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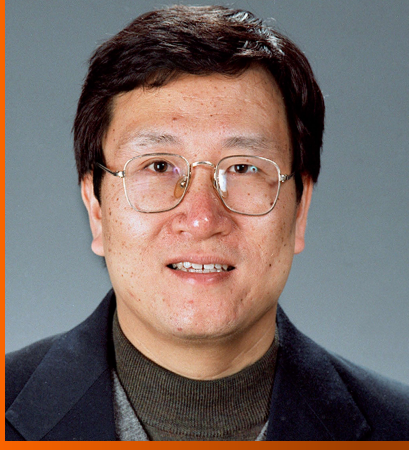


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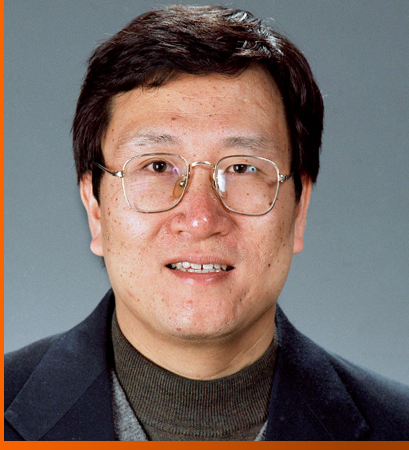


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Epithelial ovarian cancer: An overview

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logical cancer and the leading cause of death in the United States. In this article we review the diagnosis and current management of epithelial ovarian cancer which accounts for over 95 percent of the ovarian malignancies. We will present various theories about the potential origin of ovarian malignancies. We will discuss the genetic anomalies and syndromes that may cause ovarian cancers with emphasis on Breast cancer type 1/2 mutations. The pathology and pathogenesis of ovarian carcinoma will also be presented. Lastly, we provide a comprehensive overview of treatment strategies and staging of ovarian cancer, conclusions and future directions.

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Key words: Epithelial ovarian cancer; Breast cancer type 1; Chemotherapy

Core tip: Epithelial ovarian cancer (EOC) is one of the most common gynecological cancers. We present a number of theories on the origin of EOC including the recent hypothesis that the fallopian tube is the primary site of most serous carcinomas. We also discuss genetic anomalies that may cause ovarian cancer. The pathology of ovarian cancer by malignant transformation of the epithelium of the ovarian surface, peritoneum or fallopian tube is also presented. Finally we provide an overview of ovarian cancer treatment options, comparing various chemotherapy regimens and future predictive biomarkers and functional assays for targeted therapy for breast cancer type 1 associated EOC.

Abstract

Ovarian cancer is the second most common gynecological

Desai A, Xu J, Aysola K, Qin Y, Okoli C, Hariprasad R, Chinemerem U, Gates C, Reddy A, Danner O, Franklin G, Ngozi A, Cantuaria G, Singh K, Grizzle W, Landen C, Partridge EE, Rice VM, Reddy ESP, Rao VN. Epithelial ovarian cancer: An overview.

INTRODUCTION

Amongst gynecologic malignancies ovarian cancer is the second most common and the #1 cause of death^[1]. In this article we review the diagnosis and current management of epithelial ovarian cancer (EOC) which accounts for over 95 percent of the ovarian malignancies^[2]. The clinical presentation of EOC can either be acute or subacute. Acute cases present with conditions such as pleural effusion, small bowel obstruction and venous thromboembolism. Subacute cases may present with non specific symptoms such as abdominal fullness, bloating, an adnexal mass, vague pelvic or abdominal pain and gastrointestinal symptoms.

In the following review, we initially present various intriguing theories about the potential origin of ovarian malignancies. Subsequently, we discuss in depth genetic anomalies and syndromes that may cause ovarian cancers with emphasis on breast cancer type 1 (BRCA1)/2 mutations (section II). Next, the pathology and pathogenesis of ovarian carcinoma is presented (Section III). Finally, we provide a comprehensive overview of treatment strategies and staging of ovarian cancer (Section IV), conclusions (Section V) and future directions (VI).

ORIGIN

The cellular origin of EOC is not well known thus hampering the development of effective early detection methods. Sir Spencer Wells in 1872 claimed that EOCs arose from ovarian surface epithelial (OSE) cells^[3]. This hypothesis is supported by Cheng's work on *HOX* genes^[4], which shows that ectopic expression of *HOXA9*, *HOXA10* and *HOXA11* genes in mouse OSE cells followed by intraperitoneal (IP) injection into mice resulted in the establishment of cancers of serous, endometrioid and mucinous subtypes, respectively. Inclusion cysts lined by OSE were considered as precursors for EOCs in the past^[4]. However the above theory has been refuted by a number of studies^[5]. For example, a prospective study of 48230 postmenopausal women who had no family history of ovarian cancer but had annual pelvic ultrasounds was conducted in 2010. Investigators found that after a median follow-up of 6 years there was no increase in the development of EOC in women who had inclusion cysts in their initial study. Yet in another study^[6] it has been proposed that ovarian cancers arise from secondary Mullerian tract structures as they are indistinguishable from adjacent mesothelial cells lining the peritoneum.

However, in the last 5-10 years, several studies have proposed that EOCs should no longer be considered as a single disease entity. For example, high-grade serous ovarian cancers (HGSOC), which represent the most

common type of invasive EOC, are exemplified by the ubiquitous presence of TP53 mutations^[7]. In a detailed analysis of the fallopian tubes of patients who underwent prophylactic salpingo-oophorectomies, histologic studies showed that tubal lesions were present in almost 100% of cases of early serous cancers associated with familial BRCA mutations. This lends evidence to the hypothesis that fallopian tube cells may play an important role in the genesis of BRCA-associated HGSOC. Kindelberger *et al*^[8] have proposed that cancer cells are shed from the tubal epithelium and implanted on the surface of the ovary and most likely become trapped within the surface inclusion cysts to produce ovarian or primary peritoneal carcinomas.

Recently Nik *et al*^[9] hypothesized that the fallopian tube is the primary site of most serous carcinomas. Evidence supporting this hypothesis includes: (1) more than 50% of sporadic pelvic high grade serous carcinomas have serous tubal intra-epithelial cancers (STIC); (2) STICs have also been found in 10%-15% of fallopian tubes prophylactically removed from women with BRCA mutations; (3) 92% of STICs have been shown to have TP53 mutation similar to those found in concordant high grade serous carcinoma samples; (4) STIC oncogene productions such as cyclin E1, Rsf-1, and fatty acid synthase, are also overexpressed in HGSCs; and (5) STICs have been found to be present in prophylactic salpingectomy specimens in the absence of carcinoma which lends evidence against the view that STICs are formed due to metastasis from adjacent HGSCs.

GENETIC ANOMALIES AND SYNDROMES

Individuals from families with multiple cancers, cancers occurring at an early age, and two or more primary cancers in a single individual have a higher risk of hereditary ovarian cancer syndromes. A recent Cancer Genomic Atlas project analyses of mRNA expression, miRNA expression, promoter methylation, and DNA copy number in 489 high-grade serous ovarian adenocarcinomas revealed TP53 mutations in almost all tumors (65). Approximately 13% had germ line mutations in BRCA1 or BRCA2 and a small percentage had somatic mutations in NF1, RB1 and CDK12^[10]. Analysis also showed four ovarian cancer transcriptional subtypes, three miRNA subtypes, four promoter methylation subtypes and signatures associated with survival (65). Furthermore pathway analyses showed defect in homologous recombination, Notch and FOXM1 signaling was involved in the pathophysiology of serous ovarian cancer^[10].

The majority of hereditary ovarian cancers are associated with mutations in tumor suppressor genes, *BRCA1* and *BRCA2*. BRCA1 (susceptibility protein) encoded by *BRCA1* gene (located on the long arm of chromosome 17) is a nuclear-cytoplasmic shuttling protein, and many cancer-associated mutations have altered subcellular localization of BRCA1 protein^[11]. BRCA1 also plays a role in DNA repair by transcription regulation, chromatin

remodeling, homologous recombination, and cell cycle regulation. BRCA1 mutations function as either truncating or missense mutations interfering with critical regions of the gene such as the RING finger motif or BRCT region of the gene. BRCA2 (susceptibility protein) is encoded by *BRCA2* gene (located on long arm of chromosome 13), plays a crucial role in repair of double stranded DNA breaks. It has been observed that the risk of ovarian cancer is higher in women with BRCA1 mutation as compared to BRCA2 mutation^[12,13]. The cumulative risk of ovarian cancer by age 70 is estimated to be 40%-50% for BRCA1 mutation-carriers and 10%-25% for BRCA2 mutation carriers^[14]. Furthermore, BRCA1 mutations are also associated with earlier onset^[12]. In one study, the average age at diagnosis of ovarian cancer in BRCA1 and BRCA2 mutation carriers was 52 and 62 years respectively^[15].

BRCA associated cancer risks are determined by the mutation location and genetic variation of the *BRCA1* and *BRCA2* gene function. Mutations occurring within the central region of the *BRCA2* gene, called the ovarian cancer cluster region, compared to mutations in the 5' or 3' region, may be associated with a significantly higher risk of ovarian cancer in women^[16].

Research has also shown that the BRCA1 Associated Ring Domain 1 (BARD1) protein which binds to the BRCA1 protein stabilizes both proteins and targets the BRCA1 to sites where DNA strands are broken. Mutations in the *BARD1* gene may prevent the BARD1 protein from helping repair damaged DNA. Thus, BARD1 isoform expression is required for cancer cell proliferation, thereby making them cancer maintenance genes^[17]. Studies also suggest that the BARD1 protein has other functions different from its partnership with the BRCA1 protein. The BARD1 protein interacts with another protein, p53 (which is produced from the *TP53* gene) to promote controlled cell death (apoptosis) and regulate cell division. Other potential functions of the BARD1 protein are under study.

Specific genetic modifiers of cancer penetrance, sometimes called modifier genes, may influence the expression of genes like *BRCA1* or *BRCA2*. Genetic variation, particularly in the genes comprising endocrine signaling and DNA repair pathways, may modify BRCA-associated cancer risks. Another potential modifier gene in BRCA1 and BRCA2 mutation carriers is *CYP1A1*, which encodes an enzyme involved in the metabolism of polycyclic aromatic hydrocarbons and in the hydroxylation of estradiol^[18]. A particular allelic variant, found in 14 percent of carriers with cancer and 22 percent of unaffected carriers, reduced the risk of breast cancer by about 40 percent. Recently Qin *et al*^[19] have shown that BRCA1 inhibits the growth of ovarian cancers by regulating Ubc9 binding. With the aid of live imaging of YFP, RFP-tagged BRCA1 and BRCA1a proteins they showed enhanced cytoplasmic localization of mutant BRCA1 proteins in certain types of ovarian cancer cells. The mutant BRCA1 proteins were found to be impaired in

their capacity to inhibit growth of ES-2 ovarian cancer cells. Less commonly hereditary non polyposis colorectal cancer which is caused by mutations in mismatch repair genes, MSH2, MLH1, MSH6, PMS1, and PMS2 is also associated with ovarian cancer^[20]. Jones *et al*^[21] analyzed 42 cases of ovarian clear cell carcinoma and found that 57% of these cases had mutations in ARID1A which suggests that aberrant chromatin remodeling contributes to the pathogenesis of ovarian clear cell carcinoma.

PATHOLOGY

Epithelial cancer of the ovary is thought to derive from malignant transformation of the epithelium of the ovarian surface, peritoneum, or fallopian tube. The exact molecular transformation events causing EOC are not known. Baylin *et al*^[22] have shown that epigenetic phenomenon may also play a role. Mutations of the oncogenes HER2, C-myc, K-ras, Akt, and the tumor suppressor gene p53 have been observed^[23-25]. The molecular pathways underlying cancer progression are also not well understood. Most research to date have shown that factors associated with reproduction and ovulation have the largest impact^[26,27]. Some studies have shown that somatic mutations in *BRCA1*, *BRCA2* and other genes can also lead to cancer progression.

Some of the theories for the pathogenesis of EOC include: (1) repeated ovulation with trauma^[28,29]; (2) increased estrogen concentrations as a result of excess gonadotropin secretion^[30]; (3) high androgen concentrations^[30]; and (4) stromal hyperactivity^[31].

Histopathology

The classification of EOC is usually based on the origin of the tumor^[32]: (1) About 75% of EOCs are of the serous type. They simulate the lining of the fallopian tube. This histologic variant is often associated with concentric rings of calcification known as psammoma bodies. While there is no established grading scheme for serous ovarian cancer, recent work has suggested a two-tiered system-low-grade and high-grade^[33,34]. The work of Meinhold-Heerlein *et al*^[35] has shown that low-grade serous carcinomas and serous tumors of low malignant potential involve similar genes and pathways that are distinct from high-grade serous carcinomas. These findings have led some to hypothesize that low-grade carcinomas represent the natural progression of an undetected serous ovarian tumor of low malignant potential; (2) Mucinous tumors usually resemble intestinal or endocervical epithelium. Mucinous tumors are usually large with a median diameter of 18 to 20 cm and tend to remain confined to the ovaries^[36]. Furthermore, primary ovarian mucinous tumors can be difficult to distinguish from metastatic mucinous tumors from the colon/rectum, appendix, cervix or pancreas; (3) Endometrioid tumors are hypothesized to sometimes arise from the foci of endometriosis. They are associated with slightly better survival when compared to serous adenocarcinoma, regardless of disease stage^[37],

(4) Clear cell carcinomas can also sometimes arise from endometriosis. They derive their name as their histologic features include “clear cells”. Prognosis is poor due to an increased risk of venous thromboembolism and decreased response to platinum-and-taxane based chemotherapy regimens^[38]; and (5) Brenner tumors, also known as transitional cell tumors because histologically, they resemble transitional epithelium of the bladder. Majority of Brenner tumors are benign. Histologically, there are nests of transitional-type epithelial cells with longitudinal nuclear grooves lying in abundant fibrous stroma.

TREATMENT

There are basically three forms of treatment of ovarian cancer: (1) Surgery; (2) Chemotherapy; and (3) Radiation (rarely used). Surgery is considered the mainstay of treatment for ovarian cancer. Treatment is mainly based on the histologic subtype and the stage at diagnosis, which is determined surgically by a tumor reductive procedure including hysterectomy, removal of ovaries, removal of the omentum, and any other sites of disease feasible to remove. The goal of surgery is to reduce the tumor burden to no visible disease, though an “optimal” status is assigned if no residual lesions are larger than 1 cm. However, other studies^[39] designate the status of maximal cytoreduction for tumors less than 3 cm. The following table lists the different stages of EOC: stage I : Cancer is confined to one or both of the ovaries; stage II : Either one or both the ovaries are involved and the cancer has spread to uterus and/or fallopian tubes or other sites in the pelvis; stage III : Either one or both the ovaries are involved and the cancer has spread to lymph nodes and/or sites outside the pelvis but is still within the abdominal cavity; and stage IV: Either one or both the ovaries are involved with distant metastasis.

Stages I and II

For stage I epithelial cancers, surgery alone is adequate. Here surgery is used for both staging and therapeutic purposes. Surgery includes hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. Samples of the under surface of the diaphragm, peritoneal washings, pelvic/para-aortic lymph node samples are also examined for completeness. In high-grade ovarian cancer, adjuvant chemotherapy has led to better overall survival rates. Large clinical trials such as EORTC-ACTION and MRC-ICON1 involved patients with stage I and II ovarian cancer who were randomly assigned to adjuvant chemotherapy or observation^[40,41]. In EORTC-ACTION, at least 4 cycles of carboplatin/cisplatin based chemotherapy were administered whereas in MRC-ICON1 patients received 6 cycles of single agent carboplatin or cisplatin or platinum-based chemotherapy. Data from both studies showed significant improvement in recurrence-free survival and overall survival (5 year survival figures were 82% with chemotherapy and 74% with observation with a 95% confidence-interval in the difference of 2%-12%).

Stages III and IV

In the case of stages III and IV cancers, surgery includes total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and debulking of any grossly visible tumor. Cytoreduction is an independent prognostic variable for survival^[39,42]. In patients with optimal cytoreduction the median survival was 39 mo as opposed to 17 mo if the surgery was suboptimal^[43].

Treatment options for patients with optimally cytoreduced stage III disease include conventional intravenous and/or IP chemotherapy. The use of IP cisplatin has shown better progression free survival (PFS) and overall survival^[44]. One of the recent studies gynecologic oncology group (GOG)-0172 showed a higher median survival rate of 66 mo for patients receiving chemotherapy *via* the IP route as opposed to 50 mo in patients who received intra-venous cisplatin and paclitaxel ($P = 0.03$)^[44]. Another study concluded that IP cisplatin works better on small tumors (< 1 cm) that are platinum responsive^[45].

Therapeutic strategies for patients with sub-optimally cytoreduced stage III and IV disease include intravenous and/or IP chemotherapy plus additional cytoreductive surgery in some circumstances. The benefits of cytoreductive surgery have not been well established in advanced disease states. While one study performed by the EORTC showed better survival rates in patients who had debulking surgery after 4 cycles of cyclophosphamide and cisplatin^[46]. Another study, GOG-0162, in patients who received paclitaxel and cisplatin with interval cytoreductive surgery did not show any survival benefit^[47]. GOG 182-ICON5^[48] compared the effectiveness of various combination chemotherapy regimens as first line therapy for patients with stage III/IV EOC who have undergone either optimal or sub-optimal cytoreductive surgery. The study shows that combining either carboplatin or paclitaxel with one of the follow new cytotoxic agents-gemcitabine, poly-ethylene glycol liposomal doxorubicin and topotecan gave favorable results.

A combination of carboplatin and a taxane (paclitaxel or docetaxel) is the standard chemotherapeutic agent for ovarian cancer with clinical response rates > 60% and median time to recurrence usually > 1 year. The combination of carboplatin and paclitaxel regimen is the standard worldwide and is sometimes used as induction chemotherapy, if patients are poor surgical candidates, or the surgeon feels that the primary surgery is not likely to be optimal. The SCOTROC trial^[49] compared the use of either docetaxel or paclitaxel in combination with carboplatin as first line chemotherapy and found similar PFS in both cases therefore offering an alternative to paclitaxel.

Recently there have been a number of studies investigating the role of bevacizumab in first-line therapy for ovarian cancer following surgical cytoreduction. Bevacizumab is a humanized-monoclonal antibody against vascular endothelial growth factor (angiogenesis inhibitor). Two trials GOG-0218 and ICON7 have shown improvement in PFS when bevacizumab was added to initial chemotherapy and continued every three weeks for

16 and 12 additional cycles respectively^[50,51]. However, overall survival has not been demonstrated, and therefore has not been adopted outside of recurrent disease or the clinical trial setting.

Eskander *et al.*^[52] have recently proposed a new strategy for combining maximal cytoreductive surgery with intra-operative hyperthermic intraperitoneal chemotherapy (HIPEC) for treatment of advanced stage EOC resulting in promising oncologic outcomes. Currently phase III clinical trials are ongoing to test the efficacy of combining cytoreductive surgery with HIPEC.

Recurrent or persistent ovarian epithelial cancer

Recurrent cancer has a wide range of presentations. Patients may present with abdominal distention or pain or symptoms related to sites where the cancer cells have spread to. The most common follow-up procedures include bimanual pelvic examination, serial measurement of CA125, laparotomy and repeating imaging studies. Platinum sensitive cancers (recurrence more than 6 mo since completion of primary therapy) are usually re-treated with a combination of carboplatin and a taxane. The OCEANS trial^[53] was conducted to test the efficacy and safety of bevacizumab with gemcitabine and carboplatin (GC) compared with GC alone (*i.e.*, bevacizumab replaced by a placebo) in platinum sensitive recurrent ovarian cancers. Results show a significant improvement in PFS when bevacizumab was used in combination with GC. Platinum resistant disease can be treated with one of several agents that typically provide response rates in the range of 10%-20%. These include bevacizumab, topotecan, gemcitabine, liposomal doxorubicin, and others. The AURELIA trial^[54] was one of the first randomized phase III trials to demonstrate the benefit of combining bevacizumab with standard chemotherapy regimen resulting in increased PFS (6.7 mo *vs* 3.4 mo) in patients with platinum resistant ovarian cancer.

The NOVEL trial^[55] proposed the idea of a “dose-dense” therapy for treating primary ovarian cancer. It is based on the hypothesis that a shortening of the time interval between the administrations of cytotoxic agents is able to achieve better cell kill. They compared the standard 3-wk paclitaxel and carboplatin combination *vs* dose-dense weekly paclitaxel and 3-wk carboplatin for advanced EOC and found that the PFS was significantly better in the former (28 mo *vs* 17.2 mo).

CONCLUSION

Among gynecologic malignancies ovarian cancer is the most lethal. The cellular origin of EOC is not well known. Early theories hypothesized that EOC arises from OSE cells, while more recent ones have proposed that it should no longer be considered as a single disease entity but rather a diverse group of tumors with specific morphologic and genetic characteristics.

Histological differences in the ovarian cancers define a number of subtypes of EOC. Subtypes are named

based on the tissue that they closely resemble and include serous, mucinous, endometrioid, clear cell, and transitional cell. Ovarian cancer is also classified as benign, borderline or malignant depending on the degree of epithelial proliferation and stromal invasion.

Treatment of EOC is based on the combination of surgery and chemotherapy. Over the past three decades, optimal surgical cytoreduction, followed by platinum-based chemotherapy has become the standard treatment for advanced ovarian cancer.

FUTURE DIRECTIONS

A recent national institute of health study discovered genomic similarities between basal-like subtype of breast cancer (triple negative breast cancer) and serous ovarian cancer^[56]. Computational analyses show that both these cancers are susceptible to agents inhibiting blood vessel growth and chemotherapeutic drugs targeting DNA repair. However, more work is needed in this direction to determine how these findings may be used functionally and clinically.

Another area that has received attention recently is the impact of oxidative stress and BRCA1 mutations in ovarian cancer. Oxidative stress induces DNA damage (as evidenced by increased 8-hydroxydeoxyguanosine) correlates with poor outcomes in ovarian cancer^[57]. Martinez-Outschoorn *et al.*^[58] found that UWB1.289 cells, which contain mutant BRCA1, produce large amounts of hydrogen peroxide leading to oxidative stress and catabolic processes in adjacent stromal fibroblasts *via* stromal NF- κ B activation. Catabolism in stromal fibroblasts was also accompanied by the up regulation of MCT4 and decreased Cav-1 expression which signify tumor microenvironment. Furthermore, the study also showed the effect of UWB1.289 could be negated by using the antioxidant N-acetyl-cysteine or by *BRCA1* gene replacement. This suggests that new trials are needed for cancer prevention using antioxidants in hereditary BRCA1 mutations.

