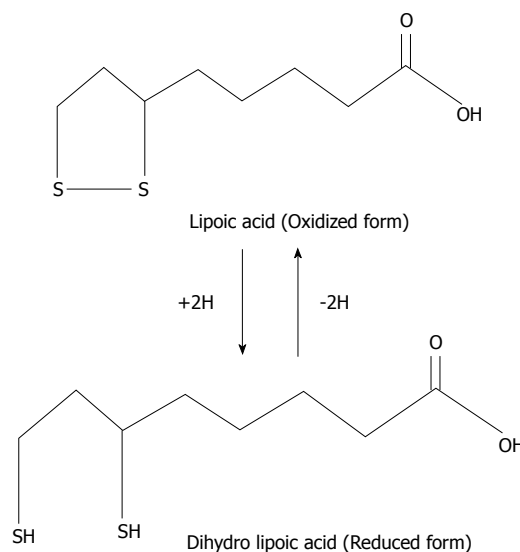


Figure 8 Reduction of lipoic acid.

protects against arsenic toxicity. An *in vivo* study showed that vitamin E treatment is effective against hepatotoxicity, nephrotoxicity and regulates altered variables of the heme synthesis pathway^[6].

Co-administration of vitamin C and E in combination with a chelator was found to be more effective than chelator alone in sub-chronically arsenic-exposed rats^[39]. Administration of vitamin C and E reduced the rate of DNA fragmentation in arsenic exposed rats^[176]. Some other vitamins such as A and B have also been reported to be effective against arsenic poisoning. Therapy with folic acid and vitamin B₁₂ alleviated oxidative damage induced by arsenic in cardiac tissue^[177]. A cross-sectional study performed in Bangladesh, reported that the intake of B-vitamins and antioxidants may reduce the risk of arsenic-related skin lesions^[178]. Other antioxidants such as taurine can be very useful in reducing oxidative stress induced by arsenic^[179].

Herbal/Natural remedies

For several years, herbal/natural remedies have been used all over the world as therapeutic and prophylactic agents. The synergistic action of a broad range of antioxidants from natural sources is better than the activity of a single or synthetic antioxidant^[180]. The use of conventional remedies, obtained from plants has been very important in managing arsenic toxicity. Numerous plants/spices or their extracts possess antioxidant effects. The administration of various plant/spice extracts, such as *Spirulina*, Curcumin, *Moringa oleifera*, *Hippophae rhamnoides*, *Centella asiatica*, *Allium sativum*, *Mentha piperita*, and *Aloe vera barbadensis*, has shown preventive and therapeutic effects against arsenic exposure in animals^[179,181].

With regard to natural and bio-available sources of antioxidants, we explored the beneficial effects of *Spirulina* in arsenic exposed diabetic rats. *Spirulina* administration was found to be associated with the alleviation of various metabolic disorders such as diabetes mellitus and

drug-metal-induced toxicities^[181-183]. Our studies on arsenic toxicity showed that the administration of *Spirulina* suspension for one week resulted in a reduction in arsenic burden, restoration of blood glucose and insulin level in rats (Kulshrestha *et al.*; Unpublished observations). *Spirulina* has significant antioxidant activity due to the presence of an enormous amount of phycobiliproteins, phycocyanin and allophycocyanin, phenolic compounds, γ -linoleic acid, minerals, tocopherols, β -carotenes, vitamin E & C and selenium^[184,185]. *Spirulina* possesses free radical scavenging properties in addition to its biosorption effect against heavy metal toxicity^[186-189]. Rahman *et al.*^[190] and Karkos *et al.*^[191] studied the efficacy of *Spirulina* in patients with chronic arsenicosis and found that *Spirulina* reversed the changes caused by arsenic^[190,191].