Another recent breakthrough in the treatment of recurrent ovarian cancer is in the use of vaccines derived from lysate-pulsed dendritic cells. Kandalaf *et al.*^[59] reported a study encompassing dendritic cell based autologous whole tumor vaccination and anti-angiogenesis therapy. They took the tumor lysate from patients with recurrent ovarian cancer. The patients were initially treated with intravenous bevacizumab and oral metronomic cyclophosphamide. This was followed by bevacizumab and the vaccination of dendritic cells with autologous tumor lysate, lymphodepletion and administration of a large number of vaccine primed T-cells. The study found that cellular immunotherapy may be used for treatment of recurrent ovarian cancer. However the study included only six subjects and the therapy was found to work for four of them. Thus more work is required in this direction to ascertain the benefits of such vaccines.

Some studies such as those by Bryant *et al.*^[60] and Farmer *et al.*^[61] have shown that BRCA-deficient cells are

especially sensitive to chemical inhibitors of poly (ADP-ribose) polymerase (PARP), which plays a critical role in single stranded DNA break repair. The most studied PARP inhibitor Olaparib has shown good results^[62] in initial studies in BRCA1 mutated and sporadic ovarian cancer but further studies are needed.

There are no clear indications for ovarian cancer screening. Testing is recommended for women at high risk such as those with a significant family history, but consultation with a genetic counselor is recommended to discuss limitations and alternatives to genetic testing. Potential screening tests include the blood test for CA-125 marker and transvaginal ultrasound. However these tests are neither sensitive nor specific for ovarian cancer. They may be abnormal in benign conditions such as endometriosis, menstruation, pregnancy, and cancers of fallopian tube, breast or the GI tract. Early results from the United Kingdom Collaborative Trial of Ovarian Cancer Screening showed that combining annual CA-125 tests with ultrasound imaging was useful for detecting ovarian cancers at an earlier stage^[63]. The full results of the trial are expected in 2015. Lastly the key to developing targeted therapies for EOC will depend on understanding the biological pathways and targets involved in the development of these cancers. Our results (unpublished work) suggest Ubc9 to be expressed at elevated levels in several ovarian cancers and BRCA1-mutant serous epithelial ovarian cancer cells^[19]. We have developed BRCA1 function-based cellular assays (Patent number United States 8372580) where loss of Ubc9 binding by BRCA1 mutants can not only predict the risk for developing Triple Negative Breast Cancers and EOC, but it may lead to the development of targeted therapies for these cancers.

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Neurological and behavioral manifestations of cerebral malaria: An update

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Abstract

Neglected tropical diseases are a group of tropical diseases endemic in poor countries even though medical treatment and cures are available. They are considered a global health problem due to the severity of the physiological changes they induce in their hosts. Malaria is a disease caused by *Plasmodium* sp. that in its cerebral form may lead to acute or long-term neurological deficits, even with effective antimalarial therapy, causing vascular obstruction, reduced cerebral blood flow and many other changes. However, *Plasmodium falciparum* infection can also develop into a cerebral malaria (CM) disease that can produce neurological damage. This review will discuss the mechanisms involved in the

neuropathology caused by CM, focusing on alterations in cognitive, behavior and neurological functions in human and experimental models.

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Key words: Malaria; Cerebral malaria; Neuropathology; *Plasmodium* sp; *Plasmodium falciparum*

Core tip: This review attempts to compile the limited current knowledge on the behavioral and cognitive effects of cerebral malaria (CM) and the possible pathological mechanisms related to neurobehavioral manifestations. CM induces acute/chronic neurological damage, affecting several Central Nervous System regions responsible for behavioral, neurological and cognitive functions which may result in motor deficits, epilepsy, blindness, speech/hearing and memory/attention disorders, hyperactivity, anxiety-like behavior, neuropsychiatric manifestations of post malaria neurological syndrome, both in humans and animal models. The action mechanisms involved in the alterations are not yet clearly defined; however proinflammatory mediators have been described with consequent axonal damage and demyelination.

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INTRODUCTION

Malaria, leishmaniasis and tuberculosis together with other neglected tropical diseases (NTDs) cause 32% of

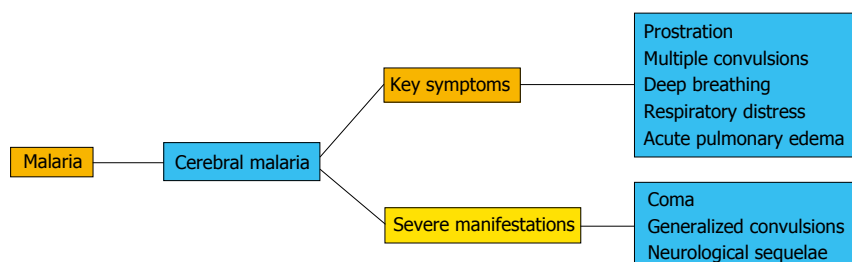


Figure 1 Clinical manifestations of cerebral malaria.

the burden of ill health in Africa and seriously impact on health outcomes in many regions of the world. NTDs share common features such as high endemicity in rural and impoverished urban areas of low-income countries. Some NTDs are disfiguring and stigmatizing, being considered poverty-promoting conditions, particularly in Africa, Asia, and the tropical regions of the Americas^[1,2].

Among various NTDs, malaria is one of the most life-threatening diseases, provided that the currently recommended interventions are not adequately implemented^[2]. In 2011, the World Health Organization (WHO) estimated that 3.3 billion people were at risk of malaria. More than 274 million clinical cases and 1.1 million deaths occurred between 2001 and 2010 worldwide, with approximately 80% of cases and 90% of deaths estimated to occur in the African Region, mostly in children under five years of age and in pregnant women^[2,3]. Kiszewski *et al.*^[4] estimated that Global resource requirements for malaria control totaling USD 38-45 billion will be spent from 2006 to 2015 for the diagnosis and treatment of malaria, mainly in countries and populations at risk of epidemic, such as sub-Saharan Africa.

Human malaria is caused by five species of obligate intraerythrocytic protozoa of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*^[2], and is transmitted by the bite of a female anopheles mosquito. At least three-dozen different species of *Anopheles* mosquitoes can transmit malaria worldwide^[5]. However, infections can also occur through exposure to infected blood products (transfusion malaria) and *via* congenital transmission^[6].

Of these, *P. falciparum* is the organism primarily responsible for severe malaria, although *P. vivax*^[3] and *P. Knowlesi*^[7,8] can also cause severe disease. According to WHO's criteria^[9], severe malaria is defined by clinical or laboratory evidence of vital organ dysfunction and/or high parasite burden; this high parasitemia can be a risk factor for death from *P. falciparum* malaria^[9].

Overall, clinical features of severe malaria include cerebral malaria (CM) with impaired consciousness (including coma), prostration, multiple convulsions, deep breathing and respiratory distress (metabolic acidosis), acute pulmonary edema and acute respiratory distress syndrome, circulatory collapse or shock and acute kidney injury^[9,10]. However, severe malaria is a complex multi-system disorder that can mimic many other diseases that are also common in malaria-endemic countries, such as central nervous system (CNS) infections, sepsis, severe pneumonia and typhoid fever^[9].

In this review, we described neurocognitive and behavioral outcomes of CM in humans and animals so as to facilitate further understanding of the disease's pathogenesis in the CNS.

CM

CM is one of the most severe and rapidly fatal neurological complications caused by *Plasmodium* species, mainly *P. falciparum*, with around one million deaths per year in children from sub-Saharan Africa^[11,12]. The first manifestations of CM are non-specific fever, chills, irritability, agitation or psychotic behavior, vomiting and cough. In adults, complications are severe jaundice, respiratory distress syndrome, and severe intravascular hemolysis leading to hemoglobinuria and anemia, which further contributes to renal failure (Figure 1). The most severe manifestations are impaired consciousness with coma, generalized convulsions and neurological sequelae. Pregnant women are also vulnerable and develop anemia, hypoglycemia, coma and pulmonary edema. In children, the main symptoms are severe anemia, metabolic acidosis, hypoglycemia, coma and gastrointestinal symptoms^[13-15], as shown in Figure 1.

CM may result in acute or long-term neurological deficits, even with effective antimalarial therapy^[16,17]. CM is a neurological complication that occurs in approximately 1% of infections caused by *P. falciparum*^[18,19]; however, a high mortality rate follows^[14,20].

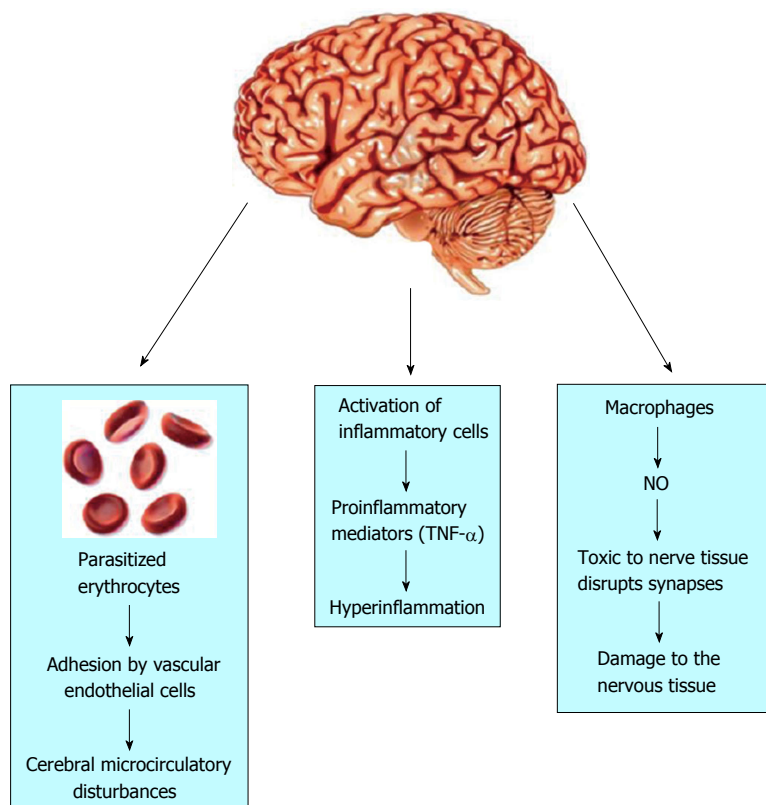
PATHOLOGICAL MECHANISMS

The pathological mechanisms that lead to neurological complications and mortality are not yet clearly defined. It is believed that in infected erythrocytes, platelets, and activated leukocytes inflammatory events occur owing to increased levels of adhesion molecules on the inflamed endothelium, leading to a reduction in microvascular blood flow, decreased delivery of nutrients to affected brain tissue and vessel walls, followed by hemorrhage and neuronal alterations^[21-23].

The blood-brain barrier (BBB) acts as a physical barrier that limits the trafficking of substances *via* transcellular transport and is responsible for regulating ion and nutrient transport into the brain, a feature that restricts the free flow of physiological molecules between the bloodstream and brain parenchyma^[18,24].

Conversely, perturbations to the BBB can lead to deregulation in any of the neurovascular components,

Figure 2 The pathological mechanisms that lead to neurological complications and mortality in patients. NO: Nitric oxide; TNF- α : Tumor necrosis factor-alpha.



which in turn can alter the brain's homeostasis leading to a multitude of neural dysfunctions and inappropriate BBB activation as observed in multiple sclerosis, Alzheimer's disease, stroke, certain depression disorders and parasitic infections, among others^[25-29]. Vascular dysfunction with subsequent BBB damage has been observed both in human CM and in animal models^[18,19,30].

The pathogenesis of CM is associated with cerebral microcirculatory disturbances resulting from the adhesion to and sequestration of parasitized erythrocytes, immune cells and platelets by vascular endothelial cells that line the small blood vessels of the brain, leading to their blockage^[30], as shown in Figure 2. In this regard, several studies provide evidence that in the erythrocytic phase the merozoites modify the surface of erythrocytes, inducing the expression of a surface protein-*Plasmodium falciparum* erythrocyte membrane protein 1-that has a strong affinity for adhesion molecules expressed on the surface of vascular endothelium, such as intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and platelet endothelial cell adhesion molecule 1, among others^[31].

In sequestration, *P. falciparum*-infected erythrocytes adhere to the brain endothelium through binding to PfEMP1^[32]. There is no evidence to date of infected erythrocyte entry into brain parenchyma, suggesting that these cells remain in the vascular space where they are sequestered. This sequestration of parasitized erythrocytes leads to multiple vascular effects, including the formation of clusters of agglomerated platelets and leukocytes, increased vasoconstriction, as well as the agglutination of erythrocytes not parasitized by generating so-called rosettes, which significantly reduce cerebral blood flow in

the capillaries and cause vascular obstruction, leading to hypoxia, brain parenchymal hemorrhage^[22] and disruption of BBB integrity^[11,33].

Moreover, hyperinflammation in the brain has also been related to CM and is another mechanism responsible for the vasculopathy observed during infection (Figure 2). Some studies report that during the inflammatory response, activation of inflammatory cells may occur accompanied by an overproduction of type-1 proinflammatory mediators, especially tumor necrosis factor-alpha (TNF- α), which is produced by microglia, astrocytes, monocytes and cerebral vascular endothelium^[34]. In humans, this cytokine induces the upregulation of adhesion molecules on endothelial cell surfaces, which contributes to the increased capture of erythrocytes in the cerebral capillaries and other organs^[25,35]. Furthermore, inflammation enhances nitric oxide (NO) production by macrophages, which seems linked to the pathogenesis of the disease, considering that it is extremely toxic to nerve tissue and disrupts synapses, contributing to the damage to the nervous tissue^[36]. In addition, inflammation can lead to micro- and ring hemorrhages and necrosis of surrounding tissues and cerebral edema, resulting in significant compression of cerebral arteries that can lead to death, as well as the various symptoms of CM, such as confusion or stupor of obtundation, or deep coma with long-term neurological deficits such as cortical blindness^[25,37].

Postmortem analyses of children who died with CM revealed that the axonal and myelin damage was associated with ring hemorrhages and vascular thrombosis in the cerebral and cerebellar white matter and brainstem. Disruption of the BBB and accumulation of monocytes

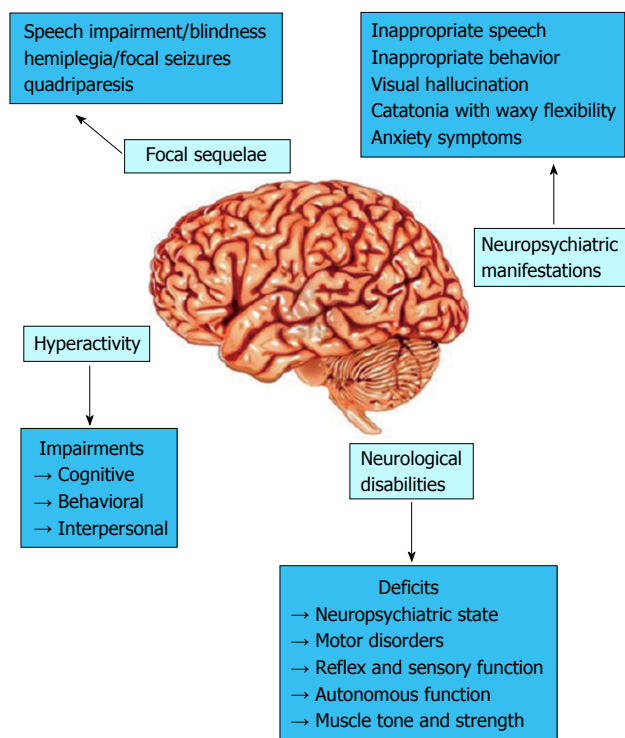


Figure 3 Neurological features of cerebral malaria.

with phagocytosed hemozoin within microvessels containing infected erythrocytes was found, suggesting a link between infected erythrocyte sequestration and intravascular/perivascular pathology in fatal pediatric CM^[38]. In animal models, the presence of apoptosis was also observed initially in endothelial cells and later in neurons and glia^[39], and may be associated with persistent cognitive impairment^[17]. These disturbances in the homeostasis of the cerebral microcirculation play an important role in the pathogenesis of CM, generating vascular obstructions, reduced cerebral blood flow and BBB disruption associated with high cerebral vasoconstriction^[24,40]. In addition, in the presence of seizures and/or fever, the metabolic demand increases with consequent risk of neural injury^[41], as shown in Figure 2.

NEUROLOGICAL FEATURES

Unfortunately, severe brain injury occurs after CM and 25% of pediatric cases result in epilepsy or long-term neurological and cognitive deficits^[42-44]. According to the time of symptom onset, CM may be classified into two patterns of neurological sequelae^[45], as shown in Figure 3. The first is immediate and characterized by coma and status epilepticus during the acute illness, resulting in focal sequelae such as hemiplegia and focal seizures, or multifocal sequelae with spastic quadriplegia, motor disorders, cognitive and behavioral impairment, blindness, speech or hearing impairment. The second pattern develops within months or years after CM, and behavioral deficits and/or epilepsy may occur.

Among gross motor deficits, hemiplegia, diplegia,

quadriplegia or quadriplegia may be observed after CM^[46]. Disorders in movement and gait can be noted, including ataxia, choreoathetosis, dystonia and poor neck control, as well as feeding difficulties^[46]. Dai *et al*^[47] demonstrated that motor coordination impairment was associated with dysregulation of Akt and GSK3 β signaling in a murine model of CM. The inhibition of the Akt pathway results in modifications in neuronal integrity, since it is a protein kinase playing a key role in the insulin signaling pathway and an important regulator of apoptosis, being consequently important for cell viability, although *via* an GSK3 β -dependent pathway. In addition, the intracranial hypertension may contribute to the motor sequelae, given that it reduces the cerebral perfusion pressure, nutrient and oxygen delivery and, where death does not occur, subsequent global ischemic injury and brainstem compression can lead to cerebral atrophy, which may result in motor and cognitive impairment^[48].

Convulsions in CM are common and inflammatory products such as quinolinic acid contribute to the neuropathology, considering that this metabolite from the kynurenine pathway is a N-methyl-D-aspartate agonist that causes neuroinflammation, convulsions, and cell death^[49-51]. Dobbie *et al*^[52] demonstrated that quinolinic acid provokes seizures in animals, possibly, altering the neurotransmission excitatory and triggering long-term deleterious effects on cognitive function and/or behavior. Sokol *et al*^[53] demonstrated irreversible neuron damage after long-term seizure activity, followed by gliosis and focal atrophy, resulting in more seizures and brain damage. The epilepsy (recurrence of seizures without apparent cause) occurs in approximately 10% of pediatric cases and may be occasioned by focal or global hypoxia or ischemia^[54,55]. The epileptogenesis mechanisms are unclear. Structural brain damage and the presence of Durck's malarial granuloma may contribute to the epileptogenesis mechanisms^[56]; however, other factors should also be considered, like genetic propensity^[57].

It has been observed that speech and language were the most common neurocognitive impairments found in Kenyan children who survived severe malaria^[58]. The authors suggested that language impairment may be part of a broad impairment that is most noted in the patterns of language, which probably contributes to deficits on verbal components of other cognitive assessments. On the other hand, Dugbartey^[59] reported that children affected by CM develop impairments in bimanual tactile discrimination, accuracy of visual scanning, visual memory, perceptual abstraction and rule learning skills, right ear auditory information processing, and dominant-hand motor speed. Other studies revealed deficits in spatial memory, mental processing, sequential processing, and attention tasks^[58,60,61]. Indeed, other kinds of memory, such as episodic memory, also seem to be affected by CM^[62].

Dai *et al*^[47,63] demonstrated that memory deficits either during or after successful treatment were associated with reduced Akt expression and dysregulation of Akt/GSK3 β signaling in a murine CM model. GSK3 β

plays a key role in the process of neurodevelopment and the transcription of brain derived neurotrophic factor, affecting long-term memory and synaptic plasticity^[64]. In this context, it has been associated with hyperphosphorylation of tau protein^[65], which is the major component in neurodegenerative disorders like Alzheimer's disease^[66]. Abnormal tau levels in the cerebral spinal fluid in CM survivors^[47,67] lead to long-term deficits in cognitive areas like memory, learning, language and psychiatric disorders^[17,42,68,69]. Specific damage in neuronal areas such as the hippocampus and sub-cortical white matter may lead to impairments in learning, memory and language function^[70-72].

Hyperactivity, impulsiveness and inattentiveness have also been observed in CM survivors^[45], similar to what occurs in attention deficit hyperactivity disorder (ADHD), which produces impairments in the cognitive, behavioral, and interpersonal domains^[73]. Dysregulated reward processing in the frontostriatal system has been proposed as a central mechanism in prevailing theoretical models of ADHD^[74,75], and altered dopamine signaling underlies a number of ADHD symptoms^[75]. Several anatomical changes in the brain are related to ADHD, including in the caudate nucleus, prefrontal cortex white matter, corpus callosum, cerebellar vermis^[76] and globus pallidus^[77], which are all areas that contain high densities of dopamine receptors. Most probably, damage occasioned by CM in the frontostriatal and cerebellar areas by a decrease in local blood flow or neuronal loss may produce impairments in dopamine signaling and consequently ADHD^[78].

Animal model parameters may reproduce some symptoms related to ADHD and stroke. In this regard, a murine study demonstrated a lower level of general activity associated with reduced response to touch escape and absent vocalization correlated with large areas of hemorrhage in animals with CM^[39]. These findings suggest an important influence of parenchymal hemorrhage distribution on the severity of neurological deficits in the late stage of the illness^[46].

An inflammatory cytokine profile has been associated with CNS dysfunction found in human and experimental CM. In the course of experimental CM induced by *Plasmodium berghei* (strain ANKA), leukocyte migration into the brain, as well as the production of TNF- α and chemokines (CCL2, CCL3, CCL5 and CXCL9) preceded neurological changes including in the neuropsychiatric state, motor behavior, autonomic function, muscle tone and strength, suggesting that the inflammatory changes may be involved in the neurological impairment^[79]. In this context, de Miranda *et al.*^[80] demonstrated an anxiety-like behavior in C57BL/6 mice infected with *P. berghei* using the elevated plus maze test. The anxiety symptoms were correlated with histopathological alterations in the brainstem, cerebrum and hippocampus and increased cerebral levels of interleukin-1 beta and TNF- α . In humans, Dugbartey *et al.*^[81] described anxiety disorders in a CM patient's recovery, suggesting that *falciparum* malaria is associated with enduring, albeit subclinical, anxiety and depressive

symptoms.

Some authors have reported correlations between neurological disabilities and glutamate levels and their contribution to the pathogenesis of these deficits, showing increased glutamate levels in cerebral spinal fluid and cerebrocortical synaptosomes from CM animals associated with alterations in neuropsychiatric state, motor behavior, reflex and sensory function, autonomic function, muscle tone and strength^[82]. Glutamate is the principal excitatory neurotransmitter in the mammalian CNS, participating in several cognitive and neurological functions under physiological conditions^[83]. Therefore, large amounts of glutamate release trigger neurotoxicity and neuronal cell death, being involved in neurodegenerative disorders^[84]. Thus, the imbalance in the neurotransmitter glutamate may be important in the establishing the pathogenesis mechanism of CM^[82].

The neuropsychiatric manifestations of post malaria neurological syndrome (PMNS) are highly variable and include an acute confusional state or acute psychosis with one or more of the following symptoms: inappropriate speech or behavior, visual hallucination, catatonia with waxy flexibility, generalized convulsion, fine postural tremor, clouding of consciousness and decreased muscle tone. It may occur within 2 mo after acute MC, with either neurologic or psychiatric symptoms^[85]. A case report on a Taiwan CM patient reported severe headache, dizziness, delirium and polyneuropathy within 2 mo after recovery^[86]; even psychotic symptoms with both visual and auditory hallucinations, aggressiveness, and inability to communicate have been related^[87,88] that can last for 12 d. The symptoms observed may not be attributed only to CM, since other factors could be responsible; however, the neurologic and psychiatric presentations were compatible with PMNS and the mechanisms are the same as those related to other neurologic CM deficits, including cerebral hypoperfusion and immunologic mechanism, which prompts psychosis in a small minority^[88].

CONCLUSION

Malaria is a parasitic disease that can affect the CNS, altering cognitive and behavioral functions. Neurological and behavioral changes described in the course of experimental or human CM are mainly a consequence of brain hyperinflammation, vascular obstruction, reduced cerebral blood flow, and disruption of the BBB associated with high levels of cerebral vasoconstriction, thrombus, ring hemorrhage, ruptured capillaries, and cerebral blood vessels filled with infected erythrocytes, with consequent axonal damage and demyelination. Additionally, neurologic alterations have been observed as motor deficits, seizures and epilepsy; neurocognitive impairment in language, speech, learning, and memory; and behavioral damage with hyperactivity, anxiety, PMNS and psychosis.

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Physics and mathematics of magnetic resonance imaging for nanomedicine: An overview

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Abstract

Magnetic resonance imaging (MRI), magnetic resonance angiography (MRA) and magnetic resonance spectroscopy (MRS) are fundamental concepts used in modern medicine to improve health care. These concepts are based on the principle of nuclear magnetic resonance (NMR). Over the years, various laboratories around the world have applied different numerical techniques based on the Bloch NMR equations to solve specific problems in physics, biology, chemistry, engineering and medicine. The ultimate goal of any physician is to obtain maximum physical, biophysical, chemical and biological information on any tissue or cell under examination. This goal can be achieved by solving the Bloch NMR flow equations analytically. In this review, we present the basic principle of NMR/MRI in a way that can be easily understood by any researcher who needs an NMR concept to solve a specific medical problems. After a very brief history of the subject, a second order, non homogeneous, time-dependent differential equation derived from the Bloch NMR equation is presented. This equation has the basic intrinsic properties of MRI, MRA and MRS that can be extracted by means of classical and quantum mechanics for possible application in nanomedicine.

reserved.

Key words: Bloch flow equations; Rotational diffusion; Molecular dynamics of biological fluids; Nuclear magnetic resonance diffusion equation; Rotational correlation time; Spherical harmonics; Molecular flow

Core tip: Magnetic resonance imaging is one of the most powerful methods for investigating structural and dynamics of biological matter. Based on quantum mechanical principles applied to Bloch nuclear magnetic resonance (NMR) flow equations, we aimed to apply the analytical solutions obtained from the Bloch NMR flow equations to nanomedicine. This may trigger research towards the design of nano devices that capable of delivering drugs directly to specifically targeted cells, with the possibility of very early diagnosis of diseases and treating them with powerful drugs at the pathological site alone, reducing any harmful side effects.

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INTRODUCTION

Nuclear magnetic resonance (NMR) is a very important analytical and experimental tool for physical, chemical and structural analysis of certain organic materials. Magnetic resonance is a branch of spectroscopy that detects the quantum-mechanical transitions induced by electromagnetic (EM) radiation in a system of discrete energy levels of electrons or nuclei placed in a static magnetic field^[1,2]. NMR employs EM waves in the radio-frequency range between 900 MHz and 2 KHz. Some nuclei experience nuclear resonance, while others do not. Exhibition

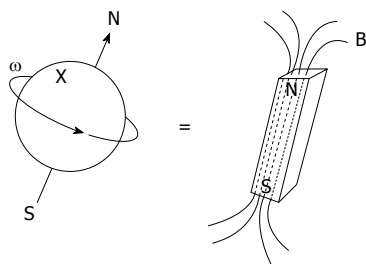


Figure 1 The charged nucleus (for example, ^1H) rotating with angular frequency $\omega = 2\pi\nu$ creates a magnetic field B and is equivalent to a small bar magnet whose axis is coincident with the spin rotation axis^[4].

of this phenomenon is dependent on whether they possess a property called “spin”^[2].

NMR is one of the most powerful methods for investigating the structure and dynamics of matter in different states of aggregation. This is due to the following features: (1) the interactions of nuclear magnetic moments are very weak compared with the thermal energy; therefore, we are dealing with para-magnetism. Moreover, the energy delivered by the radio-frequency generator are much larger compared with the strength of these inter-nuclear couplings. That leads to the possibility of manipulating these interactions in a specific way and simplifying the spectral response; (2) the radio-frequency photons have much lower energy compared with the energy of chemical bonds. Therefore, the interaction of EM radiation with matter, particularly biomolecules, is non-ionizing; and (3) the number of radio-frequency photons with a specific frequency is very large. Hence, the phase of the associated EM wave is very well defined. The high degree of coherence of radio-frequency radiation is essential to implement NMR experiments, including magnetic resonance imaging (MRI)^[3].

SPIN

Spin is a fundamental property of nature, like electrical charge or mass. Spin comes in multiples of $1/2$ and can be positive (+) or negative (-). Protons, electrons and neutrons possess spins. Individual unpaired electrons, protons and neutrons each possess a spin of $1/2$. In the deuterium atom (^2H), for example, with one unpaired electron, one unpaired proton and one unpaired neutron, the total electronic spin is equal to $1/2$ and the total nuclear spin is equal to 1. Two or more particles with spins having opposite signs can pair up to eliminate the observable manifestations of spin. An example is helium, (^4He). In NMR, it is the unpaired nuclear spins that are important. When placed in a magnetic field of strength B , a particle with a net spin can absorb a photon, of frequency ω . The frequency of ω depends on the gyromagnetic ratio γ , of the particle [as shown in equation (1)], given by the expression:

$$\omega = \gamma B \quad (1)$$

For hydrogen nuclei, the gyromagnetic ratio $\gamma = 42.58$ MHz/T^[4]. Nuclei are composed of positively charged

Table 1 Properties of nuclei most useful for biological studies^[5]

Nucleus	Spin quantum number (I)	Natural abundance (%)	Gyromagnetic ratio γ (10^{-7} rad/T sec)	Sensitivity ¹ (% vs ^1H)	Electric quadrupole moment (Q) ($\text{e} \cdot 10^{-24} \text{ cm}^2$)
^1H	1/2	99.9844	26.7520	100.000	-
^2H	1/1	0.0156	4.1067	0.965	0.00277
^{13}C	1/2	1.1080	6.7265	1.590	-
^{15}N	1/2	0.3650	-2.7108	0.104	-
^{19}F	1/2	100.0000	25.167	83.300	-
^{31}P	1/2	100.0000	10.829	6.630	-

¹Relative sensitivity for equal number of nuclei at constant magnetic field strength.

protons and uncharged neutrons held together by nuclear forces^[4,5], as shown in Figure 1.

The shell model for the nucleus tells us that nucleons, just like electrons, fill orbitals. When the number of protons or neutrons equals 2, 8, 20, 28, 50, 82 and 126, the orbitals are filled, because nucleons have spin, just like electrons do, and their spins can pair up when the orbitals are being filled and cancel out. Almost every element in the periodic table has an isotope with a non-zero nuclear spin^[4,5]. NMR can only be performed on isotopes whose natural abundance is high enough to be detected; some of the nuclei that are of interest in NMR/MRI are listed in Table 1.

We have seen that $\omega = \gamma B$ and hence the energy of the radio waves needed to cause a transition between the two spin states is given by equation (2):

$$E = \eta \gamma B \quad (2)$$

When the energy of the photon matches the energy difference between the two spin states, absorption of energy occurs. In an NMR experiment, the frequency of the photon is in the radio frequency (RF) range. In NMR spectroscopy, ω is between 600 and 800 MHz for hydrogen nuclei. However, in clinical MRI, ω is typically between 15 and 80 MHz for hydrogen imaging^[6] (Table 2).

To get a better understanding of how particles with spin behave under a magnetic field, we consider a proton that has a spin property. If we imagine the spin of this proton as a magnetic moment vector, causing the proton to behave like a tiny magnet with a North and South Poles. When the proton is placed in an external magnetic field, the spin vector of the particle aligns itself with the external field, just like a magnet would. There is a low energy configuration or state where the poles are aligned N-S-N-S and a high energy state N-N-S-S.

This particle can undergo a transition between the two energy states by the absorption of a photon. A particle in the lower energy state absorbs a photon and ends up in the higher energy state. The energy of this photon must exactly match the energy difference between the two states. The energy E , of a photon is related to its frequency ω , by Planck's constant ($\eta = h/2\pi$, $h = 6.626 \times 10^{-34}$ Js).

Table 2 Nuclear Spin values and gyromagnetic ratios of some nuclei^[5]

Nuclei	Unpaired protons	Unpaired neutrons	Net spin	γ (MHz/T)
^1H	1	0	1/2	42.58
^2H	1	1	1/1	6.54
^{31}P	1	0	1/2	17.25
^{23}Na	1	2	3/2	11.27
^{14}N	1	1	1/1	3.08
^{13}C	0	1	1/2	10.71
^{19}F	1	0	1/2	40.08

$$E = \hbar\omega \quad (3)$$

In NMR and MRI, the quantity ω is called the resonance frequency or the Larmor Frequency^[6].

MRI

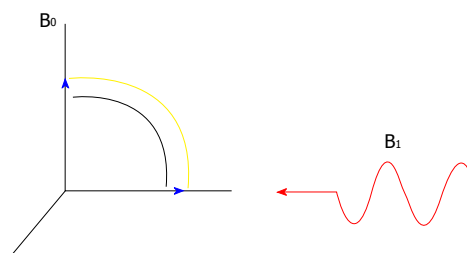
MRI is an imaging technique used primarily in medical settings to produce high quality images of the inside of the human body. MRI is based on the principles of NMR, a spectroscopic technique used by scientists to obtain microscopic chemical and physical information about molecules. The technique was called MRI rather than nuclear MRI because of the negative connotations associated with the word nuclear in the late 1970's. MRI started as a tomographic imaging technique; that is, it produced an image of the NMR signal in a thin slice through the human body. MRI has advanced from a tomographic imaging technique to a volume imaging technique. MRI is based on the absorption and emission of energy in the RF range of the EM spectrum^[3,7].

In the past, many scientists were taught that one cannot obtain an image smaller than the wavelength of the energy being used to image it. MRI gets around this limitation by producing images based on spatial variations in the phase and frequency of the RF energy being absorbed and emitted by the imaged object.

Clinical MRI uses the magnetic properties of hydrogen and its interaction with both a large external magnetic field and radio waves to produce highly detailed images of the human body. Hydrogen has a significant magnetic moment and is the most abundant nucleus in the human body. For these reasons, we use only the hydrogen proton in routine clinical imaging^[7].

To perform MRI, we first need a strong magnetic field. The field strength of the magnets used for MR is measured in units of Tesla. One (1) Tesla is equal to 10000 Gauss. The magnetic field of the earth is approximately 0.5 Gauss. Given that relationship, a 1.0 T magnet has a magnetic field approximately 20000 times stronger than that of the earth. The type of magnets used for MRI usually belongs to one of three types; permanent, resistive, and superconductive.

A permanent magnet is sometimes referred to as a vertical field magnet. These magnets are constructed of two magnets (one at each pole). The patient lies on a scanning table between these two plates^[8].

**Figure 2** A 90-degree flip of the net magnetization.

The advantages of these systems are: relatively low cost, no electricity or cryogenic liquids are needed to maintain the magnetic field, their more open design may help alleviate some patient anxiety and their nearly non-existent fringe field. It should be noted that not all vertical field magnets are permanent magnets.

Resistive magnets are constructed from a coil of wire. The more turns to the coil, and the more current in the coil, the higher the magnetic field. These types of magnets are most often designed to produce a horizontal field because of their solenoid design. As previously mentioned, some vertical field systems are based on resistive magnets. The main advantages of these types of magnets are: no liquid cryogen, the ability to “turn off” the magnetic field and their relatively small fringe field.

Superconducting magnets are the most common. They are made from coils of wire (as are resistive magnets) and thus produce a horizontal field. They use liquid helium to keep the magnet wire at 4 degrees Kelvin where there is no resistance. The current flows through the wire without having to be connected to an external power source. The main advantage of superconducting magnets is their ability to attain field strengths of up to 3 Tesla for clinical imagers and up to 10 Tesla or more for small bore spectroscopy magnets^[9].

CREATION OF AN MR SIGNAL

A radio wave is actually an oscillating EM field. The RF field is also referred to as the B_1 field. It is oriented perpendicular to the main magnetic field (B_0). If we apply a pulse of RF energy into the tissue at the Larmor frequency, we first find the individual spins begin to precess in phase, as will the net magnetization vector. As the RF pulse continues, some of the spins in the lower energy state absorb energy from the RF field and make a transition into the higher energy state. This has the effect of “tipping” the net magnetization toward the transverse plane. This phenomenon is illustrated in Figure 2. For the purpose of this explanation, we will assume sufficient energy is applied to produce a 90-degree flip of the net magnetization. In such an example, it is said that a 90-degree flip angle or a 90-degree pulse has been applied^[10].

Oriented perpendicular to B_0 is a receiver coil. As the magnetization (now referred to as transverse magnetization, or M_{xy}) precesses through the receiver coil, a current or signal is induced in the coil. The principle behind

this signal induction is Faraday's Law of Induction. This states that if a magnetic field is moved through a conductor, a current will be produced in the conductor. If we increase the size of the magnetic field, or increase the speed with which it moves, we will increase the size of the signal (current) induced in the conductor.

To detect the signal produced in the coil, the transmitter must be turned off. When the RF pulse is discontinued, the signal in the coil begins at given amplitude (determined by the amount of magnetization precessing in the transverse plane (Figure 2) and the precessional frequency) and fades rapidly away. This initial signal is referred to as the Free Induction Decay (FID). The signal fades as the individual spins contributing to the net magnetization lose their phase coherence, making the vector sum equal to zero. At the same time, but independently, some of the spins that had moved into the higher energy state give off their energy to their lattice and return to the lower energy state, causing the net magnetization to re-grow along the z axis. This re-growth occurs at a rate given by the tissue relaxation parameter, known as T_1 ^[9,10].

DEFINITION OF TERMS IN NMR/MRI

Spin packets

A spin packet is a group of spins experiencing the same magnetic field strength. At any instant in time, the magnetic field caused by the spins in each spin packet can be represented by a magnetization vector; \vec{M} . The vector sum of the magnetization vectors from all the spin packets is the net magnetization. Adapting the conventional NMR coordinate system, the external magnetic field and the net magnetization vector at equilibrium are both along the Z axis.

T_1 relaxation time

The time constant that describes how M_z returns to its equilibrium value is called the spin lattice relaxation time (T_1). The equation governing this behavior as a function of time t after its displacement is:

$$M_z = M_0 (1 - e^{-t/T_1}) \quad (4)$$

At equilibrium, the net magnetization vector lies along the direction of the applied magnetic field B_0 and is called the equilibrium magnetization M_0 . In this configuration, the Z component of magnetization M_z equals M_0 . M_z is referred to as the longitudinal magnetization. There is no transverse (M_x or M_y) magnetization here.

Larmor frequency

The resonant frequency of a nucleus is determined by a combination of nuclear characteristics and the strength of the magnetic field. The specific relationship between resonant frequency and the field strength is an inherent characteristic of each nuclide and is generally designated as gyromagnetic ratio γ . The resonant frequency is also known as the Larmor frequency.

T_2 relaxation time

The time constant that describes the return to equilib-

rium of the transverse magnetization, M_{xy} , is called the spin-spin relaxation time, T_2 .

$$M_{xy} = M_{xy0} e^{-t/T_2} \quad (5)$$

T_2 is always less than or equal to T_1 . The net magnetization in the XY plane goes to zero and then the longitudinal magnetization grows in until we have M_0 along Z.

Excitation

If a pulse of RF energy with a frequency corresponding to the nuclear precession rate is applied to a material, some of the energy will be absorbed by the individual nuclei. The absorption of energy by a nucleus flips its alignment away from the direction of the magnetic field. This increased energy places the nucleus in an excited state. In this excited state, the precession is now transformed into a spinning motion of the nucleus around the axis of the magnetic field^[11-14].

BRIEF HISTORY ON THE DEVELOPMENT OF NMR AND MRI

The history of the development of the concept of NMR started with Felix Bloch at Harvard, and Edward Purcell at Stanford, both of whom were awarded the Nobel Prize in 1952, discovered the magnetic resonance phenomenon independently in 1946, using different instrumentation. In the period between 1950 and 1970, NMR was developed and used for chemical and physical molecular analysis. In 1971 Raymond Damadian (an Armenian-American medical practitioner and inventor of the first MR Scanning Machine) showed that the nuclear magnetic relaxation times of tissues and tumors differed, thus motivating scientists to consider magnetic resonance for the detection of disease. In 1973 the X-ray-based computerized tomography (CT) was introduced by Hounsfield. MRI was first demonstrated on small test tube samples that same year by Paul Lauterbur. He used a technique similar to that used in CT. In 1975 Richard Ernst, a Swiss physical chemist, proposed MRI using phase and frequency encoding, and the Fourier Transform. This technique is the basis of current MRI techniques. A few years later, in 1977, Raymond Damadian demonstrated MRI called field-focusing NMR. In this same year, Peter Mansfield developed the echo-planar imaging (EPI) technique. This technique was later developed to produce images at video rates (30 ms/image). Edelstein and coworkers demonstrated imaging of the body using Ernst's technique in 1980. A single image could be acquired in approximately five minutes by this technique. By 1986, imaging time was reduced to about five seconds, without sacrificing significant image quality. In the same year, the NMR microscope was developed, which allowed approximately 10m resolution on approximately one cm samples. In 1987 EPI was used to perform real-time moving imaging of a single cardiac cycle. In this same year, Charles Dumoulin perfected magnetic resonance angiography (MRA), which allowed imaging of flowing blood without the use of contrast agents.

In 1991, Richard Ernst was rewarded for his achievements in pulsed Fourier Transform NMR and MRI with the Nobel Prize in Chemistry. In 1992 functional MRI (fMRI) was developed. This technique allows the mapping of the functions of the various regions of the human brain. Five years earlier, many clinicians thought EPI's primary application was to be in real-time cardiac imaging. The development of fMRI opened up a new application for EPI in mapping the regions of the brain responsible for thought and motor control. In 1994, researchers at the State University of New York at Stony Brook and Princeton University demonstrated the imaging of hyperpolarized ^{129}Xe gas for respiration studies.

In 2003, Paul C Lauterbur of the University of Illinois and Sir Peter Mansfield of the University of Nottingham were awarded the Nobel Prize in Medicine for their discoveries concerning MRI. MRI is clearly a young, but growing science^[1,3,5-16].

THE THEORY OF NMR

The appearance of NMR spectra, and consequently the molecular structure they are able to provide, arises from the discrete nature of the energy levels pertaining to a nuclear spin system. The energy levels are mainly a result of Zeeman interaction, $-\vec{\mu}\vec{B}_0$ between the static magnetic field of induction \vec{B}_0 and nuclear magnetic moment $\vec{\mu}$. The quantum-mechanical quantity called spin momentum, \vec{I} is related to magnetic moment by $\vec{\mu} = \gamma\eta\vec{I}$, where γ is the gyromagnetic ratio and η is the Planck's constant divided by 2π .

In the absence of the magnetic nuclear, the spin states are generated. The application of a static magnetic field \vec{B}_0 which induces a magnetic interaction, is described by Zeeman Hamiltonian $H = -\vec{\mu}\vec{B}_0$. Taking the magnetic field orientation to be along the z-direction we get:

$$H = -\gamma\eta B_0 L_z \quad (6)$$

The Eigen values E_m of this Hamiltonian can be evaluated from the Schrodinger equation

$$H|m\rangle = -\gamma\eta B_0 m|m\rangle \quad (7)$$

where $|m\rangle$ is the Eigen state corresponding to the Eigen value $E_m = -\gamma\eta B_0 m$. The magnetic quantum number is m , where $m = l, l-1, \dots, -l$. Therefore, the equidistant energy differences are for the single-quantum transitions $m = \pm 1$ given by^[1,4,5,7]

$$\Delta E = \eta\omega_0 \quad (8)$$

where the Larmor frequency is defined as^[1,4,5,7]

$$\nu_0 = \nu_L = \omega_0/2\pi$$

Another important ingredient for a magnetic resonance experiment is represented by the presence of the RF field. Only the magnetic component of the EM field, *i.e.*, $B_1(t) = B_{10} \cos(2\pi\nu t)$ interacts with the magnetic moment of the nuclei. The amplitude of the RF field is B_{10} and ν is the carrier frequency. This field is produced by an RF coil and leads to a perturbation Hamiltonian:

$$\vec{H}_p = -\gamma\eta\vec{B}_{10}\vec{I} \cos(2\pi\nu t) \quad (9)$$

From the time-dependent perturbation theory of quantum mechanics, it can be stated that a transition between two states $|\psi\rangle$ and $|\phi\rangle$ is allowed, provided that $\langle\psi|H_p|\phi\rangle \neq 0$. This takes place if $\nu \approx \nu_0$ (*i.e.*, the resonance condition) and the alternative magnetic field \vec{B}_0 is polarized perpendicular to the static magnetic field \vec{B}_{10} ^[11].

In general, NMR experiments are performed at high temperatures, employing a large number of spins. These features lead to the possibility to treat classically some aspects of the experiments. The excess of spins oriented along the static magnetic field \vec{B}_0 with respect to those oriented in the opposite direction results in a macroscopic nuclear magnetization \vec{M} , aligned along the static magnetic field, which is called the equilibrium magnetization. It can be displaced from this equilibrium by an appropriate perturbation, for instance, by an RF excitation. It is then subject to a precessional motion around \vec{B}_0 with the Larmor frequency ν_L . The EM perturbation that brings \vec{M} into a plane perpendicular to \vec{B}_0 allows the observation of the Larmor precession through an electromotive force that occurs in a coil whose axis is contained in that plane. This can be done by rotation of the magnetization using a resonant 90° RF pulse. The nuclear magnetization \vec{M} can be oriented antiparallel to \vec{B}_0 by the action of a 180° pulse. The majority of NMR experiments used pulse sequences composed of 90° and 180° RF pulses^[9-18].