Centella or Indian Pennywort, *Centella asiatica* (L.) Urban Syn. and *Hydrocotyle asiatica* L. belong to the family Apiaceae. *C. asiatica* is useful for restoring biochemical alterations in arsenic-induced toxicity. It depletes tissue arsenic concentrations, to some extent, in rats^[192]. Sea buckthorn (*Hippophae rhamnoides* L.) Elaeagnaceae, is a nitrogen fixing shrub found in Europe and Asia. It is now cultivated in various parts of the world for nutritional and remedial purposes. The whole plant is an excellent source of various bioactive compounds such as carotenoids (α , β , δ -carotene, lycopene), vitamins (A, C, E, K, riboflavin, folic acid), organic acids (malic acid, oxalic acid), phytosterols (ergosterol, stigmasterol, lanosterol, amyris), and a few vital amino acids^[193-195]. *In vitro* and *in vivo* studies have shown the antioxidant and immunomodulatory properties of Sea buckthorn^[196]. The antioxidant activities of *H. rhamnoides* extract is due to the presence of flavanoids and phenolic compounds which exhibit free radical scavenging properties^[197]. Gupta and Flora evaluated the protective role of the aqueous extract of *H. rhamnoides* fruit against arsenic toxicity. However, this extract does not have the ability to chelate arsenic, and it is recommended that it should be administered along with an effective chelating agent to achieve the best possible outcome in chelation treatment^[198].

Garlic, *Allium sativum* L. belongs to the family Alliaceae, and contains a high concentration of sulfur compounds. Some biologically active sulfur-containing lipophilic compounds are allicin (diallyl thiosulfinate or diallyl disulfide, DADS), S-allylcysteine (SAC), and diallylsulfide (DAS) and hydrophilic compounds include s-ethyl cysteine (SEC) and N-acetylcysteine (NAC), which are responsible for antioxidant activities due to the stimulation and modification of enzymes such as 3-hydroxy-3-methylglutaryl-CoA reductase, glutathione-s-transferase and catalase^[199,200]. *In vitro* and *in vivo* studies demonstrated that administration of the aqueous extract of garlic resulted in the reduction of tissue arsenic burden and enhanced urinary arsenic excretion, which was due to the chelating properties of thiosulfur components such as allicin^[165,201].

Moringa oleifera is another plant belonging to the Moringaceae family, which exhibits antioxidant and chelating

properties. The seed powder of *M. oleifera* protected animals from arsenic-induced oxidative damage and reduced arsenic concentrations^[202]. This protection may be due to the presence of ascorbic acid, and cysteine and methionine rich proteins in the seed powder^[203,204]. Curcumin, a polyphenolic compound, is another herbal product which is a major constituent of *Curcuma longa* (Zingiberaceae family). It possesses numerous pharmacological activities including antioxidant and anti-inflammatory. Curcumin protects the hepatic tissues from arsenic-induced imbalance in antioxidants and oxidants. It also reduces hepatic arsenic burden^[205]. Curcumin prevents arsenic-induced neurotoxicity and hepatotoxicity during embryonic development^[206,207]. Other herbal products including *Mentha piperita* leaf extract and *Aloe vera barbadensis* also showed protective effects against arsenic toxicity^[208,209].

CONCLUSION

Arsenic is omnipresent in the environment, however, drinking water, including both groundwater and surface water supplies, is regarded as a major route of human exposure to iAs in arsenic-contaminated regions. Arsenic ingestion through the food chain may affect physiological and biochemical processes in the body. Although human exposure to arsenic is known to induce adverse health effects, the level and time of exposure as well as genetic susceptibility are important factors in outcome. Arsenic exposure plays an etiological role in diabetes development. Low or moderate arsenic exposure plays a positive role, while a high level of arsenic is associated with the risk of developing type-2 diabetes. Studies associated with the biochemical mechanism(s) in relation to arsenic exposure and risk of developing diabetes are still contentious and need to be delineated further. Although various therapeutic and nutritional strategies are available to alleviate arsenic toxicity, more preventive and therapeutic measures against arsenic toxicity are required.

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Effects of USPSTF guidelines on patterns of screening and treatment outcomes for prostate cancer

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and prompted the physicians to initiate conversation of informed screening. Younger patients were inclined towards aggressive treatment and older patients opted towards watchful waiting both with emphasis on the importance of evidence-based information provided by the physician. Decision aids were useful in making informed decisions and could be used to educate patients on screening purposes and treatment options. However, even with well-created decision aids and physician influence, patients' own belief system played a major part in healthcare decision making in either screening or treatment for prostate cancer.

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Key words: Prostate cancer screening; United States Preventive Services Task Force guidelines; Prostate cancer treatment outcomes

Abstract

The updated United States Preventive Services Task Force (USPSTF) for prostate cancer in 2012 recommends against prostate-specific antigen (PSA) based screening for men of all ages. Prostate cancer is the second most common and second most deadly cancer in American men. PSA screening for prostate cancer has been present since 1994 leading to an over diagnosis and over treatment of low volume disease. There is an overall agreement of men towards the guidelines but even with the understanding of the USPSTF, these men tend to follow more personal beliefs that have been influenced by their knowledge of the disease process and physician influence. Physicians also followed the directions of the patients and opted not to change their current practice of PSA screening despite the new guidelines. Time, legal, and ethical issues were some of the barriers that physicians faced in tailoring their practice towards screening. The importance of informed consent is highlighted by both the patients and the physicians and clearly more effective when the patient was pre-informed of the disease process

Core tip: Prostate cancer screening has never been more controversial since publication of large randomized trials showing conflicting results with some demonstrating beneficial mortality effects from the European trials but the American screening trial showing no mortality benefit. At the core of the prostate cancer screening debate is not only the overdiagnosis, but rather over-treatment of men with low-risk prostate cancer. This review explores the literature regarding these patterns of screening especially post publication of the United States Preventive Services Task Force guidelines. The use of enhanced risk-adapted approach, perhaps with decision aids, may serve as useful tools to help in the decision for continued screening for men who would benefit.

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INTRODUCTION

In May 2012, the United States Preventive Services Task Force (USPSTF) presented new guidelines for prostate cancer screening with recommendation against prostate-specific antigen (PSA) based screening for men of all ages^[1]. This was a Grade D recommendation which suggests that there was no net benefit from screening or that harm outweighs the benefit from PSA screening. The new guidelines comes 4 years after the previous guideline in August 2008 which recommended against PSA screening for men mainly 75 years or older and concluding that there is insufficient data to assess the benefit *vs* harm in PSA screening in men younger than 75 years^[2]. Prostate cancer is the second most common cancer in American men with American Cancer Society estimating 233000 new cases being diagnosed in 2014 and 29480 men dying of prostate cancer^[3]. The average age for diagnosis is about 67 years of age and about 1 out of 6 men will be diagnosed with prostate cancer in their lifetime. Prostate cancer is the second leading cause of cancer death in American men with 1 in 36 men who will die of their disease^[3]. Screening for prostate cancer in men 50 years or older by PSA testing and digital rectal exam were approved by The Food and Drug Administration in 1994. Since then, there has been an increase in diagnosed cases of prostate cancer especially those with low risk and low volume disease. There has been a corresponding decline in mortality from prostate cancer which can be attributed to newer therapies and not entirely due to screening alone. However, PSA screening has been faced with controversy regarding over-diagnosis and over-treatment^[4]. Given the ambiguity of PSA screening, many organizations have recommended “informed decision making” where the patient is allowed to make the decision to undergo the test or not with knowledge gathered from physician, social, as well as cultural input. The American Urological Association (AUA) has also supported informed decision making until May 2013 when the new guidelines from the AUA presented that PSA screening is recommended against in men ages 40-54 and 70 years or older. They continued to recommend informed decision making for men 55-69 years which is the core group that would benefit from screening^[5]. This review will focus on how the new guidelines presented by USPSTF for screening for prostate cancer has affected the decision making in choosing to screen for prostate cancer and treatment outcomes with men diagnosed with prostate cancer with exploration into new biomarkers used in disease diagnosis and progression. A literature review was performed using MEDLINE and Pubmed using key words: prostate cancer screening; USPSTF guidelines; informed decision making; decision aids for screening; prostate cancer treatment outcomes. The time frame was set at 2000-2014 and focused on studies done in United States.