PULSED NMR SPECTROSCOPY

A coil of wire placed around the x-axis will provide a magnetic field along the x-axis when a direct current is passed through the coil. An alternating current will produce a magnetic field that alternates in direction. In a frame of reference rotating about the z-axis at a frequency equal to that of the alternating current, the magnetic field along the x'-axis will be constant, just as in the direct current case in the laboratory frame. This is the same as moving the coil about the rotating frame coordinate system at the Larmor Frequency. In magnetic resonance, the magnetic field created by the coil passing an alternating current at the Larmor frequency is called the B_1 magnetic field. When the alternating current through the coil is turned on and off it creates a pulsed B_1 magnetic field along the x'-axis. The spins respond to this pulse in such a way as to cause the net magnetization vector to rotate about the direction of the applied B_1 field. The rotation angle depends on the length of time τ for which the field is switched on and its magnitude, B_1 ^[2,6,7,9].

A 90° pulse is one that rotates the magnetization vector clockwise by 90° about the x'-axis and rotates the equilibrium magnetization down to the y'-axis. In the laboratory frame, the equilibrium magnetization spirals down around the z-axis to the xy-plane. One can now see why the rotating frame of reference is helpful in describing the behavior of magnetization in response to a pulsed magnetic field. A 180° pulse will rotate the magnetization vector by 180° and rotates the equilibrium magnetization

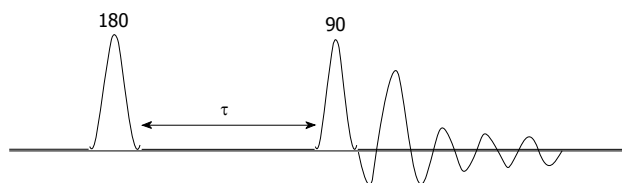


Figure 3 A series of spectra recorded with different values of τ to map out the recovery of the magnetization.

down along the z-axis.

The net magnetization at any orientation will behave according to the rotation equation. For example, a net magnetization vector along the y' -axis will end up along the y' -axis when acted upon by a 180° pulse of B_1 along the x' -axis. A net magnetization vector between x' - and y' - will end up between x' and y' after the application of 180° pulse of B_1 applied along the x' -axis^[9].

Longitudinal magnetization is aligned along the field axis B_0 (that is, the z-axis) and has a finite equilibrium value of M_{z0} . M_{z0} represents the equilibrium magnetization at the start of any NMR experiment.

Transverse magnetization is perpendicular to B_0 , precesses around the z-axis at the Larmor frequency and has an equilibrium value of zero^[13].

$$\omega = 2\pi\nu = -\gamma B_0$$

A simple 1D NMR spectrum is obtained by a 90° pulse, followed by detection of the FID and Fourier transformation of the FID. More complicated pulse sequences allow measurement of specific parameters, 2D and multi-dimensional data.

MEASUREMENT OF T_1 RELAXATION TIMES

The inversion-recovery (IR) pulse sequence can be used to measure the T_1 relaxation times of all the resonances in a spectrum. IR pulse sequence is pictorially represented in Figure 3.

Alternatively, the zero crossing point for each peak can be used to estimate the T_1 . Using the time τ_0 at which the signal I is zero:

$$\tau_0 = T_1 \ln 2 = 0.693 T_1 \quad (10)$$

This provides a very useful spot check for the value of T_1 in any sample. We must note that the relaxation delay between subsequent scans must be set to at least $5 T_1$ for experiments for good estimates of T_1 .

The saturation-recovery pulse sequence starts from perfectly equal populations of α and β spin states ($M_z = 0$, achievable by saturation). During a subsequent waiting period t , the z-magnetization reappears in an exponential recovery until it has assumed equilibrium value M_{z0} for a very long time t :

$$M_z = M_{z0} (1 - e^{-t/T_1}) \quad (11)$$

An experiment of this nature does not require long recovery delays between scans; however, saturation of the spin states is not always easy^[10].

PRACTICAL IMPORTANCE OF T_1 RELAXATION

Recovery delays

Any pulsed NMR experiment is repeated many times, and the scans added together to improve signal-to-noise (S/N) ratio. If the signals are not fully relaxed before each pulse/acquisition, then the signal in each scan will progressively decrease.

A recovery delay of about $5 \times T_1$ between subsequent scans almost completely restores M_{z0} , yielding reliable integrals. Recovery delays of about $1.4 T_1$ maximizes S/N ratio, but the integrals of slowly relaxing signals will be smaller than those of the rapidly relaxing signals.

Solvent saturation

If a signal, or a whole spectrum, is irradiated continuously with RF, then the populations N_α and N_β will equalize and no signal can be observed. This can be used for selective removal of a peak, e.g., a solvent peak from a spectrum. For example, in biological samples, H_2O would yield a huge signal without solvent suppression. A simple way of solvent suppression is presented by selective saturation by pre-irradiation = weak irradiation of the solvent signal during the recovery delay before the 90° pulse^[10,18].

Weak interactions between the small magnetic moments of nuclear spins with the environment result in slow relaxation rates and allow the design of long and complicated pulse sequences. In contrast to optical spectroscopy, nuclear spins can access only a finite number of energy levels. This allows the accurate calculation of the outcome of pulse sequences. Transverse magnetization can be destroyed by a "pulsed field gradient": an electric current is sent through a gradient coil for a few milliseconds. This results in a wide distribution of Larmor frequencies during the gradient and, hence, averaging of the transverse magnetization vectors to zero^[18].

NUCLEAR ENERGIES IN A MAGNETIC FIELD

The energy of the magnetic moment of a nuclear spin in a magnetic field is given by

$$E = -\mu B \quad (12)$$

where μ is the spin angular momentum and B is the magnetic field (in general B is a vector quantity). To conduct an NMR experiment, a sample is first placed in a static field. By convention, the direction of the static magnetic field is along the z-axis, and the magnitude of the magnetic field is given by B_0 (no longer a vector because it points only along the z-axis). In this case, the energy of a nuclear spin in an NMR magnet becomes

$$E = -\mu_z B_0 = -\gamma I_z B_0 \quad (13)$$

where γ is the gyromagnetic ratio (sometimes called the magnetogyric ratio) and I_z is the spin quantum number in the z-axis direction. The energy associated with a particu-

lar quantum number m is given as:

$$E_m = -m\hbar\gamma B_0 \quad (14)$$

NMR transition energies are very small. These small energies translate into low sensitivity. When samples are placed into a magnetic field, a small excess of nuclei fall into the α state. This excess of spins in the α over the β states accounts for the entire net magnetization that is used in the NMR experiment. The ratio of the number of spins in the β state to those in the α state is given by a Boltzmann distribution:

$$N_\alpha/N_\beta = e^{\Delta E/(\hbar\gamma T)} \quad (15)$$

where ΔE is the difference of energies of the α and β states, $\hbar\gamma$ is Boltzmann constant, and T is the absolute temperature. Higher magnetic fields produce correspondingly larger differences in spin states, leading to greater sensitivity^[12,14,15].

BULK MAGNETIZATION IN AN ELECTRIC FIELD

If the bulk magnetization is along the field direction, as it is at equilibrium, then there is no torque and hence no motion. As expected, at equilibrium the system is stationary. If the system is away from equilibrium and the bulk magnetization vector is oriented other than along the z-axis, then the magnetization precesses (rotates) about the z-axis with an angular velocity given by the energy separation of the two states (γB_0). This torque will not change the length of the magnetization vector; it only varies its orientation. This orientation cannot be the only motion, since the system would never return to equilibrium. Therefore, along with the rotation, there is a relaxation of the vector to bring it back along the z-axis. Therefore the x- and y-components of the nuclear magnetization decay towards zero, and the z-component decays towards the equilibrium value (M_0)^[4,10,14].

Considering bulk magnetization M that arises from all the magnetic moments in a sample, M experiences a torque when placed in a magnetic field, according to the expression given below:

$$dJ(t)/dt = M(t) \times B(t) \quad (16)$$

where $J(t)$ is the bulk spin angular momentum. The vector quantities in equation (16) are time dependent. The time-dependence of the magnetic field comes about when we apply RF pulses along the x- or y-axis. Equation (16) is essentially identical to an equation that describes the motion of a gyroscope^[18-20]:

$$dL(t)/dt = r \times mg \quad (17)$$

where $L(t)$ is the gyroscope's angular momentum, r the radius from the fixed point of rotation, m is the mass, and g is gravity. Thus, a nuclear spin in a magnetic field will behave much like a gyroscope in a gravitational field. To make equation (16) useful, we use the relationship for the z-component of the magnetic moment:

$$\mu_z = \gamma L_z = \gamma \hbar m \quad (18)$$

Then multiply each side by γ to yield

$$dM(t)/dt = M(t) \times \gamma B(t) \quad (19)$$

Equation (19) is the basis of the Bloch equations^[18-20].

BLOCH EQUATIONS

In 1946 Felix Bloch formulated a set of equations that describe the behavior of nuclear spin in a magnetic field under the influence of RF pulses. He modified equation (19), given above, to account for the observation that nuclear spins “relax” to equilibrium values following the application of RF pulses. Bloch assumed they relax along the z-axis and in the x-y plane at different rates, but following first order kinetics. These rates are designated $1/T_1$ and $1/T_2$ for the z-axis and x-y plane, respectively. T_1 is called spin-lattice relaxation and T_2 the spin-spin relaxation. With the addition of relaxation, equation (19) becomes:

$$dM(t)/dt = M(t) \times \gamma B(t) - R [M(t) - M_0] \quad (20)$$

where R is the “relaxation matrix”. Equation (20) can best be explained by considering each of its components:

$$\begin{aligned} dM_z(t)/dt &= \gamma [M_x(t) B_y(t) \times M_y(t) B_x(t)] - [M_z(t) - M_0]/T_1 \\ dM_x(t)/dt &= \gamma [M_y(t) B_z(t) \times M_z(t) B_y(t)] - M_x(t)/T_2 \\ dM_y(t)/dt &= \gamma [M_z(t) B_x(t) \times M_x(t) B_z(t)] - M_y(t)/T_2 \end{aligned} \quad (21)$$

The terms in equation (21) that do not involve either T_1 or T_2 are the result of the cross product in equation (20). Equation (21) describes the motion of magnetization in the “laboratory frame”, an ordinary coordinate system is stationary. Mathematically, the laboratory frame is not the simplest coordinate system, because the magnetization is moving at a frequency $\omega_0 = \gamma B_0$ in the x-y (transverse) plane. A simpler coordinate system is the “rotating frame”, in which the x-y plane rotates around the z-axis at a frequency $\Omega = -\gamma B_0$. In the rotating frame, magnetization “on resonance” does not precess in the transverse plane. The transformation of equation (21) to the rotating frame is achieved by replacing each B_z (defined as B_0) by Ω/λ :

$$\begin{aligned} dM_z(t)/dt &= \gamma [M_x(t) B_y^r(t) - M_y(t) B_x^r(t)] - [M_z(t) - M_0]/T_1 \\ dM_x(t)/dt &= -\Omega M_y(t) - \gamma M_z(t) B_y^r(t) - M_x(t)/T_2 \\ dM_y(t)/dt &= \gamma M_z(t) B_x^r(t) + \Omega M_x(t) - M_y(t)/T_2 \end{aligned} \quad (22)$$

In equation (22), the components of B have been written with r superscripts to denote that it is a rotating frame^[17-23].

PHYSICAL INTERPRETATION OF BLOCH EQUATIONS

We shall examine the behavior of equation (22) under two different limiting conditions, the effect of a short RF pulse and free precession. The RF pulse will be assumed to be very short compared to either relaxation times T_1 and T_2 , as well as the angular frequency Ω . This assumption is valid for many typical pulsed NMR experiments, in which the pulse lengths can be as short as 5 μ s. If the

RF pulse is applied along the x-axis, these conditions will allow us to neglect terms in equation (22) that contain T_1 , T_2 , Ω , and B_y .

$$\begin{aligned} dM_z/dt &= -M_y(t) \gamma B_x(t) \\ dM_x(t)/dt &= 0 \\ dM_y(t)/dt &= M_z(t) \gamma B_x(t) \end{aligned} \quad (23)$$

Before solving equation (23), we need to discuss the meaning of $B_x(t)$ and $B_y(t)$. We can recall that B_0 is the static magnetic field strength oriented along the z-axis. $B_x(t)$ and $B_y(t)$ are magnetic fields oriented along the x- and y-axes that are generated by rf pulses. By analogy to $\omega_0 = \gamma B_0$ defining the frequency of the NMR transitions in the static magnetic field, we can see that the terms $\gamma B_x(t)$ and $\gamma B_y(t)$ are frequencies of the magnetization rotating around the x- or y-axis. Thus, applying these frequencies for different periods of time will allow for different degrees of rotation around the x- or y-axis. If we introduce a frequency of rotation about the x-axis as $\omega_x = \gamma B_x(t)$, solutions to equation (23) can now be given as:

$$\begin{aligned} M_z(t) &= M_0 \cos(\omega_x t) \\ M_x(t) &= 0 \\ M_y(t) &= M_0 \sin(\omega_x t) \end{aligned} \quad (24)$$

Finally, if we let $\theta = \omega_x t$ be the pulse angle, equation (24) shows that application of a magnetic field (RF pulse) along the x-axis causes the magnetization that was originally along the z-axis to rotate toward the y-axis by an angle θ . Note that when $\theta = 0$, $M_z(t) = M_0$ and $M_y(t) = 0$ (all the magnetization is still pointing along the z-axis). When $\theta = 90^\circ$, $M_y(t) = M_0$ and $M_z(t) = 0$ (all the magnetization is still pointing along the y-axis). These have described the effects of a simple RF pulse. The second limiting condition for equation (22) is free precession in the absence of any applied pulse. In that case, B_x and B_y are both equal to zero, and equation (22) becomes:

$$\begin{aligned} dM_z(t)/dt &= -[M_z(t) - M_0]/T_1 \\ dM_x(t)/dt &= -\Omega M_y(t) - M_x(t)/T_2 \\ dM_y(t)/dt &= -\Omega M_x(t) - M_y(t)/T_2 \end{aligned} \quad (25)$$

The solutions to equation (25) can be given as:

$$\begin{aligned} M_z(t) &= M_0 (1 - e^{-t/T_1}) \\ M_x(t) &= M_0 \cos(\Omega t) e^{-t/T_2} \\ M_y(t) &= M_0 \sin(\Omega t) e^{-t/T_2} \end{aligned} \quad (26)$$

Equation (26) describes magnetization precessing in the x-y plane at a frequency Ω , while it is relaxing along the z-axis at a rate of $1/T_1$ and relaxing in the x-y plane at a rate $1/T_2$ ^[17-19,21,24].

THE GENERAL BLOCH NMR FLOW EQUATION

The Bloch NMR flow equations can be written as^[25,26]:

$$\partial M_x / \partial t + v \partial M_x / \partial x = -M_x / T_2 \quad (27)$$

$$\partial M_y / \partial t + v \partial M_y / \partial x = \gamma M_z B_1(x) - M_y / T_2 \quad (28)$$

$$\partial M_z / \partial t + v \partial M_z / \partial x = -\gamma M_x B_1(x) + (M_0 - M_z) / T_1 \quad (29)$$

From equation (28 and 29), we have

$$\begin{aligned} v^2 \frac{\partial^2 M_y}{\partial x^2} + 2v \frac{\partial^2 M_y}{\partial x \partial t} + v \left(\frac{1}{T_1} + \frac{1}{T_2} \right) \frac{\partial M_y}{\partial x} + \left(\frac{1}{T_1} + \frac{1}{T_2} \right) \frac{\partial M_y}{\partial t} \\ + \frac{\partial^2 M_y}{\partial t^2} + \left[-\frac{1}{T_1 T_2} + \gamma^2 B_1^2(x, t) \right] M_y = \frac{\gamma B_1(x, t) M_0}{T_1} \end{aligned} \quad (30)$$

Equation (30) is a general second order, non-homogeneous, time dependent differential equation that can be applied to any fluid flow problem. At any given time t , we can obtain information about the system, provided that appropriate boundary conditions are applied. From equation (30), we can obtain the diffusion equation, the wave equation, telephone and telegraph equations, Schrödinger's equation, Legendre's equation, *etc.*, and solve them in terms of NMR parameters by the application of appropriate initial or boundary conditions. Hence, we can obtain very important information about the dynamics of the system. It should be noted, however, that the term $F_0 \gamma B_1(x, t)$ is the forcing function ($F_0 = M_0/T_1$). If the function is zero, we have a freely vibrating system; otherwise, the system is undergoing a forced vibration.

NMR DIFFUSION EQUATION

A diffusion equation can easily be obtained from equation (30) if we assume that the NMR wave is a plane wave such that:

$$M_y(x, t) = A e^{\mu x + \eta t} \quad (31)$$

subject to the following MRI experimental conditions:

$$\gamma^2 B_1^2(x, t) < 1/(T_1 T_2) \quad (32)$$

where μ and η are dependent on the NMR parameters. Taking

$$\eta^2 = T_2 \text{ and } 2\eta = T_0 \quad (33)$$

Equation (30) becomes

$$v^2 \frac{\partial^2 M_y}{\partial x^2} + T_0 \frac{\partial M_y}{\partial t} = F_0 \gamma B_1(x, t) \quad (34)$$

If we write

$$\begin{aligned} D &= -v^2/T_0 \\ v &= (-D T_0)^{1/2} \end{aligned} \quad (35)$$

Then equation (34) becomes

$$\partial M_y / \partial t = D \frac{\partial^2 M_y}{\partial x^2} + F_0/T_0 \gamma B_1(x, t) \quad (36a)$$

Equation (36a) can be written in generalized co-ordinates as:

$$\partial M_y / \partial t = D \nabla^2 M_y + F_0/T_0 \gamma B_1(t) \quad (36b)$$

If D represents the diffusion coefficient, then Equation (36) is the equation of diffusion of magnetization as the nuclear spins move. The function $F_0/T_0 \gamma B_1(x, t)$ is the forcing function, which shows that application of the RF B_1 field has an influence on the diffusion of magnetization within a voxel. It is interesting to note that the dimension of equation (35) exactly matches that of the diffusion coefficient.

Equation (36) is only applicable when D is non-directional. That is, we have a constant diffusion coefficient (isotropic medium). Equation (36) can be considered for

restricted diffusion in various geometries^[25,26]. This model would work quite well for molecules that move very short distances over a very considerable amount of time; where

$$\begin{aligned}\Omega &= T_g + \gamma^2 B_1^2(x, t); \\ F_0 &= M_0/T_1; \\ T_g &= 1/(T_1 T_2) \\ \text{and } T_0 &= 1/T_1 + 1/T_2\end{aligned}\quad (37)$$

where γ is the gyromagnetic ratio, D is the diffusion coefficient, v is the fluid velocity, T_1 is the spin lattice relaxation time, T_2 is the spin relaxation time, M_0 is the equilibrium magnetization, $B_1(x, t)$ is the applied magnetic field and M_y is the transverse magnetization. Solutions to equation (36) have been discussed by applying a number of analytical methods^[26], and for the present purpose it is sufficient to design the NMR system in such a way that the transverse magnetization M_y , takes the form of a plane wave.

MATHEMATICAL CONCEPT OF ROTATIONAL DIFFUSION MRI AND MOLECULAR DYNAMICS OF BIOLOGICAL FLUIDS

The random re-orientation of molecules (or larger systems) is an important process for many biophysical probes. By the equipartition theorem, larger molecules re-orient more slowly than do smaller objects and, hence, measurements of the rotational diffusion constants can give insight into the overall mass and its distribution within an object. In this study, the mathematical concept of rotational diffusion MRI and molecular dynamics of biological fluids is presented. This approach ensures the analytical solution of the Bloch NMR flow equation, which enables us to obtain the NMR transverse magnetization in terms of spherical harmonic functions and NMR relaxation parameters for measuring rotational diffusion at the molecular level.

Theoretical and experimental studies to determine rotational diffusion coefficients using Fluorescence Correlation Spectroscopy, fluorescence anisotropy, flow birefringence, dielectric spectroscopy, NMR relaxation and other biophysical methods that are sensitive to picosecond or slower rotational processes have been published earlier studies^[1-12].

In this study, we have presented a new method based on the Bloch NMR flow equation to measure rotational diffusion of biological fluids. The approach ensures that analytical solutions to the Bloch NMR flow equation yield the NMR transverse magnetization in terms of spherical harmonic functions and NMR relaxation parameters. The NMR/MRI technique can generate exquisite images of the soft tissue anatomy of the human body; therefore, this method is expected to become an efficient and reliable technique for measuring rotational diffusion at the molecular level for application in nanomedicine.