DECISION MAKING IN PSA SCREENING

The response of men to the new guideline was looked

into by Squiers *et al*^[6] in a study where 1089 males were inquired about decision making about PSA testing with regard to the new changes from 2012. These were non-institutionalized men between the ages of 40-74, residing in the United States, who have never been diagnosed with prostate cancer. The men were given questionnaires assessing their knowledge about the PSA testing, their opinions on the new recommendations and whether or not they would follow the guidelines. Forty-four percent of the men in the study group have had a PSA test done in the preceding two years while 70% of the subjects responded that they had not discussed the benefits and potential harms with their healthcare providers. The study also revealed that the majority of the men were not aware of USPSTF but when explained, 69% reported that they felt confident that the recommendation was based on latest research. After the introduction of the new guidelines, 62% of the men stated that they agreed with the guidelines. However, among those who agreed with the guidelines, 54% intended to not follow them in the future. Most of these men tended to be African-Americans, income over > \$100000 and have had previous PSA testing. The younger men (40-49 years) tended to agree more with recommendations and were less worried about getting the disease compared to men aged 50-59 years of age. Overall, 61% of the men stated that the new recommendation did not affect their decision on getting PSA testing in the future. The study cites Pollack *et al*^[7] where primary care physicians were surveyed on the effect of the new recommendations on their practice. Not surprisingly, only 2% of the physicians would no longer order routine PSAs and 38% indicated that they would not change their practice. These studies shed some light into the fact that even after the presentation of the new guidelines, informed decision making would still continue on with input from both the physicians’ practice and patients’ beliefs.

It is important to assess the informed decision making in the view of the physicians as well. Wilkes *et al*^[8] focused on primary care physicians’ ability to educate or activate patients’ informed decision about routine PSA testing. It has been established that factors such as legal fears, lack of time educating the patient, and difficulty understanding each patient’s personal belief system were among the barriers physicians usually face in choosing to follow the guidelines as stated. The study group consisted of 120 California-based primary care physicians and 712 of their male patients were between ages of 55 to 65 years, who have no history of cancer. Majority (80%) of the patients had undergone a PSA test in the past 2 years and had expressed strong preferences in being involved in their health decision making. Among the parameters investigated in the study, was the response from the physician about PSA testing when prompted by the patient. Majority of the physicians opted towards education and screening when prompted by these patients and had long term sustained education even beyond the 3 mo after prompting by the patient. According to the authors, this

method might be more effective and sustainable than using continued medical education for physicians. It is to be noted that a major limitation that is mentioned in the study is that the patient population were of higher education and socioeconomic status and therefore it would be unclear if these results could be extrapolated to patients who are from a lower socioeconomic status. Even though physicians were more stimulated when the discussion about decision making was initiated by the patient, the patients themselves were educated on prostate cancer and screening prior to the physician encounter. Therefore, the patient initiation of the discussion is dependent on the patient's knowledge and his willingness to bring the discussion to the physician.

Some populations were more affected by the new guidelines than others. In Cohn *et al.*^[9]'s study, a population of men chosen before and after the USPSTF recommendation were brought in and analyzed how the new recommendations impacted the decision making of PSA screening by their primary care physicians. The number of men chose to undergo testing post recommendation was statistically significantly ($P < 0.0001$) less (7.6%) than men who tested prior to the recommendations (8.6%). Some factors that influenced decision making included patients who have had prior benign prostatic hyperplasia (BPH), had previous PSA status and time since previous testing. Men with BPH had increased screening even post recommendations and men were more likely to continue testing if they had done so previously. In contrast, the study also resulted in increased PSA testing in men who had never undergone a PSA (5.1% *vs* 4.5%, $P = 0.03$). The study observed an abrupt decrease in testing in the group of men who had previous PSA values > 4.0 ng/mL. Men in this population were of mostly ages 70-79 years and could reflect decrease due to chronically elevated PSA. The changes in frequency of PSA screening also differed with the age of the population. Men 70-79 years had an increase in frequency of testing up until 2008, when more research was presented that showed less benefit in testing in this age population. Men ages 50-69 years, followed an overall general trend. The ER-SPC study (Table 1) suggested that men in this age range are more likely to receive mortality benefit with PSA screening^[10]. The younger population of men 40-49 years had a plateau in 2010 and a significant decrease in 2012. The NCCN clinical practice guidelines in oncology suggest discussion of PSA screening at age 40. Even though younger men are choosing to opt out of PSA screening, the long term effect of decreased screening is unknown. The overall decrease in testing shown in the study corresponds to Veterans Health Administration (VHA) Pacific Northwest Network and the linked Surveillance, Epidemiology and End Results-Medicare databases looking at the influence of new USPSTF recommendations. Interestingly, the study states that independent of previous PSA screening or age, African American men tend to receive less PSA testing. African American men tend to have more aggressive tumors and would benefit

from regular screening. Given the social notion of "over-screening" in the general population, this concept seems to result in "under-screening" of African-American men.

Aslani *et al.*^[4] used retrospective data from health care systems in northeastern Ohio from January 2008 to December 2013 to assess the outcome changes given the new guidelines. The study indicated that the PSA screening has been significantly increasing from the beginning of the research period up until March of 2009 and slightly declined up until May 2012, when the guidelines were published. The decline since March 2009 is attributed by the authors to the PLCO trials (Prostate, Lung, Colorectal and Ovarian cancer screening trial) indicating no difference in mortality with the control group in PSA screening. The rate of testing declined, mentioned a statically insignificant by the author from May 2012 to end of research period. With regard to the age, the most significant decrease in testing was observed in men older than 60 years. Similar results were presented by Zeliadt *et al.*^[11] where a 3% decline in PSA testing among men of all ages was seen after the PLCO trial. The data from Zeliadt *et al.*^[11] ranged from August 2004 to March 2010 in practices from the Veterans Health administration Pacific Northwest network.

EFFECT OF TREATMENT OUTCOMES FOR PROSTATE CANCER

In Xu *et al.*^[12]'s study, the main focus was on the perspective of men when choosing their prostate cancer treatment. Men who were younger than 75 years who were recently diagnosed with prostate cancer were given information about the different options of treatment and was interviewed on their decision making process. The study included 21 men who consisted of both Caucasian and African American males. Compared to the other studies in the review, this study focused on the emotional perspective rather than evidence or system-based information, that goes towards discussing newly diagnosed cancer and choosing treatment. Younger men opted towards more aggressive treatment and there were few men who initially chose surgery but decided on radiation or watchful waiting after adverse effects of surgery was mentioned. Majority of the men appreciated the depth of resources provided by the physician and wanted the physician to give a personalized recommendation on the treatment type. The patients felt more secure if the physician provided an evidence based treatment option for them to consider. It is important to note that many personal, emotional factors go into decision making for these patients and even distrust of physicians in a small number of patients. However, the decisions were based on a foundation set forth by the physician using evidence-based recommendations.

Similar results were shown in Holmboe *et al.*^[13] where men were asked about their prostate cancer treatment decisions. Most men cited popular data and good research

Table 1 Comparison between the prostate, lung, colorectal and ovarian and European randomized study of screening for prostate cancer trials

| Participants | PLCO | | ERSPC | |
|---------------------|--|--------------|-------------|-------------|
| | Screening | Control | Screening | Control |
| Age | 55-74 yr | | 55-69 yr | |
| Contamination rates | 40% (1 st year) to 52% (subsequent years) | | 15% | |
| Total | 38343 | 38350 | 72890 | 89353 |
| Cancer incidence | 3452 (9%) | 2974 (7.75%) | 5990 (8.2%) | 4307 (4.8%) |
| Cancer mortality | 92 | 82 | 214 | 326 |

PLCO: Prostate, lung, colorectal and ovarian; ERSPC: European randomized study of screening for prostate cancer.

as a decision factor. It is possible that the ultimate decisions were influenced by the physicians, yet confirmed by the patient's own beliefs. This sets the standard for the physicians to provide latest and most pertinent data for the patients given the strong influence they have on the decision process.