We consider a tumbling molecule that can be completely described by a rotational diffusion equation where the radius is fixed ($r = R$). It would be very important to derive the diffusion system directly from equations (36). Equations (36) within a spherical cavity is given by

$$\frac{\partial M_y}{\partial t} = D \left[\frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial M_y}{\partial r} \right) + \frac{1}{r^2 \sin^2 \theta} \frac{\partial^2 M_y}{\partial \phi^2} + \frac{1}{r^2 \sin \theta} \frac{\partial}{\partial \theta} \left(\sin \theta \frac{\partial M_y}{\partial \theta} \right) \right] \quad (38)$$

A tumbling molecule exhibits rotational diffusion that describes the tumbling motion of the molecules. In this case, the radius is fixed ($r = R$) and equation (38) becomes the rotational diffusion equation (the radial differential terms disappear):

$$\frac{\partial M_y}{\partial t} = D_r \left[\frac{1}{\sin^2 \theta} \frac{\partial^2 M_y}{\partial \phi^2} + \frac{1}{\sin \theta} \frac{\partial}{\partial \theta} \left(\sin \theta \frac{\partial M_y}{\partial \theta} \right) \right] \quad (38)$$

where $D_r = D/R^2$ is the rotational diffusion coefficient and R is the fixed radius of a sphere. The parameter D is called translational diffusion coefficient. The NMR transverse magnetization M_y of the diffusing molecule which is making a tumbling motion is the general solution of equation (39) obtained by the method of separation of variables:

$$M_y(\phi, \theta, t) = A e^{-D_r l(l+1)t} Y_l^m(\phi, \theta) \quad (40)$$

Equation (40) can be related to the correlation time constant τ :

$$1/\tau_l = D/R^2 l(l+1) = D_r l(l+1) \quad (41)$$

Equation (40) becomes:

$$M_y(\phi, \theta, t) = A e^{-t/\tau_l} Y_l^m(\phi, \theta) \quad (42)$$

MOLECULAR HYDRODYNAMICS IN NMR OF PROTEINS

If we sum over all possible values of m and l , equation (42) gives

$$M_y(\phi, \theta, t) = \sum_{l,m} A e^{-t/\tau_l} Y_l^m(\phi, \theta) \quad (43)$$

The rotational correlation time τ_l , is the characteristic time constant associated with the Brownian rotation diffusion of a particle in a solution. This is the time it takes the particle to rotate by one radian and it is a function of the particle size. For globular proteins, a spherical approximation can be used and the rotational correlation time is given by equation (44)^[21]

$$\tau_l = 4\pi \eta R^3 / (3kT) \quad (44)$$

where η is the viscosity of the solvent, R is the effective hydrodynamic radius of the protein molecule, k is the Boltzmann constant and T is the temperature. From the molecular weight (MW) of the protein (M), the hydrodynamic radius can be calculated as follows:

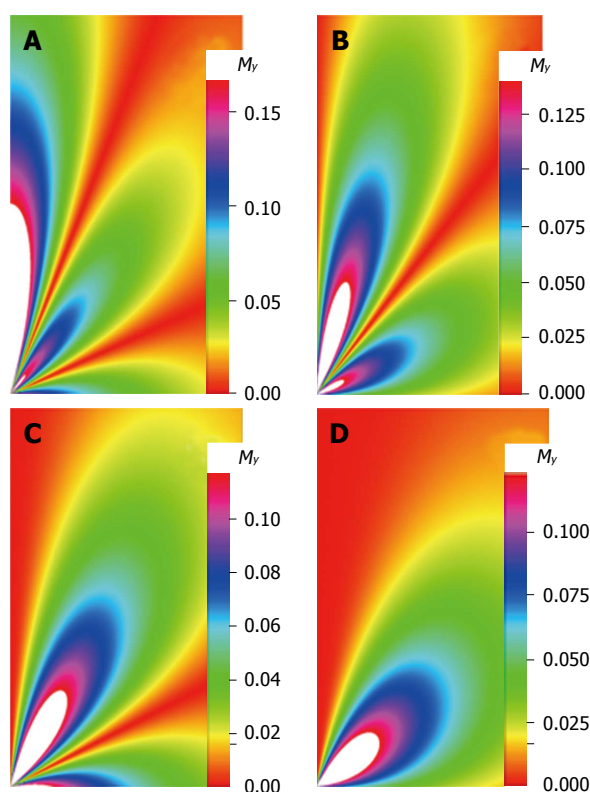
$$R = [3M/(4\pi \rho N_a)]^{1/3} + R_w \quad (45)$$

where ρ is the average density for proteins (1.37 g/cm^3), N_a is the Avogadro's number and R_w is the hydration ra-

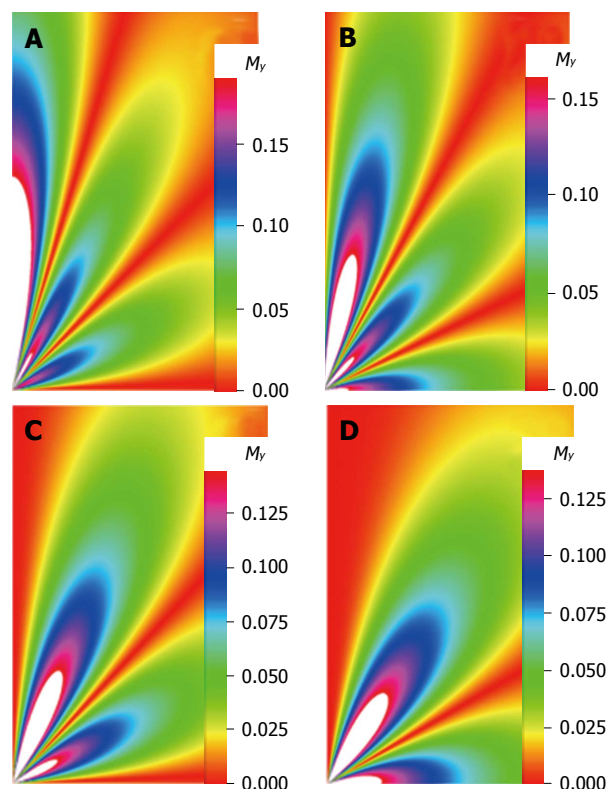
Table 3 Nuclear magnetic resonance determined rotational correlation time values for known monomeric NESG targets^[19]

NESG target (isotope labeling)	MW (kDa)	¹⁵ N T ₁ (ms)	¹⁵ N T ₂ (ms)	<i>I</i> = T ₁ /T ₂	τ _{<i>l</i>} (ns)
PsR76A (NC5)	7.2	478	128	3.734375	5.1
VfR117 (NC)	11.2	605	119	5.084034	6.3
SyR11 (NC5)	12.4	630	104	6.057692	7.1
ER541-37-162 (NC5)	15.8	729	66.5	10.96241	10.0
ER540 (NC5)	18.8	909	66.5	13.66917	11.3
SoR190 (NC)	13.8	697.5	100.9	6.912785	7.7
TR80 (NC5)	10.5	612.8	102.9	5.955296	7.0
Ubiquitin (NC)	9	441.8	144.6	3.055325	4.4
HR2873B (NC)	10.7	492	115	4.278261	5.7
B-domain (NC)	7.2	423.5	153.3	2.762557	4.05
BcR97A (NC)	13.1	705.8	80.6	8.756824	8.8
PfR193A (NC)	13.6	733.9	80.9	9.071693	9.0
MvR76 (NC)	20.2	1015	64.5	15.73643	12.2
DvR115G (NC)	10.9	608.7	115.6	5.265571	6.5
MrR110B (NC5)	11.8	707	99.2	7.127016	7.8
VpR247 (NC5)	12.5	661.2	88.3	7.488109	8.05
BcR147A (NC)	11.9	645	104	6.201923	7.2
WR73 (NC5)	21.9	1261	41.3	30.53269	13.0
NsR431C (NC5)	16.8	855.5	71.2	12.01545	10.6
StR82 (NC)	9.2	537.3	100.4	5.351594	6.6

MW: Molecular weight.

**Figure 4** Image from the transverse magnetization as it varies with time, *t* = 3 ns, and the relaxation parameters τ_{*l*} = 5.1 ns, *I* = 3.734375 for (A) *m* = 0; (B) *m* = 1; (C) *m* = 2; (D) *m* = 3.

dus (1.6Å to 3.2Å). For rigid protein molecules, in the limit of slow molecular motion (τ_{*l*} >> 0.5 ns) and high magnetic field (500 MHz or greater), a closed-form solution for τ_{*l*} as a function of the ratio of the longitudinal (T₁) and transverse (T₂) ¹⁵N relaxation times is

**Figure 5** Image from the transverse magnetization as it varies with time, *t* = 3 ns, and the relaxation parameters τ_{*l*} = 6.3 ns, *I* = 5.084034 for (A) *m* = 0; (B) *m* = 1; (C) *m* = 2; (D) *m* = 3.

$$\tau_l = 1 / (4\pi \omega_N) (6T_1/T_2 - 7)^{1/2} \quad (46a)$$

where ω_N is the ¹⁵N resonance frequency (Hz). Average ¹⁵N T₁ and T₂ relaxation times for a given protein can be measured using 1D ¹⁵N-edited relaxation experiments. To minimize contributions from unfolded segments, each 1D spectrum is integrated over the downfield backbone amide ¹H region (typically 10.5 to 8.5 ppm) and the results are used to fit an exponential decay as a function of delay time. One then computes the correlation time and compares it to a standard curve of τ_{*l*} vs protein MW obtained at the same temperature on a series of known monomeric proteins of varying size. The T₁/T₂ method is suitable for proteins with MW of up to MW ≈ 25 kDa. Accurate measurement of the diminishing ¹⁵N T₂ becomes difficult for larger proteins and cross-correlated relaxation rates are measured instead^[21].

The parameter *I* is a dimensionless constant; therefore, it may be appropriate in this study to define *I* as

$$I = T_1/T_2 \quad (46b)$$

Values of rotational correlated time for some monomeric NESG (North East Structural Genomics Consortium) targets are shown in Table 1.

The density images below are obtained for the first three isotopes of Table 3, for M₀ = 1, *m* = 0, 1, 2, 3, and a time of 3 ns. The plots shown in Figures 4-7 are made with the assumption that the spins move across rigid spheres whose radii (= R) are in the range {0, 8^{1/2}}. Figures 4-7 give the density mapping of the transverse magnetization for specific correlation times (*i.e.*, for selected

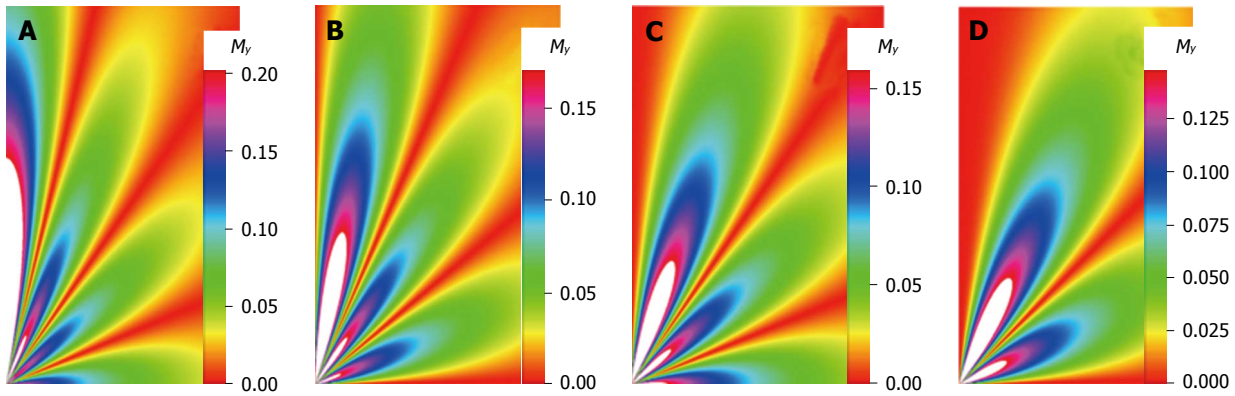


Figure 6 Image from the transverse magnetization as it varies with time, $t = 3$ ns, and the relaxation parameters $\tau_r = 7.1$ ns, $l = 6.057692$ for (A) $m = 0$; (B) $m = 1$; (C) $m = 2$; (D) $m = 3$.

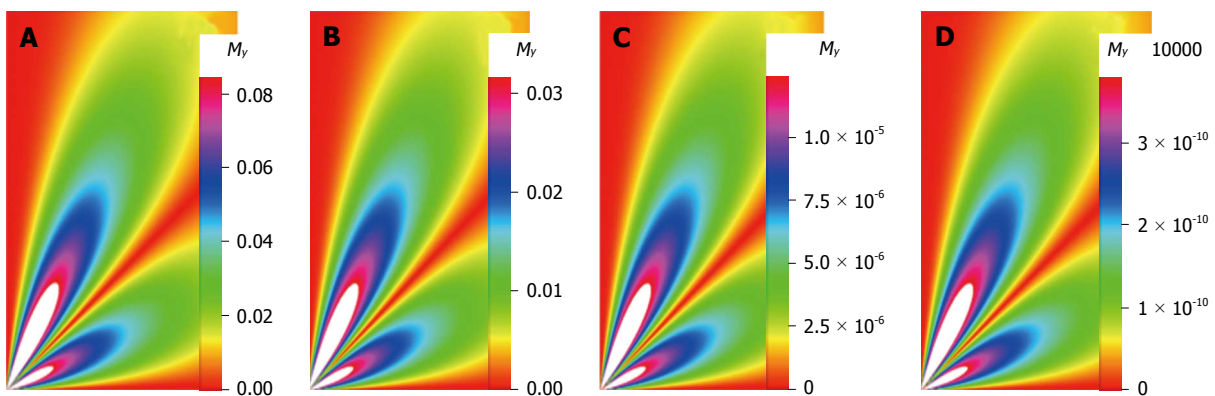


Figure 7 Image from the transverse magnetization as it varies with $m = 3$, and the relaxation parameters $\tau_r = 7.1$ ns, $l = 6.057692$ for (A) $t = 5$ ns; (B) $t = 10$ ns; (C) $t = 50$ ns; (D) $t = 150$ ns.

NESG targets) and NMR relaxation parameters.

ROTATIONAL FRICTIONAL COEFFICIENT AND MOLECULAR NMR

When a particle moves in a fluid, either under the influence of an applied force or torque, or due to Brownian motion, it experiences frictional resistance. The proportionality between particle velocity and frictional resistance is the frictional coefficient.

It may be significant to note that the rotational diffusion coefficient D_r can be defined from equation (39) as

$$D_r = D/R^2 = k_B T / (R^2 f_r) \quad (47)$$

where k_B is the Boltzmann's constant, f_r is the rotational frictional coefficient, R is the hydrodynamic radius of the molecule being observed and T is the absolute temperature. Equation (47) becomes discretized when the time constant is introduced:

$$1/\tau_l = k_B T / (R^2 f_r) l(l+1) \quad (48)$$

$$f_r = k_B T \tau_l / R^2 l(l+1) \quad (49)$$

Therefore, for the NESG target PsR76A (NC5)^[21], the rotational friction coefficient can easily be calculated:

$$f_r = 90.16765 \times 10^{-9} k_B T / R^2$$

It may be very important to note from equations (35,

39) that

$$D = -v^2 / T_0 = D_r R^2 \quad (50a)$$

And

$$D_r = -v^2 / (T_0 R^2) = -\omega^2 / T_0 \quad (50b)$$

where

$$\omega = v/R$$

is the angular velocity. Hence, we have:

$$f_r = k_B T T_0 / v^2 \quad (50c)$$

The angular drift velocity can be defined as

$$\Omega_d = d\omega / dT_0 = F_\omega / f_r \quad (51)$$

Equation (50b) defines the angular deviation in terms of the T_1 and T_2 relaxation parameters for rotational diffusion about a single axis

$$\omega^2 = D_r T_0 \quad (52)$$

Equation (52) describes the response of the angular drift velocity to an external torque F_ω assuming that the flow stays non-turbulent and that inertial effects can be neglected.

RELAXATION STUDIES OF DIATOMIC MOLECULES IN ROTATIONAL DIFFUSION

Rotational diffusion is a process by which the equilib-

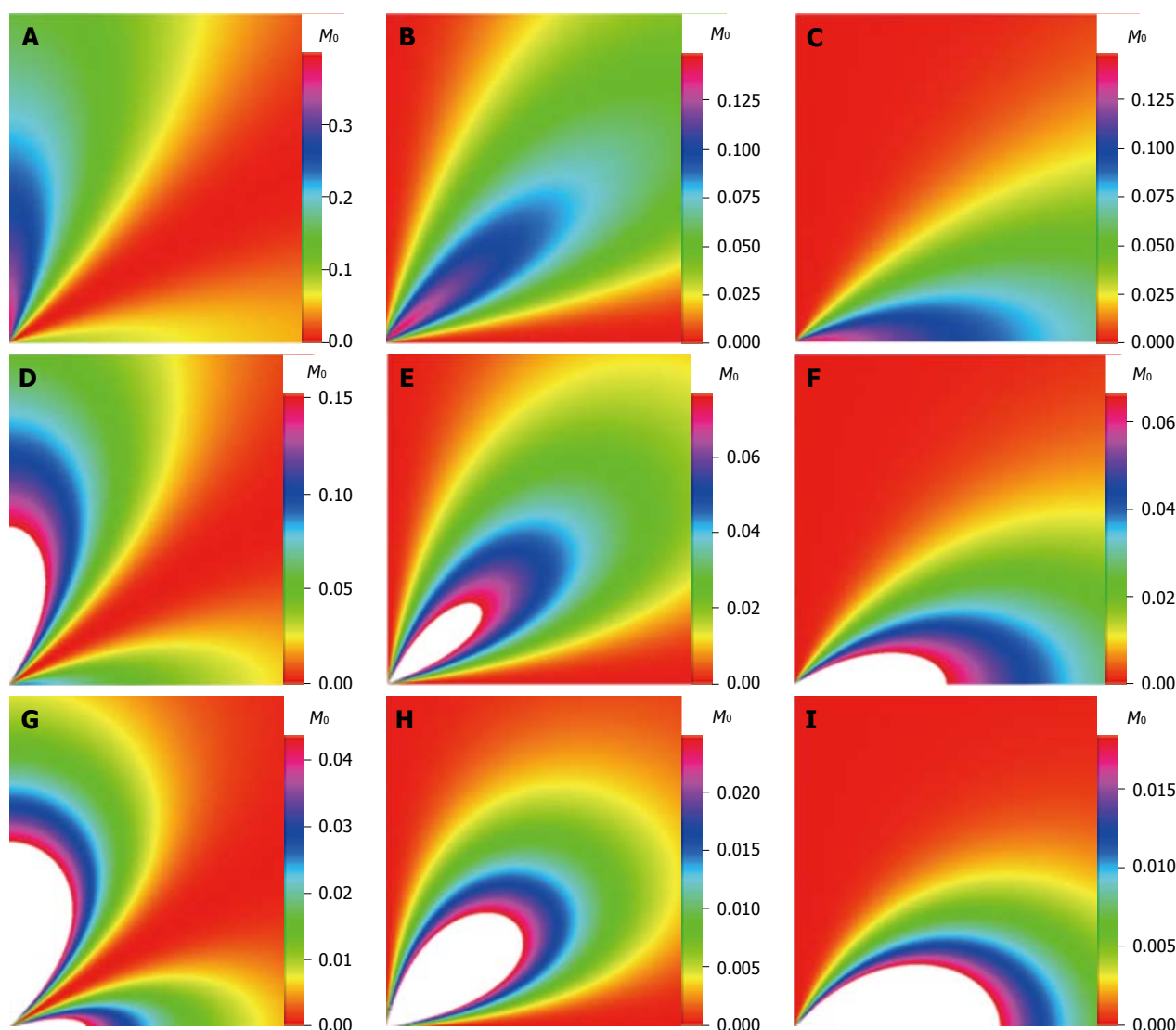


Figure 8 Density maps of M_0 using Equation (8) for $l = 2$ and (A) $m = 0, 0 \leq R \leq 2^{1/2}$; (B) $m = 1, 0 \leq R \leq 2^{1/2}$; (C) $m = 2, 0 \leq R \leq 2^{1/2}$; (D) $m = 0, 0 \leq R \leq 8^{1/2}$; (E) $m = 1, 0 \leq R \leq 8^{1/2}$; (F) $m = 2, 0 \leq R \leq 8^{1/2}$; (G) $m = 0, 0 \leq R \leq 32^{1/2}$; (H) $m = 1, 0 \leq R \leq 32^{1/2}$; (I) $m = 2, 0 \leq R \leq 32^{1/2}$.

rium statistical distribution of the overall orientation of molecules is maintained or restored. The random re-orientation of molecules is an important process for many biophysical probes. The rotational diffusion of molecules in the presence of static magnetic and RF fields can be described by the NMR diffusion equation. The NMR diffusion equation within a spherical cavity has been described in equation (42).

The radial parameter R is constant; therefore, we may assume that $\mathcal{A} = M_0 e^{-R}$. If we sum over all m and l , we have:

$$M_y(\phi, \theta, t) = \sum_{l,m} M_0 e^{-R_{lm}} e^{-t/\tau_l} Y_l^m(\phi, \theta) \quad (53)$$

If at $t = 0$, $(\phi, \theta) = (\phi_0, \theta_0)$, we write:

$$M_y(\phi, \theta, 0) = \sum_{l,m} M_0 e^{-R_{lm}} Y_l^m(\phi, \theta) = \delta[(\phi, \theta) - (\phi_0, \theta_0)] \quad (54)$$

Then, the delta function may be expanded such that:

$$\begin{aligned} \delta[(\phi, \theta) - (\phi_0, \theta_0)] &= \sum_{l,m} Y_l^{m*}(\phi_0, \theta_0) Y_l^m(\phi, \theta); \\ M_0 e^{-R_{lm}} &= Y_l^{m*}(\phi_0, \theta_0) \end{aligned} \quad (55)$$

$$M_y(\phi, \theta, t) = \sum_{l,m} Y_l^{m*}(\phi_0, \theta_0) Y_l^m(\phi, \theta) e^{-t/\tau_l} \quad (56)$$

For this system, the autocorrelation function may be given as:

$$G(t) = 12\pi/20 [\mu_0/(4\pi)]^2 \eta^2 \gamma^4 / r^6 [Y_2^{m*}(\phi, \theta) Y_2^m(\phi, \theta) e^{-t/\tau_l}] \quad (57)$$

The angle bracket is the average over the transverse magnetization of the rotating molecules. This average is given as:

$$\begin{aligned} 1/4 \int d(\phi, \theta) \int d(\phi_0, \theta_0) \sum_{l,m} Y_l^{m*}(\phi_0, \theta_0) Y_l^m(\phi, \theta) \\ Y_2^{m*}(\phi_0, \theta_0) Y_2^m(\phi, \theta) e^{-t/\tau_l} \end{aligned} \quad (58)$$

If we perform the integral, we obtain^[27-31]:

$$G(t) = K/3 e^{-t/\tau_l} \quad (59)$$

where $\tau_l = R^2/(6D) = 1/(6D)$, and $K = 9/20 [\mu_0/(4\pi)]^2 \eta^2 \gamma^4 / r^6$ is the second moment of interaction and r is the separation between two nuclear spins. The spectral density function is the Fourier transformation^[27-31] of equation (53):

$$J(\omega) = 2/3 [K\tau_l/(1 + \omega^2\tau_l^2)] \quad (60)$$

$$\frac{1}{T_1} = K\tau_l \left(\frac{2/3}{1 + \omega^2\tau_l^2} + \frac{8/3}{1 + 4\omega^2\tau_l^2} \right)$$

$$\frac{1}{T_2} = K\tau_l \left(1 + \frac{5/3}{1 + \omega^2\tau_l^2} + \frac{2/3}{1 + 4\omega^2\tau_l^2} \right) \quad (61)$$

MAPPING OF EQUILIBRIUM MAGNETIZATION

From equation (55), we can map M_0 as a function of the radius of the rigid rotator R (which is also dependent on θ_0 and ϕ_0). At the point when RF B_1 field is just removed, M_0 starts building up from its lowest value. For multi-voxel imaging, R may be changing with different tissue conditions. This may have very important influence on the changes in M_0 . Figure 8 show the changes in M_0 with assumed ranges for R .

CONCLUSION

We have presented the basic principle of NMR/MRI in a way that can be easily understood and that may fascinate researchers into the field of NMR/MRI. After a very brief history of the subject, a second order non-homogeneous, time dependent differential equation derived from the Bloch NMR equation was presented. Note that equation (30) uniquely assembles all the NMR, MRI, MRA and magnetic resonance spectroscopy parameters in an exciting way ready to be explored. The NMR signals as represented by equations (40-42) and Figures 4-7 are greatly influenced by the T_1 and T_2 relation times and the NMR parameter m . As l increases the motion gets faster and as m is increased, the particle's motion moves closer to orbiting the equator. This can greatly motivate further research into the use of rotational motion of nanoparticles to perform medical procedures inside the human body, noninvasively. Equations (40-42) are also the solutions for a rigidly rotating diatomic molecule. They are the angular parts of the hydrogen atom wave functions. These functions are important in many theoretical and practical applications, particularly in the computation of atomic orbital electron configurations, representation of gravitational fields, geoids, and the magnetic fields of planetary bodies and stars, and characterization of the cosmic microwave background radiation. In 3D computer graphics, spherical harmonics play a special role in a wide variety of topics including indirect lighting (ambient occlusion, global illumination, pre computed radiance transfer, *etc.*) and recognition of 3D shapes. The concept presented in this study can also be used to analyze the Earth's magnetic resonance. Application of this concept to nanomedicine will be the focus of our next investigation. Towards this goal, we derived the standard parameters of NMR relaxometry of diatomic molecules directly from the NMR diffusion equation. The advantage of this is that we are able to obtain the autocorrelation function and the spectral density function without the use of the

rigorous method of probability distribution function.

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Translational medical mycology guides clinical and laboratory practice on fungal diseases

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Abstract

Patients with fungal infection having skin lesions may consult a dermatologist, which is a diagnostic and therapeutic challenge. Dermatologists take samples from the lesion to check the fungal elements under a microscope by KOH preparation and then treat the patient. This model has advanced from bedside to bench and from bench to bedside (B to B to B), which is defined as Translational Medical Mycology. Dermatologists have an advantageous position in finding, isolating and identifying the pathogenic fungi and treating the patient with antifungal drugs. Samples should be cultured in different media with or without chloramphenicol and cycloheximide and incubated at room temperature or 37 °C. Non-culture techniques such as polymerase chain reaction based molecular identification, transmission electron microscopy, scanning electron microscopy, biochemistry tests and histopathology are also necessary to confirm the identification of the species, especially when the routine culture is negative. We start treatment upon obtaining evidence of fungal infection,

i.e., positive KOH examination. Antifungal drugs such as itraconazole, fluconazole, terbinafine and amphotericin B can be used alone or in combination based on the fungal species and the location of the lesion. Practice on fungal infection includes screening of the patient, merging all of the laboratory techniques and methods from the microbiologists, pathologists, molecular researchers, identification of the pathogen and determination of the optimum antifungal drug.

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Key words: Translational; Mycology; Bedside; Bench; Fungal infections

Core tip: "Translational medical mycology" has transformed vastly in recent years, which is aimed to solve clinical problems. This transformation is a dynamic, multi-level, multi-directional and continuously improving process. There are different processes in translational medicine, with the main objective being translation journey from bench to bedside to the community. We can solve a clinical problem by using a number of new technologies with the help of advanced laboratories and professionals, which will eventually promote the overall progress of medical mycology and ultimately provide an access to the effective treatment and prevention of the fungal disease.

Lama J, Ran X, Ran YP. Translational medical mycology guides clinical and laboratory practice on fungal diseases. *World J Transl Med* 2014; 3(1): 31-36 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v3/i1/31.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v3.i1.31>

INTRODUCTION

As modern science continuously spawns even more dra-

matic discoveries about human health, more research with the help of new technology and advanced laboratories is increasingly encouraged to find the practical application that can improve human lives. The problems encountered in clinical situation undergo laboratory studies through the problem-solving process (such as finding etiology and pathogenesis to find a key factor against the development of the cause or drugs) and then return to clinical guided treatment, and this process is summarized as from the bedside to the laboratory and vice-versa (Bench to Bedside and Bedside to Bench, *i.e.*, B to B to B, defined as Translational Medicine). Furthermore there are different processes in translational medicine, with the main objective being translation journey from bench to bedside to community. As the medicine enters the era of translational medicine, medical mycology as an important part of medicine has become translational medical mycology^[1,2].

Although in translational medical mycology the patient's history and clinical manifestations are important clues to clinical diagnosis, sample collection from lesion sites is a crucial step in order to identify patients with fungal infection specimens (scales, pus, sputum, blood, cerebrospinal fluid, *etc.*). Obtaining high quality specimens to meet the laboratory requirements for etiological diagnosis is critical, including professional training in mycology, biologically secure laboratory conditions, preparation of different media, culture incubator (24 °C-26 °C and 37 °C), and most importantly avoidance of contamination during the procedure. Microscopic computer image acquisition and processing system is the basic premise of fungal microscopic examination, specimens are processed (KOH preparation) to observe fungi (hyphae/spores, different shapes and size), and for further inquiry there is also molecular identification. It is estimated that there are more than 1.5 million kinds of fungi on the Earth. So far more than 100000 kinds have been described, but only just over 100 kinds are known as common pathogenic fungi, suggesting that there is a great development space for medical mycology.

The rapid developed molecular biology techniques are used for molecular identification of not only fungi isolated in culture but also those from non-culture specimens, like direct DNA extraction using universal fungal primers by polymerase chain reaction (PCR) amplification/sequencing; further pathological examination (Periodic acid-Schiff or Methenamine-silver staining) can be used to identify the fungus and its special structures. Electron microscopy is used to observe the ultra-structure and fungal pathogens for species identification and study of pathogenesis. For translational medical mycology, molecular biology and individualized treatment should include both the host and the fungus. To perform host susceptibility analysis to the pathogenic species and the virulence factors, molecular biology techniques are required to analyze the host susceptibility genes, environmental and exposure factors. DNA from the fungal specimens (isolated colonies in culture, non-cultured clinical specimens or pathological paraffin block specimens) for molecular am-

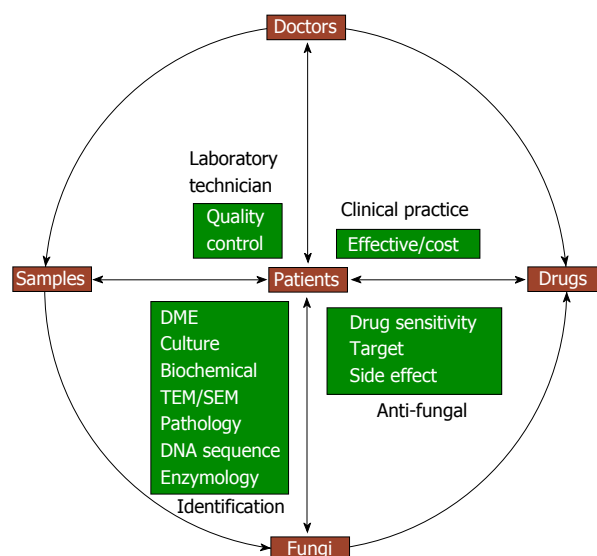


Figure 1 Essential factors for translational medical mycology. DME: Direct microscopic examination; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy.

plification and sequencing of pathogenic fungi is equally important. For identification, common fungal universal primers to amplify the internal transcribed spacer 1/4 region and D1/D2 domain are used, but species-specific primers can also be designed.

Various comprehensive detection techniques are designed to quickly identify pathogenic fungi for administration of antifungal therapy on time. Antifungal drug sensitivity test is a superior choice to select drugs to which the particular strain is highly sensitive, but at the same time, drug interactions, safety and drug prices should be taken into consideration. Each link has a puzzle challenge with a variety of problems and each clinical problem is transformed into medical mycology research topic. The essential factors for translational medical mycology and their relationship are summarized in Figure 1.

With the improvement in patient acceptance and the rate of positive fungal results^[3], we can improve the traditional methods for sample collection. In one patient with Candidosis intertrigo in the groin, specimens were collected using a cotton swab, tape and blunt scraping of scales, respectively, for direct DNA extraction by PCR/sequencing. The results confirmed that the three different methods all identified the pathogenic fungi as *Candida albicans*, and the results were verified by cultured colony identification. The new method for sample collection by using a cotton swab, tape and using blunt scraping of scales for direct DNA extraction by PCR/sequencing does not only solve the problem of sample collection from sensitive parts, but also can be used for early identification of pathologic fungi and treatment accordingly, which in turn saves time^[4]. Direct DNA extraction from the clinical samples (hair or nail) for identification of the pathogenic strains has also been successfully developed to distinguish between tinea capitis^[5] or onychomycosis. In one case, hair samples collected from a 6-year-old girl

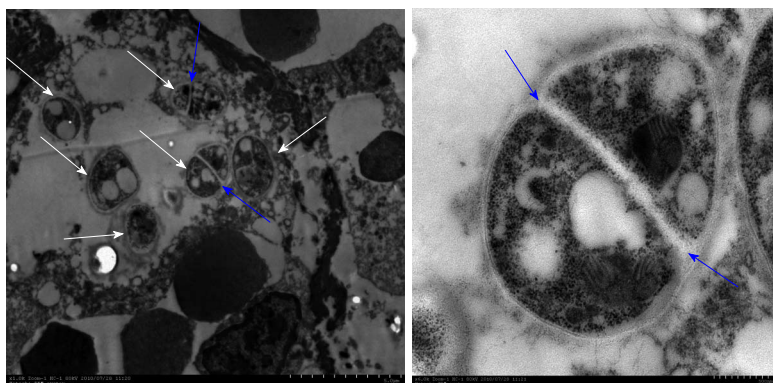


Figure 2 In transmission electron microscopic examination, there are 6 organisms with cell wall (white arrows), of which 2 have septated wall (blue arrows) within a macrophage. This specific structure of *Penicillium marneffei* helped to clarify the pathogen as *Penicillium marneffei*.

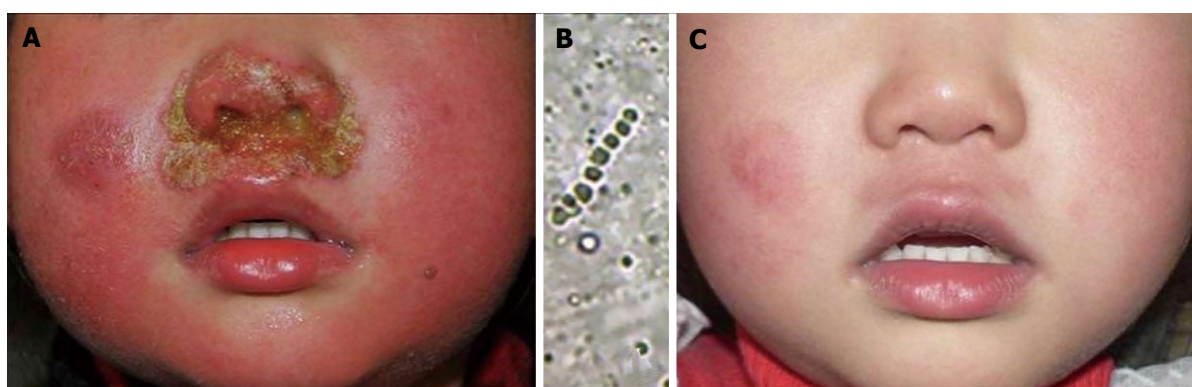


Figure 3 A 3-year-old girl was consulted with impetigo-like lesions around the nostrils. A: Pustules and erythema around nostrils; B: Hyphae and clustered spores in the crust but the culture was negative. The pathogenic agent was identified as *Arthroderma vanbreuseghemii* (a teleomorph of *Trichophyton interdigitale*) using polymerase chain reaction-based sequencing of the crusts; C: The pustules and erythema disappeared after 35 d of antifungal treatment.

who suffered from kerion were divided into two parts for culture dependent and independent methods, respectively. Since the patient was already on terbinafine, the culture grew slowly. Two sequences from the hair and the culture were both identified as *Arthroderma vanbreuseghemii*. Hence, PCR based molecular biological validation of DNA directly from the sample can rapidly identify the pathogenic fungus, especially after the use of antifungal drugs. Pathological examination is important in the field of medical mycology, but it has its own limitations. It is important to verify the results since specific fungal strains cannot be determined by pathological reports. In a patient with oral ulcers, histopathology reported a diagnosis of “Leishmaniasis”, but the review of the pathological section found that “*Leishmania*” is more like *Penicillium marneffei* yeast cells. The oral specimens were negative for fungal culture. To rule out the “Leishmaniasis” and to establish a new diagnosis as “Penicilliosis marneffei”, paraffin-embedded tissue was used to extract DNA using universal primers for PCR, and the obtained sequence was compared with the sequences in database by Blast and found to share a 99% similarity to the *Penicillium marneffei*. In addition, the paraffin-embedded tissue was prepared for transmission electron microscopy observation, which showed the septated wall, the specific structure for *Penicillium marneffei* yeast in tissue (Figure 2). The diagnosis was

confirmed as a case of HIV-negative primary, localized, penicilliosis marneffei by the non-culture method^[6].

The fungal culture result can be influenced by various factors like culture media, incubation conditions, the volume and quality of inoculation, previous history of use of antifungal drugs and the technique of the inoculation, which lead to unsuccessful attempt to isolate the pathogenic fungi. Direct extraction of DNA is a useful method for guiding treatment in these cases where microscopic tests are positive and cultures are negative. We have encountered some cases in which there was no growth in fungal culture but a significant treatment effect was achieved with antifungal agents^[7]. A 3-year-old girl was consulted with impetigo-like lesions around the nostrils. KOH examination showed hyphae and clustered spores in the crust but the culture was negative. The pathogenic agent was identified as *Arthroderma vanbreuseghemii*, a teleomorph of *Trichophyton interdigitale* using PCR-based sequencing of the crusts taken from the lesion. She recovered from the infection after oral and topical antifungal agents (Figure 3).

Regarding the treatment, whenever the direct microscopic examination with KOH is positive, the antifungal treatment should be started immediately without waiting for the fungus to be isolated or the species be identified. As this might take weeks and the patient's

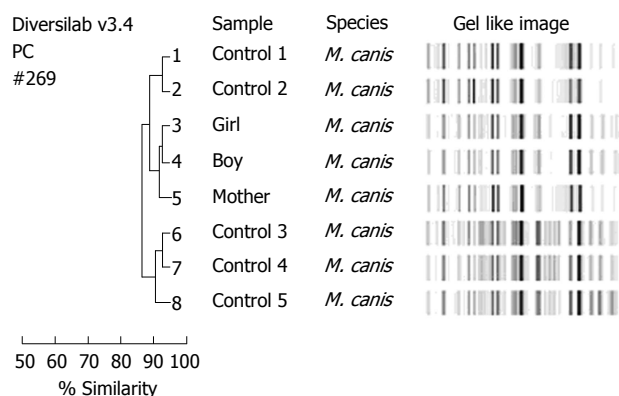


Figure 4 Molecular typing of eight *Microsporium canis* isolates showed distinct and similar fingerprint patterns visualized by the gel-like image and as indicated by a > 90% similarity coefficient in the dendrogram. The three isolates from patients of a same family (number 3, 4, 5) were grouped together with a $\geq 98\%$ similarity compared with the control isolates of *M. canis*. *M. canis*: *Microsporium canis*.

condition might deteriorate. More importantly, sample collection from the lesions for culture and pathology should be conducted just before starting the antifungal agents. As the treatment progresses, a fungal culture (the conventional method and subsequent identification and susceptibility testing) and direct extraction of DNA for molecular identification should be performed in addition to the weekly follow-up. Samples should be collected and examined until the culture becomes negative before stopping the antifungal treatment^[8].

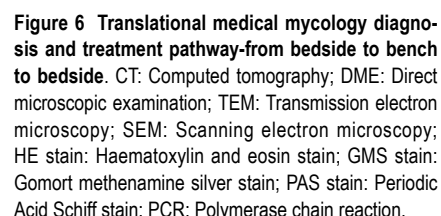
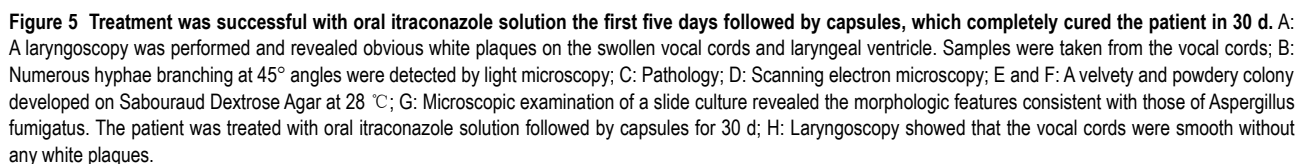
Antifungal drugs are the key factor in the treatment of fungal infection. Commonly used topical antifungal drugs consist of allylamines (such as terbinafine, naftifine, butenafine), imidazoles (such as ketoconazole and bifonazole) or a mixture of the two (naftifine with ketoconazole), and some are even mixed with steroids (triamcinolone acetonide with econazole or miconazole nitrate). Topical drugs are made by mixing various concentrations of antifungal agents with matrices composed of different vehicles and formula. The antifungal spectrum and activity of this mixture (final product) are determined by applying a fixed volume directly to the pathogenic fungus, and then observing and comparing the size of the inhibition zone around the well containing the tested cream. This modified agar diffusion method is a practical agar-based method, which enables determination of the activity of various antifungal drugs against an array of fungal genera and species. It is useful to assess antifungal activity and spectrum against fungal skin infection and to determine the *in vitro* spectrum and activity of different topical antifungal creams by studying their direct inhibition activity against clinical isolates. We can select the product with the best effect/cost potency ratio from the commercial antifungal creams based on the comparison of the inhibition zone in the same condition to the same species of pathogenic fungus^[9,10].

Sometimes in clinical practice we might encounter multiple mixed fungal infections, which will require different media. The samples should be incubated at differ-

ent temperatures to distinguish them and we must not jump into a premature conclusion that there is a “contaminated fungus”. Generally, the fungus which invades the skin during trauma at the initial stage is considered contaminated. When the local condition becomes suitable for its growth, this “contaminated fungus” could become as the pathogenic fungus (opportunistic fungal infection). This idea leads us to find out the uncommon pathogen and the cure to the difficult cases^[11,12].

It is important to use techniques of molecular biology and electron microscopy to identify fungi from all the lesions, particularly in cases of rare fungi, where there is not much of a morphological characteristic. In one case, several samples were collected from patient’s face, neck, back, buttocks, foot as well as toenail lesions and all were isolated as *Trichophyton rubrum*. By using *Trichophyton rubrum* specific random amplified polymorphic DNA (RAPD) and tandem repeat subunit Trs-1/Trs-2 PCR product analysis, we found that the patient was infected with six different strains at various sites of the infection, proving that various strains did not occur by autoinoculation^[13]. RAPD and Trs-1/Trs-2 analysis of 8 family members showed that although *Trichophyton rubrum* can infect one member of the family to another (2 families), but more cases showed that the infections are beyond the families^[14]. The members from one family had kerion (son) and tinea corporis (father and mother), and *Arthroderma vanbreuseghemii* was isolated from their lesions. By using the morphology, rapid urease test, hair perforation experiment, random primers polymorphism and zymogram analysis, it was proved that the pathogen was the same and it was transmitted from the neighbor’s rabbit to the son and later to his parents^[15]. In another case, two children (tinea capitis) and mother (tinea corporis) were diagnosed based on the positive KOH examination. Morphological characteristics and sequencing of the internal transcribed spacers 1 and 2 amplified from primary culture isolates confirmed that their infections were caused by the zoophilic *Microsporium canis*. Repetitive sequence-based molecular typing using the DiversiLab system, secreted enzymatic activity analysis and antifungal susceptibility indicated that these isolates might share the same source (Figure 4)^[16]. Therefore, molecular identification can give us clues about the source of infection whether the infection is caused by autoinoculation, by some external sources or by close contact within the family members.

In recent years we have seen several cases of common fungi causing debilitating rare diseases, which give us a new thought about how people with normal immune system can get infected with common pathogens. In one case, a boy had scalp laceration from a road accident, and the treatment included debridement and scalp transplantation in orthopedic surgery. After the treatment, the scalp had honeycomb nodules and abscesses. Culture, morphology and molecular identification showed that it was *Microsporium gypseum* (*M. gypseum*) infection. *M. gypseum* is a geophilic fungus commonly found in soil, and it may have lurked in the scalp when the wound came in contact



that there were plaques on the vocal cord. By using microscopic examination, culture and molecular identification, the results showed *Aspergillus fumigatus* (*A. fumigatus*) and later she was cured with itraconazole^[19]. In another a woman who had hoarseness because of a lump on her vocal cord, examination of tissues extracted for scanning electron microscopy revealed that they were destroyed by the hyphae of *A. fumigatus*. In addition, we also discovered several kinds of exocrine protease activities in the specimen^[20]. The third female patient with a similar pathogen had severe hoarseness and a medical history of oral sex. The pathogen was identified by morphology and molecular identification. Treatment was successful by oral itraconazole solution for the first five days and then it was followed by capsules, which completely cured the patient in 30 d (Figure 5). This case gives us a new thought about how people with normal immune system can be infected with common airborne pathogenic *A. fumigatus*.

The vocal mucous membrane barrier which was damaged by oral sex made it easy for the colonization of the spore which later invades the tissue^[21].

Over the past one century medical mycology has had a typical “transformation” characteristic: we used to take samples from the lesion of a patient to check the fungal elements under the microscope to make the diagnosis and then treat the patient. Translational Medical Mycology is not just an empty theory and slogan, it should be in the classic “transformation” based on constantly enhanced (electron microscopy, *etc.*) and enriched contents (molecular biology, *etc.*) and individual medicine. In summary, in “translational medicine mycology” the starting point and end point are the clinical and laboratory studies, which are aimed to solve clinical problems (Figure 6). Patient is the “center” and problems that are encountered during the diagnosis and treatment are transforming medical mycology. For specific diseases, we might need to integrate microbiologists, pathologists, molecular biologists and other laboratory experts with all techniques and methods to determine the pathogenic fungi, help with susceptibility testing, remote consultation, so that we can start the treatment as soon as possible to save patient lives. Transformation is a dynamic, multi-level, multi-directional and continuously improving process. We can solve a clinical problem by using a number of new technologies, new methods and training, and then there will be an improvement in the treatment of fungal diseases. It will eventually promote the overall progress of medical mycology and ultimately provide an access to the effective treatment of the disease as well as prevention.