Another aspect of the new recommendations is the treatment outcomes of men who have already been diagnosed with prostate cancer. The PIVOT trial (Prostate cancer Intervention Versus Observation) found no disease specific survival benefit for radical prostatectomy at 12 years compared to watchful waiting^[14]. However, one of the thought processes brought about in this trial is that most of these cases of prostate cancer, which was clinically non-apparent, would not have been diagnosed with the previous thoughts about PSA screenings and majority of men were older for whom contemporary recommendation would probably dictate active surveillance rather than surgery. There is no optimal treatment for prostate cancer especially at early stages and the radical surgical treatments are not without adverse outcomes. It is important for the physician to guide the patient to make informed decisions^[15].

A systemic review done to analyze the decision making of men with prostate cancer showed that controlling cancer was one of the major decision factors in choosing the treatment. This was either defined as extending survival or preserving the quality of life, depending on the patient values. However, efficacy of the treatment was not given gravity in the decision making process. Relying on published research varied among the patients and avoiding adverse effects were more commonly cited by those who chose watchful waiting. It appears that the decision making mostly relied on the content and information provided to them by their provider over the patient's own belief system. In addition, psychological factors play a role where younger men's perception of early stage of cancer would warrant more aggressive treatment. The decision for over-diagnosis or over-treatment rests partly in the hands of physicians. Therefore, shared decision-making may help since majority of men would report no major physician-patient interactions regarding PSA screening^[16].

However, lack of physical, social support and misinformation often resulted in patients choosing more aggressive treatment over more non-invasive treatments. One method of providing proper, balanced information is the use of decision making aids^[17].

USING DECISION AIDS IN THE PROCESS OF PSA SCREENING AND PROSTATE CANCER TREATMENT

Given the ambiguity in the benefit of PSA screening for prostate cancer, the decision making to undergo screening is at times left at the discretion of the patient. Evans *et al*^[18] reviewed aids that served to assist patients in deciding to undergo the screening based on input from the patient including the patient's health status in the era prior to the published USPSTF guidelines. The study utilized 7 decision aids where 2 were specified for men over 50 years with others presented to the general population of men in United States and Canada. Presence of prostate cancer and/or urological symptoms varied among the sample populations. The decision aids were from Cancer Information Services, NHS Centre for Reviews and Dissemination, Foundation for Informed Medical Decision-Making, Minneapolis VA Medical Center, prostate-specific antigen information script, Cancer Research United Kingdom and American Institute for Cancer Research. The decision aids included knowledge assessing about PSA testing, screening guidelines, test interpretation and prostate cancer disease process. The aids also gave information about treatment options including surgery, radiation and watchful waiting. The results concluded an overall 3.5% absolute reduction in the number of patients who had a PSA test 12 or 18 mo following the decision aid intervention. The knowledge of prostate cancer and PSA screening was also tested in these trials. The results indicated that there was a short term increase of 19.5% more correct answers compared with control group at 2 wk after the intervention. This was less prominent in long term knowledge retention at a year or more with only a difference of 3.4% more correctly answered questions. However, the relationship of the knowledge about prostate cancer and screening and the outcome of decision making to undergo PSA screening were not clearly stated. There were personal values, linguistic and cultural influences that affected the decision making that the authors considered a limitation to the study.