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WJTM publishes articles that report the results of translational medicine-related applied and basic research in fields such as immunology, physiopathology, cell biology, pharmacology, medical genetics, and pharmacology of Chinese herbs. The current columns of WJTM include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of translational medicine diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors:

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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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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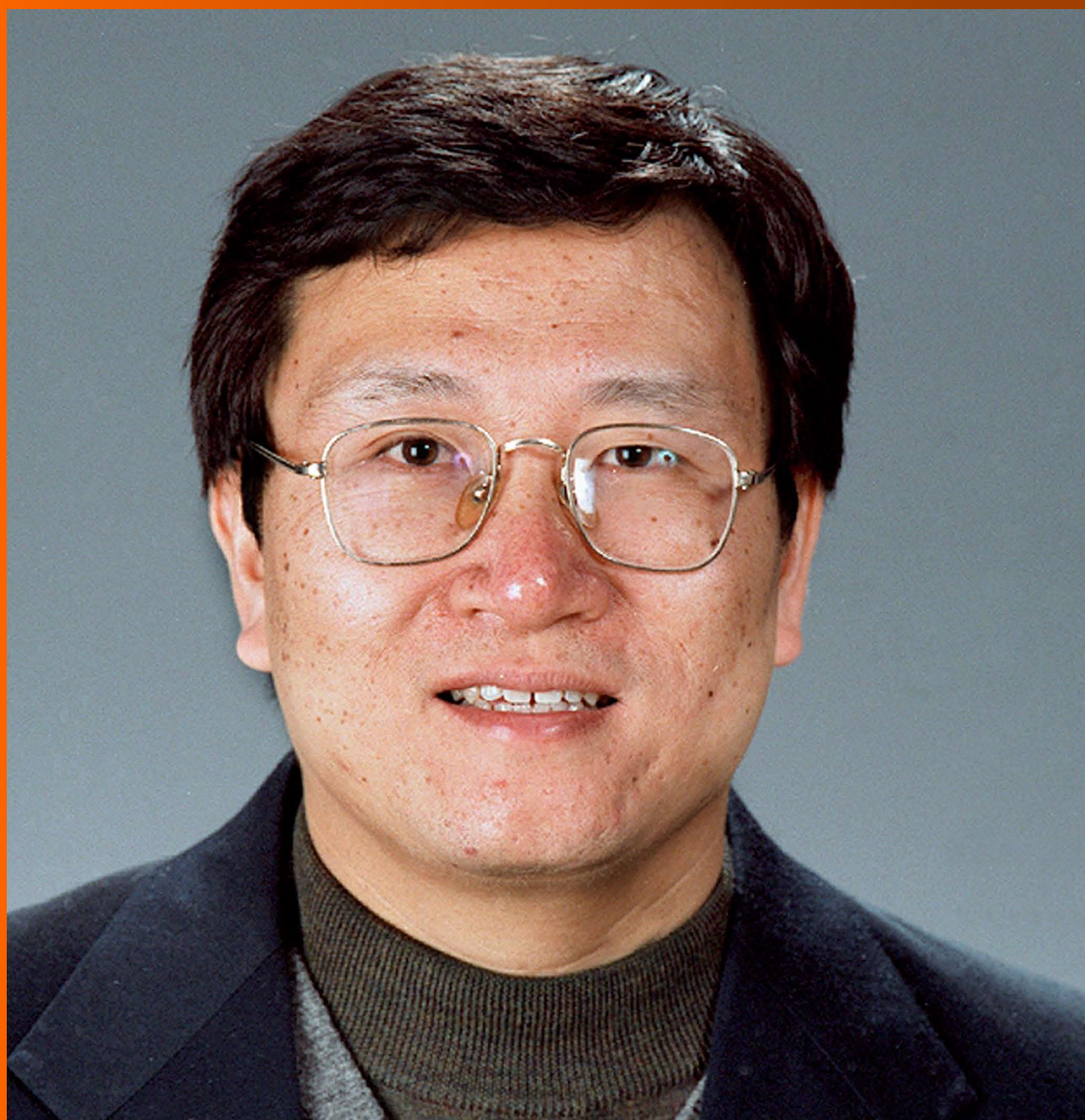
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Inhibitors of glucose transport and glycolysis as novel anticancer therapeutics

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Author contributions: Qian Y screened glucose transport inhibitory compounds and wrote sections for glycolysis, glycolysis inhibitors and glucose transporters of the manuscript; Wang X assisted in compound screening and wrote the section of glucose transporter inhibitors of the manuscript; Chen X supervised compound screening and wrote summary, introduction, the Warburg effect, and future direction of the manuscript and finalized the manuscript.

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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.

Qian Y, Wang X, Chen X. Inhibitors of glucose transport and glycolysis as novel anticancer therapeutics. *World J Transl Med* 2014; 3(2): 37-57 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v3/i2/37.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v3.i2.37>

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

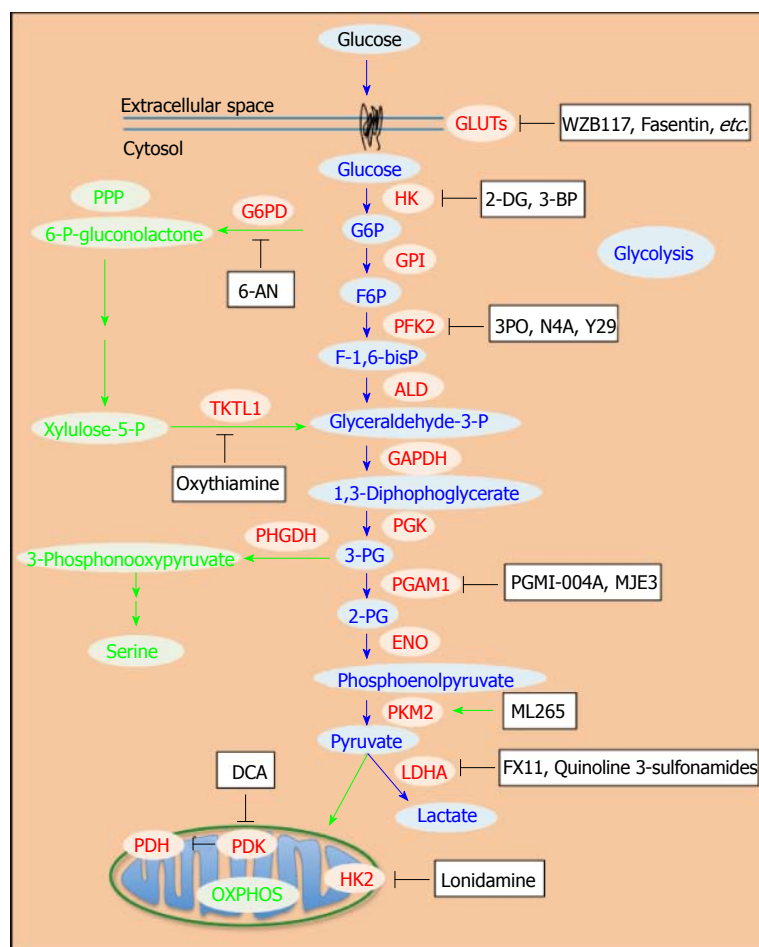


Figure 1 Glycolysis and inhibitors/activators of glycolysis as potential anti-cancer therapeutics.

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: Elongase; 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
3PO	Inhibits PFK2	Pre-clinical	NCT00435448
N4A, YZ9	Inhibits PFK2	Pre-clinical	[90]
PGMI-004A	Inhibits PGAM1	Pre-clinical	[91]
MJE3	Inhibits PGAM1	Pre-clinical	[96]
TT-232	Inhibits PKM2	Pre-clinical	[98]
		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 μmol/L^[125]. However, oxamate also works as an inhibitor of aspartate aminotransferase with an even lower K_i of 28 μmol/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 μmol/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 μmol/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

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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

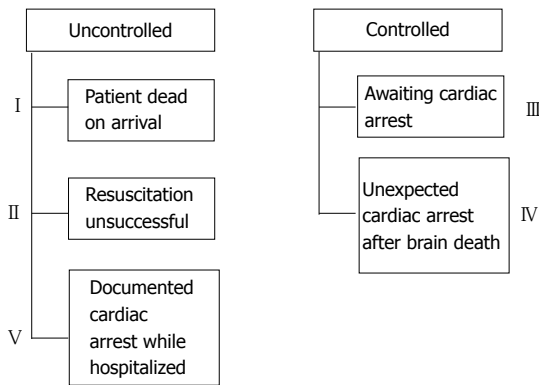


Figure 1 The maastricht classification of donation after cardiac death donors.

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

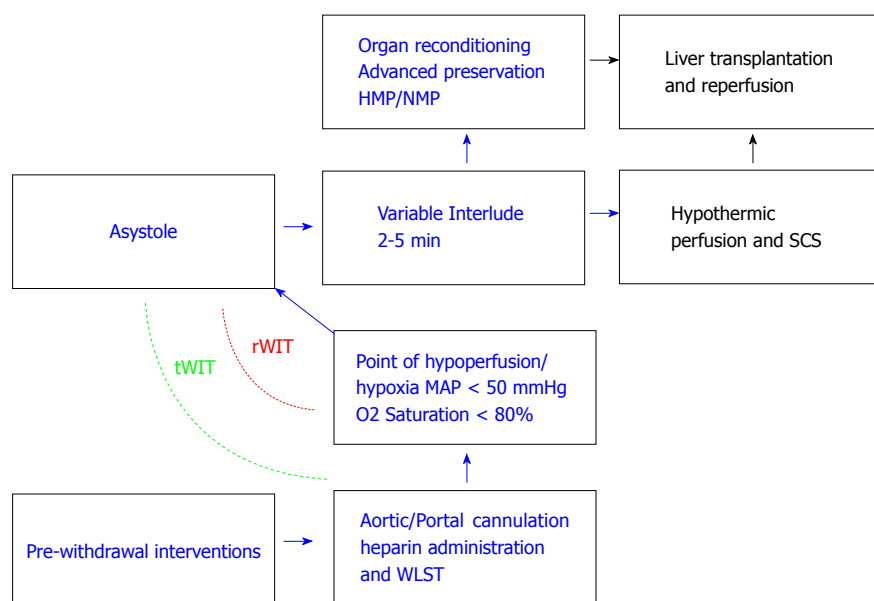


Figure 2 Donation after cardiac death organ procurement algorithm. Modified from Abradelo De Usera *et al.*^[24]. HMP: Hypothermic machine perfusion; NMP: Normothermic perfusion; SCS: Static cold storage; WLST: Withdrawal of life support therapies.

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

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 IIIa-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]
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.	Hayward <i>et al</i> ^[25] 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]
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).	Chauleur <i>et al</i> ^[113] 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 thrombasthenia, 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 adenylylase 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 μ m) 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)N2CCCCC2</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]1Nc2ccccc2N1C(=O)CCc3ccc(OC(=O)O)cc3</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]. Proinsulin, 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.>

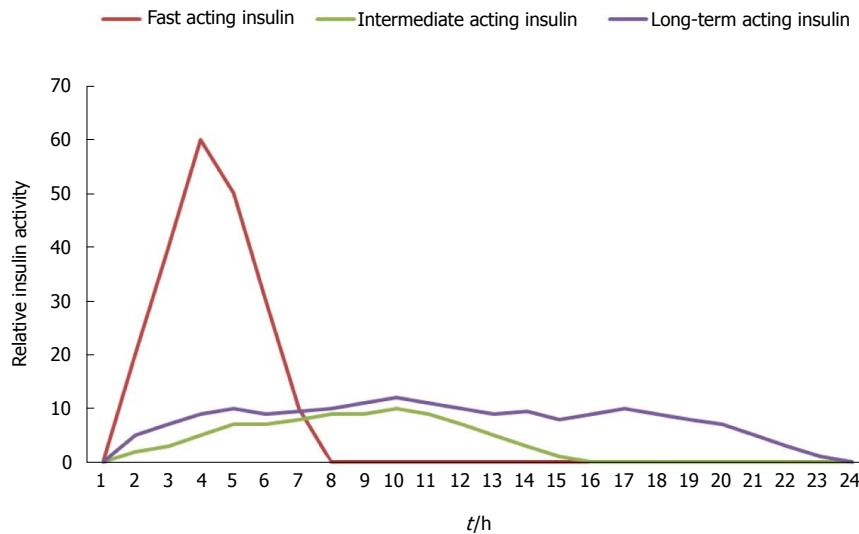


Figure 2 Diagrammatic representation of insulin activity profile.

ncbi.nlm.nih.gov/pubmedhealth/PMHT0010728/) and insulin detemir (<http://www.ncbi.nlm.nih.gov/pubmed-health/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-

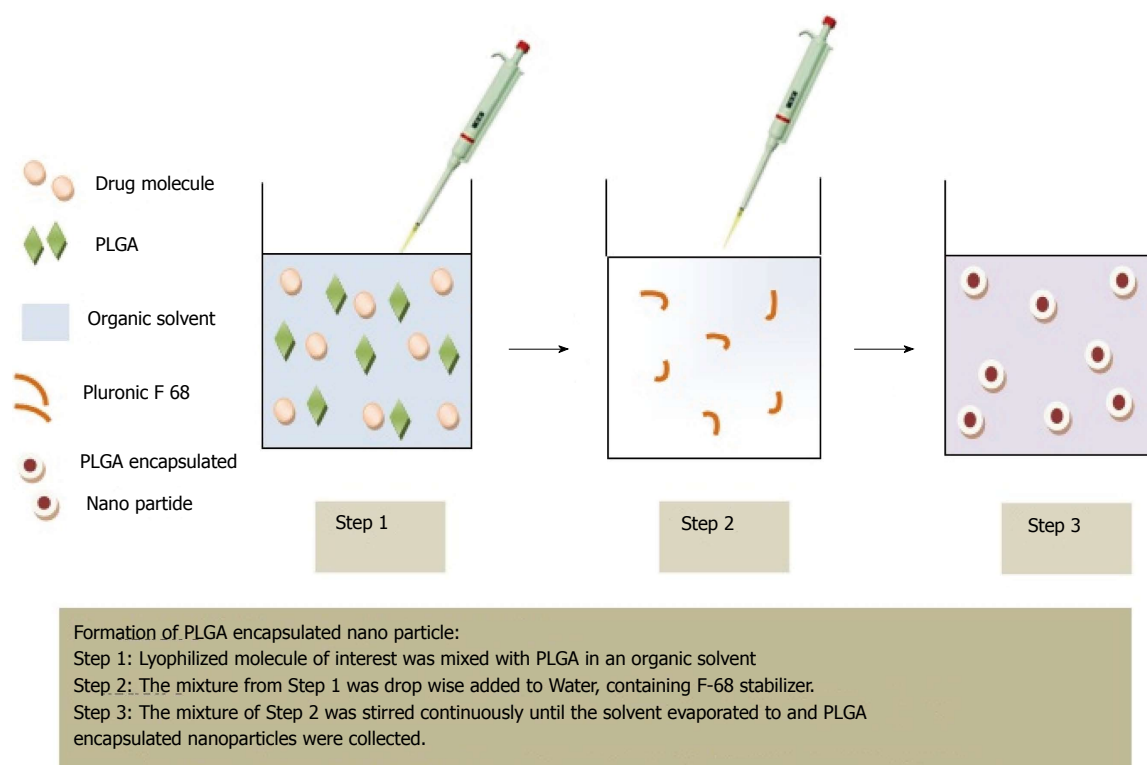


Figure 3 Steps involved during the formulation of [poly (lactide-co-glycolide)] polymers encapsulated nanoparticles. PLGA: [Poly (lactide-co-glycolide)] polymers.

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	Morrioniside	Anti-oxidant, regeneration of β cells, drug-DNA interaction, regulates signal proteins	Samadder <i>et al</i> ^[33]
Eugenia jambolana	1-0-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

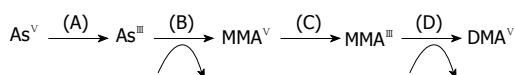


Figure 1 Arsenic methylation pathway in the human body^[25]. A: Arsenate reductase or purine nucleoside phosphorylase (PNP); B: Arsenite methyl transferase (As3MT); C: Glutathione S-transferase omega 1 or 2 (GSTO1, GSTO2); D: Arsenite methyl transferase (As3MT). SAHC: S-adenosylhomocysteine; SAM: S-adenosylmethionine;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

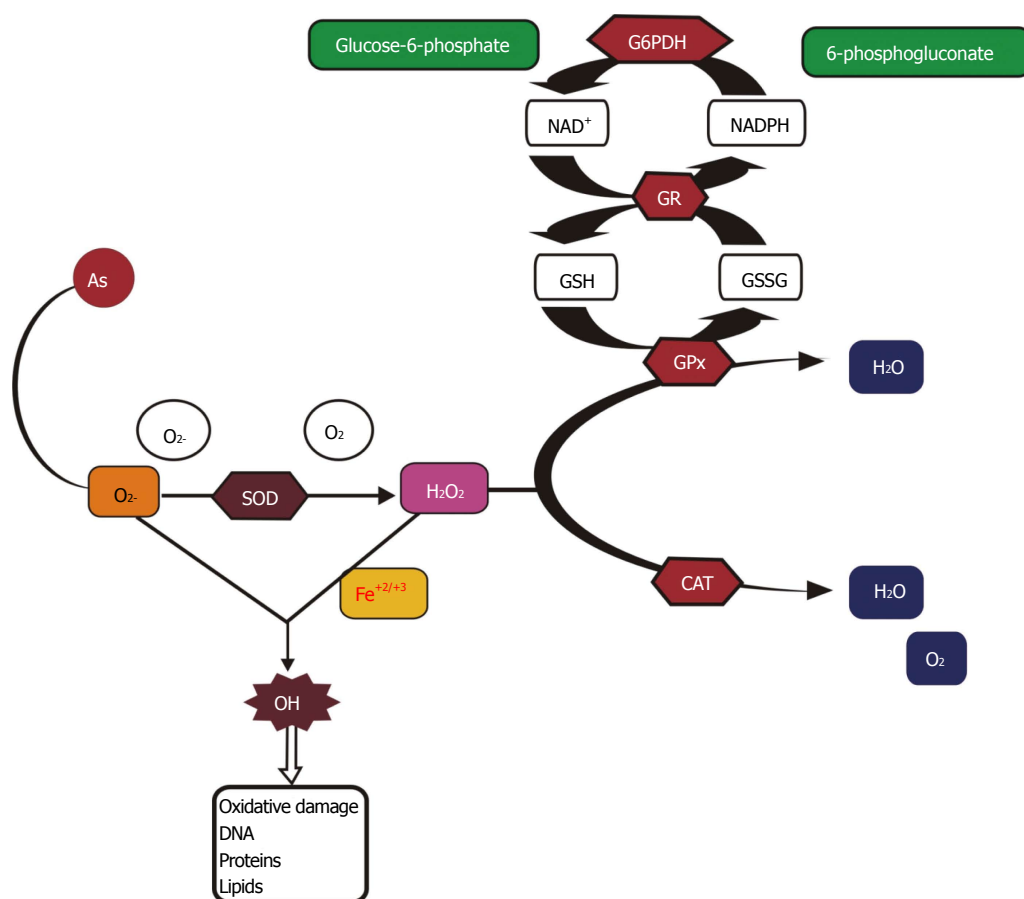


Figure 2 Mechanism of arsenic toxicity: Arsenic enhances the production of superoxide anion radical which results in a higher oxidant level than antioxidant enzymes involved in the detoxification of superoxide anion radical viz., superoxide dismutase, catalase, glutathione reductase and glucose-6-phosphate 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.

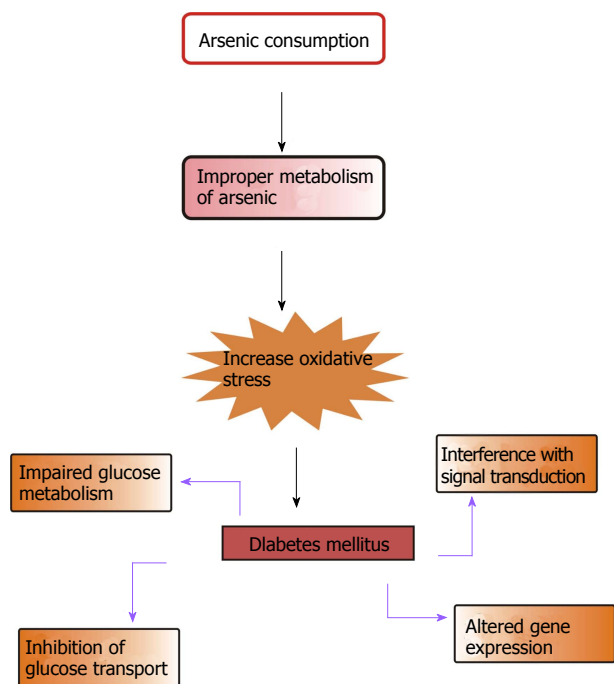


Figure 3 Possible biochemical approaches by which arsenic induces diabetes.

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-

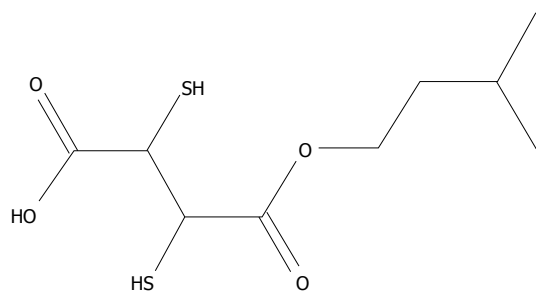


Figure 4 Mono isoamyl 2,3-dimercaptosuccinic acid.

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

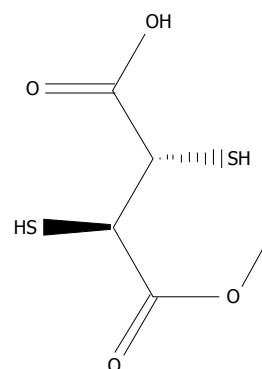


Figure 5 Mono methyl 2,3-dimercaptosuccinic acid.

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

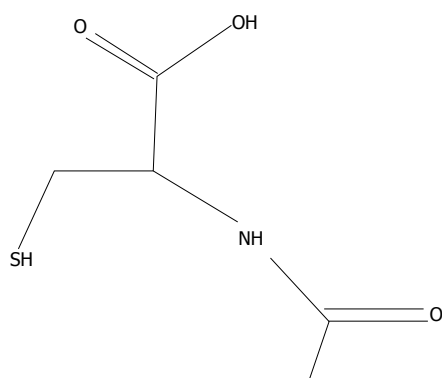


Figure 6 N-acetylcysteine.