Fagerlin *et al*^[19] analyzed patient information aids that were supplied to prostate cancer patient and found that only 44 out of 546 had all the treatment options provided (surgery, radiation therapy, hormone therapy and watchful waiting). Only about half of the aids described surgery and radiation therapy in full detail and about one third discussed the risks and benefits of each type of treatment. Most aids had a biased towards one specific type of treatment or overall biased towards the more aggressive treatment options. Patients who received well

prepared decision aids were more likely to select watchful waiting over active treatment [relative risk ratio (RR) = 1.53, 95% confidence interval (CI) = 1.31 to 1.77]. Several online decision aids are currently available and Knight presented key characteristics that offer guidance in the delivery of these decision aids^[20]. A limitation to these aids is the fact that watchful waiting is written in the same connotation as palliative approach at times and this leads the patients to select inappropriate treatment. As a result, older men tend to opt for inappropriate hormonal therapy and younger men with low risk disease attempted invasive curative therapy. In addition, the clinical utility of decision aids resides in its ability to increase patients' involvement, improve knowledge and realistic perception of outcomes. However, it remains uncertain whether it is truly cost-effective or would work in patients with lower health literacy^[21].

USING EMERGENT BIOMARKERS IN FURTHER REFINING PSA SCREENING AND DIAGNOSIS

The topic of PSA screening has also brought about an upsurge of techniques to better refine the use of a biomarker other than the PSA for improvement in PSA screening or diagnosis. To this end, several promising biomarkers have emerged in the market and while a comprehensive discussion can be found elsewhere^[22], recent discovery and commercial availability of a few may illustrate these points. Traditional serum biomarkers such as the PSA has long been heralded as the mainstay of screening biomarker but some would advocate that instead of seeking a new and better marker, a more prudent approach may be to use a panel that incorporates already existing features which led to the development of the PHI or prostate health index which comprises of analyses of the PSA, free PSA (fPSA), and [-2]proPSA^[23-26], the latter showing increased specificity for aggressive prostate cancer detection^[27]. Instead of serum biomarkers, a promising approach could be the use of molecular signatures such as the fusion product TMPRSS2-ERG which makes biological sense since TMPRSS2 is androgen-regulated and coming under control of the transcription factor ETS family would be a driver for prostate cancer growth and it is prevalent in prostate cancers^[28]. Perhaps a search for a more economical and sensitive way of diagnosing prostate cancer but ultimately distinguishing the benign from aggressive ones in a single or combination of tests^[29], could impact the screening landscape for this disease.

DISCUSSION

Prostate cancer is the second most common cancer seen in American men. The 2012 USPSTF guidelines recommended against PSA screening for all men. Patients tend to agree with the new USPSTF guidelines for PSA

screening. However, the ultimate decision was not solely based on the guidelines and was more focused around their medical or social beliefs. Patients who were older, with previous prostate pathology continued to follow their regular screening despite agreeing with the guidelines. Same outcomes were seen from the physicians' point of view. Most physicians agreed to the guidelines but opted not to change their routine PSA testing given legal, time and knowledge constraints. The populations that saw a decrease in the PSA screening were younger men, with no prior prostate disease and who have a low risk of prostate cancer. The treatment outcomes for prostate cancer were based on personal expectations and physician recommendations. Using new research and guidelines were part of the decision making but not the sole determining factor. There is also the potential utility for the use of decision aids that provides information about prostate cancer disease process, different treatment options and the benefit/risk of each option. The decision aids overall improved the knowledge of the patients and assisted in the decision making process for treatment options. However, the choice of treatment could be affected by any biases presented in the aids and whether a certain treatment is written in a positive or negative connotation.

CONCLUSION

Patients had a general trend of continuing their regular screening for prostate cancer despite the new USPSTF guidelines although certainly, time will tell whether the uptake in the community with both physicians and patients alike, would ultimately show the trend towards decreased overall screening. The use of decision aids can be used to improve the patients' knowledge of the patient on the disease process and treatment options but ultimately patients tend to put forth personal belief and personal outcome expectations as more determining factors in choosing treatment.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA*

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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Volume with supplement

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Books

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

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Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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