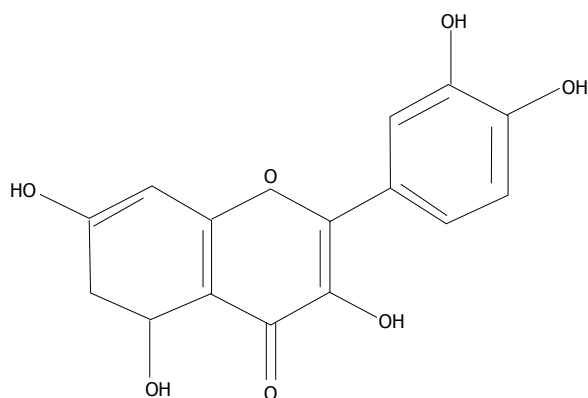


Figure 7 Quercetin.

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

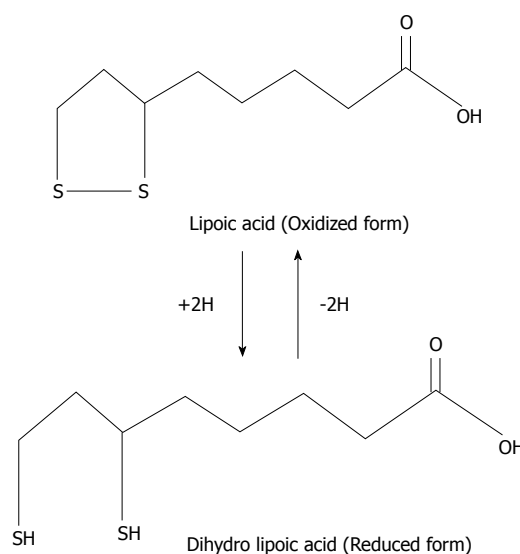


Figure 8 Reduction of lipoic acid.

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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Obesity research: Status quo and future outlooks

Alexander T El Gammal, Anna Dupree, Stefan Wolter, Jens Aberle, Jakob R Izbicki, Cenap Güngör, Oliver Mann

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Sleeve Gastrectomy, and other bariatric procedures are therefore under intense investigation. In this review, however, we will focus on obesity treatment, highlighting new insights and future trends of gut hormone research, the relation of obesity and cancer development *via* the obesity induced chronic state of inflammation, and new potential concepts of interventional and conservative obesity treatment.

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Key words: Obesity; Cancer; Gut hormones; Bariatric surgery

Core tip: This review focuses on the latest obesity research breakthroughs, current therapy options, future outlooks, also from a view of a surgeon as well as recently identified molecules that promote obesity and its comorbidities, outlining their great potential as new target molecules in the fight against the global pandemic, called "obesity".

Abstract

Obesity is a multifactorial disease showing a pandemic increase within the last decades in developing, and developed countries. It is associated with several severe comorbidities such as type II diabetes, hypertension, sleep apnea, non-alcoholic steatosis hepatitis and cancer. Due to the increasing number of overweight individuals worldwide, research in the field of obesity has become more vital than ever. Currently, great efforts are spent to understand this complex disease from a biological, psychological and sociological angle. Further insights of obesity research come from bariatric surgery that provides new information regarding hormonal changes during weight loss. The initiation of programs for obesity treatment, both interventional and pharmaceutical, are being pursued with the fullest intensity. Currently, bariatric surgery is the most effective therapy for weight loss and resolution of comorbidities in morbid obese patients. Reasons for weight loss and remission of comorbidities following Roux-en-Y-Gastric Bypass,

El Gammal AT, Dupree A, Wolter S, Aberle J, Izbicki JR, Güngör C, Mann O. Obesity research: Status quo and future outlooks. *World J Transl Med* 2014; 3(3): 119-132 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v3/i3/119.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v3.i3.119>

INTRODUCTION

Obesity is a multifactorial disease caused by an energy sparing lifestyle on a predisposed polygenetic background. An obese person is defined as having a body mass index (BMI) greater than 30 kg/m². Within the last decades, there has been an extraordinary increase in the worldwide prevalence of obesity becoming a major human health threat especially in developing and developed countries with a tendency to rise. Being referred to as a global pandemic^[1], the number of overweight or obese

individuals increased up to 2.1 billion worldwide. Unfortunately, no single country announced decreasing numbers of obese individuals during the last three decades^[2]. Obesity is associated with several severe comorbidities (Figure 1) such as type II diabetes mellitus (T2DM), hypertension, sleep apnea, non-alcoholic steatosis hepatitis (NASH) and cancer. Obesity-related diabetes can lead to coronary heart disease, apoplex or kidney failure. Over 80% of all patients with type II diabetes in the United States are overweight and up to 20% of United States health expenditures are estimated to be spent on treating obesity-related diseases^[3].

It is expected that NASH will be the leading cause of liver transplantation within the next years^[4]. Additionally, obesity is associated with an increased risk of developing various cancer entities such as colorectal-, esophageal-, liver- and breast cancer^[5]. Visceral-, orthopedic or cardiac surgical treatment of obese patients is associated with higher complication rates^[6-9]. Subsequently, obesity is the origin of a wide spectrum of diseases and a confounding factor hindering adequate treatment. Due to this reasons, obesity and overweight are associated with an increased risk of death. Thus, therapy for obesity should be individually tailored and various factors such as sex, obesity degree, individual health risks should be taken into account^[10,11].

Secondary causes for obesity like endocrine disorders (*e.g.*, hypothyroidism, cushing disease), drug-induced obesity (*e.g.*, glucocorticoids, psychoactive drugs), inherited syndromes (*e.g.*, Prader-Willi syndrome, Bardet-Biedl syndrome) or monogenetic disorders (leptin receptor, melanocortin receptor) play a minor role or are cofactors in causation of obesity in daily practice. Therefore, identifying single reasons for obesity is a complex task. Intervention strategies for weight loss and maintenance at the individual and community level are strongly needed to reduce general health risks as well as health expenditures.

STATUS QUO

Due to the increasing number of overweight individuals worldwide, research in the field of obesity has become more vital than ever. As a multifactorial disease, research is conducted at a wide variety of areas. Currently, great efforts are spend to understand this complex disease from a biological, psychological and sociological angle. Further insights of obesity research come from bariatric surgery, which display new information regarding the hormonal changes during weight loss. The initiation of programs aiming to treat obesity, both interventional and pharmaceutical, are being pursued with the fullest intensity. There are various scopes of possible research activities. In this review, however, we will focus on obesity treatment, highlighting new insights into gut hormones and the relation of obesity and cancer development.

Multidisciplinary Treatment Modalities-or, how to lock the stable door after the horse had bolted

Among physicians there is consensus, to treat obese patients multidisciplinary. After diagnosis, the patient

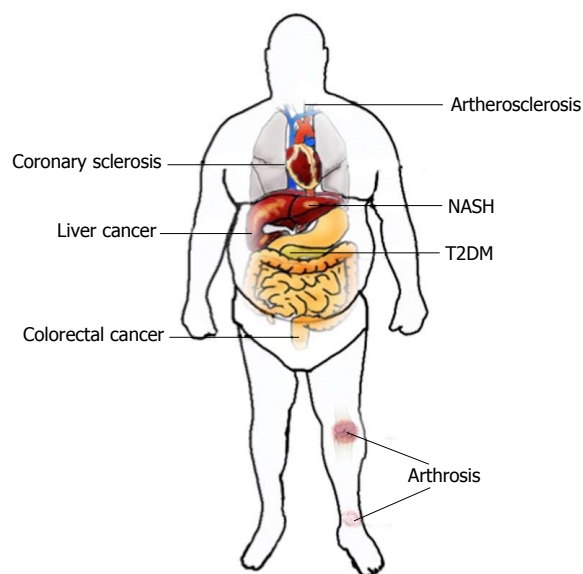


Figure 1 Obesity related diseases. T2DM: Type 2 diabetes mellitus; NASH: Non-alcoholic steatosis hepatitis.

should undergo a multimodal therapy concept based on individualized dietary education focusing on reducing energy intake, physical exercising, pharmacological therapy and psychological attendance with behavioral therapy. For the latter, many efforts to modify the behavior of obese individuals through encouragement of changes in dietary intake along with physical activity have not declined the obesity epidemic, unfortunately. The primary causes are high rates of therapy abandonment and poor patient compliance.

Patients who completed a comprehensive program including a low-calorie diet are able to lose approximately 15%-25% of their initial body weight during 3 to 6 mo of treatment. After therapy, most patients maintain a weight loss of 8% one year after treatment, 7% three years after treatment, and 5% four years after treatment^[12]. These results represent the best-case scenario, excluding patients who dropped out of their programs. It was already shown that patients who have completed structured weight loss programs, maintained their weight loss of less than 3 kg on average after 5 years; patients who accomplished more radical low-calorie diets had significantly higher weight loss of up to 20 kg and maintained more weight loss over time^[13]. In a randomized study, Jeffery *et al*^[14] evaluated the efficacy of long-term weight loss comparing one group with behavior therapy and an energy expenditure goal of 1000 kcal per week to a group of patients with high physical activity treatment and an energy expenditure goal of 2500 kcal per week. The high activity group showed significant higher weight loss and long term weight loss maintenance, reflecting that mobility is of high importance^[14].

Is bariatric surgery the best choice for long-term weight loss accompanied by remission of comorbidities in severe obese (BMI > 40 kg/m²) patients?

Bariatric surgery is more effective for weight loss and

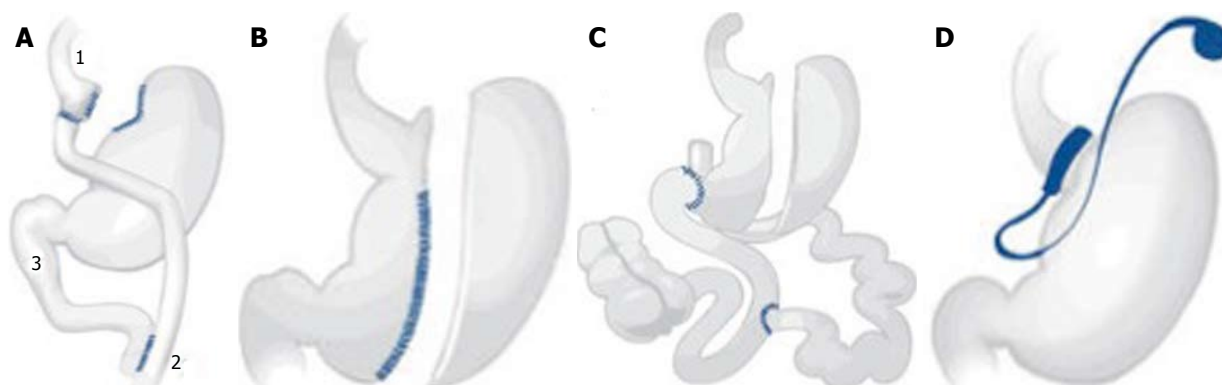


Figure 2 Bariatric surgical procedures. A: Roux-Y gastric bypass; B: Sleeve gastrectomy; C: Biliopancreatic diversion with duodenal switch; D: Gastric band.

resolution of comorbidities than conventional medical treatment modalities^[15,16]. A variety of procedures are described in the literature but only Roux-Y gastric bypass (RYGB) (46.6% of all bariatric procedures worldwide), sleeve gastrectomy (SG) (27.8%), adjustable gastric banding (GB) (17.8%) and in a smaller proportion biliopancreatic diversion with duodenal switch (BPD/DS) (2.2%) are performed in a notable quantity^[17].

Gastric bypass was first performed in the 1960s by Mason *et al.*^[18]. It was modified to a RYGB in the 1970s^[19]. It is still the most common bariatric surgical procedure. The combination of food-intake restriction by a small pouch and malabsorption through the smaller common channel (Figure 2A) leads to long-term weight loss^[15]. Also, hormonal changes after surgery may have a great impact on weight loss and diabetes remission.

SG (Figure 2B), includes the resection of the greater curve of the stomach. It is the first step of the BPD/DS (Figure 2C). BPD/DS can be performed by a two step procedure; a minority of patients do not need the second surgical step for weight loss^[20]. While SG is described as a primary restrictive procedure, including minor hormonal changes, BPD/DS causes malabsorption and leads to a higher rate of deficiencies^[21]. GB, which was developed in the 1970s, restricts food-intake by an inflatable, adjustable gastric band resulting in a small gastric pouch (Figure 2D). Since it is based on restriction only, it is the most insufficient bariatric surgical procedure regarding long-term weight loss^[22].

Overall, a preoperative multidisciplinary program is recommended. Our own clinical experiences and a review of the literature revealed that bariatric surgery for severe obese patients results in extensive weight loss and long-term comorbidity remission in a very short time frame.

There is no evidence for conventional treatment leading to sufficient excess weight loss in severe obese patients.

Padwal *et al.*^[23] performed an observational study of 500 patients with a two years follow-up. Three patient cohorts were included in which 200 patients received medical treatment, 150 patients received bariatric surgical treatment, and 200 patients received no therapy and were grouped as being waitlisted. Medically treated patients

received individualized and intensive medical management consisting of a 24-36 wk life style counseling (diet education, physical exercise, and behavioral therapy) and were observed by a multidisciplinary staff which is mandatory before bariatric surgery. Mean weight loss in the waitlisted group was 0.9%, 1.8% in the medically treated group and 22% in the surgery group. The proportion of patients who achieved at least 5% weight loss was 17% in the waitlisted group, 32% in the medically treated group and 75% in the surgery group. The prevalence of hypertension, diabetes and dyslipidemia was reduced in the surgical group, but remained unchanged or increased in the medically treated and waitlisted group^[23].

A large meta-analysis included 164 studies (37 randomized controlled trials and 127 observational studies). A total of 161756 patients were analyzed regarding effectiveness and outcome after bariatric surgery.

One year after surgery the patients showed 60% excess weight loss (EWL), and 57% EWL after 3 years. T2DM remission after surgery was 92%, hypertension remission was 75%, dyslipidemia remission was 76%, cardiovascular diseases remission was 58% and remission of sleep apnea was 96%, reflecting that surgical intervention may increase the long-term quality of life^[24].

Interestingly, 75.3% of patients that received bariatric surgery showed excess weight loss, whereas patients that had received conventional therapy showed only 11.3% excess weight loss. Moreover, remission of T2DM was reported in 63.5% of cases in surgery group, compared to 15.6% of patients in the conventional therapy group^[25]. Subsequently, there is no evidence for conventional treatment leading to sufficient EWL in obese patients with a BMI greater 40 kg/m². In fact, the only efficient treatment showing results in EWL and release of obesity associated diseases results from bariatric surgery. However, there is a strong recommendation to include the patients to a perioperative multidisciplinary medical treatment consisting of dietary changes, exercising and behavioral therapies. There is evidence that preoperative multidisciplinary preparation and education may lead to better long-term effects of bariatric surgery.

In sum, bariatric surgery is currently the only effective treatment for morbid obesity^[26]. Reasons for weight loss

Table 1 Gut hormones and their clinical relevance

Peptide	Production site	Effect	After bariatric surgery	Potential pharmaceutical intervention
Ghrelin	Stomach, mainly fundus	Appetite stimulating Growth hormone releasing	↓	Receptor antagonists GOAT inhibition Vaccination
GLP-1	L-cells of the distal small bowel	Postprandial insulin secretion Suppresses glucagon secretion Delays gastric emptying Suppresses appetite	↑	Weight loss in patients with diabetes Off-label use in obese patients
GIP	Duodenum, jejunum	Postprandial insulin secretion Energy expenditure	↓	GIP receptor antagonist
CCK	Duodenum, jejunum	Delays gastric emptying Suppresses appetite	↑	CCK analogue substance
PYY	Distal small bowel	Delays gastric emptying Suppresses appetite	↑	Long-acting analogue substance
PP	Distal small bowel	Suppresses appetite	↔	PP analogue substance
OXM	L-cells of the distal small bowel	Delays gastric emptying Suppresses appetite Increase energy expenditure	↑	Receptor agonist

Modified according to Kim *et al.*^[35]. GLP-1: Glucagon-like peptide-1; GIP: Glucose-dependent insulintropic polypeptide; CCK: Cholecystokinin; PYY: Peptide YY; PP: Pancreatic polypeptide; OXM: Oxyntomodulin; GOAT: Ghrelin O-acyltransferase.

and remission of comorbidities following RYGB, SG, and other bariatric procedures are therefore in a strong research focus.

Gut hormones and their impact on weight loss

Alterations of gut hormone serum levels after RYGB influence appetite, satiety, energy expenditure, and glucose homeostasis^[27-29]. Several hormones and peptides are considered to be involved in weight loss in bariatric patients (Table 1).

Incretins are gut-derived peptides that increase pancreatic insulin secretion. The Glucagon-like peptide (GLP-1) and Glucose-dependent insulintropic polypeptide (GIP) are well explored. GLP-1 and its analogues are used to treat diabetes. Beside its stimulating effects on β -cells of pancreatic Langerhans' islets, GLP-1 also suppresses glucagon secretion, delays gastric emptying and suppresses appetite^[30,31]. Therefore, GLP-1 is currently under intense discussion to become a potential therapeutic drug for obesity treatment^[32].

Ghrelin is mainly produced in the fundus of the stomach and plays an important role in satiety. When administered to humans, it increases food intake. Several studies showed that postprandial reduction of Ghrelin after bariatric surgery led to weight loss and T2DM remission^[33,34]. Therefore, lowering of Ghrelin plasma levels by non-surgical interventions might be a useful approach for obesity treatment. Different approaches already exist in the development of anti-obesity drugs. Pharmacological molecules like Ghrelin antagonists or Ghrelin receptor antagonists showed heterogeneous results in food intake reduction^[35]. Other strategies are the inhibition of Ghrelin O-acyltransferase (GOAT) that is required for activation of Ghrelin^[36] or lowering body weight by a vaccination targeting Ghrelin^[37].

Administration of Oxyntomodulin (OXM) decreases food intake and reduces body weight in rats^[38]. Further-

more, OXM has been shown to increase energy expenditure^[39]. The combination of decreasing energy intake and increasing energy consumption qualifies OXM to be a potential agent for bariatric treatment. Moreover, a plethora of other gut hormones and peptides are currently under intense investigation regarding weight loss. Interestingly, there is also evidence that various gut hormones are related to cancer growth and cancer development making their physiological understanding even more alluring^[40].

Obesity and cancer

Morbid obesity is associated with various types of cancer: Epidemiological studies identified an association of morbid obesity and several types of cancer disease, such as colorectal cancer, endometrium carcinoma, postmenopausal breast cancer, kidney cancer, esophageal cancer, pancreatic cancer, gallbladder cancer, liver cancer, and hematological malignancies^[41,42]. Obese patients have a tendency for worse prognosis and outcome after cancer treatment and an increased risk of cancer related morbidity^[43]. Calle *et al.*^[5] conducted a prospective study to examine the association of obesity and cancer related mortality. They concluded that increased body weight is associated with increased death rates for all cancers combined.

The link between obesity and cancer is still poorly understood. Several adipokines, growth factors, signaling pathways, inflammatory processes as well as the general demodulation of energy-balance and the lack of calorie restriction are being intensively discussed.

Adipokines are involved in cancer development: Traditionally, the adipose tissue was considered to be an energy storage organ. In recent years, however, it became evident that it also functions as an endocrine organ. Besides estrogen, it produces and secretes various adipokines and cytokines. Leptin and adiponectin, two well characterized adipokines, are associated with cancer development^[44].

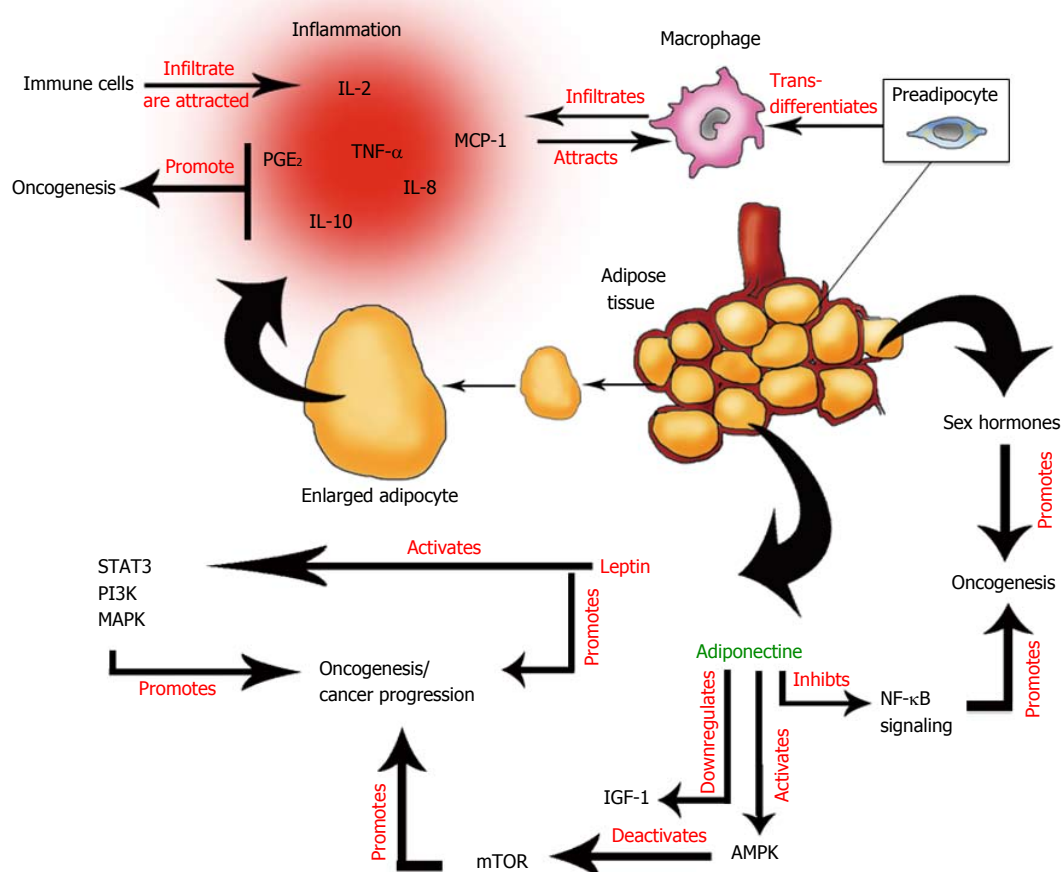


Figure 3 Endocrine, inflammatory, and cancer promoting effects of adipose tissue. IL-8: Interleukin 8; PGE₂: Prostaglandin E₂; TNF- α : Tumor necrosis factor- α ; MCP-1: Monocyte chemoattractant protein-1; STAT3: Signal transducer and activator of transcription 3; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; MAPK: Mitogen-activated protein kinase; NF- κ B: Nuclear-factor-kappa-light-chain-enhancer activated B cells; IGF-1: Insulin-growth factor-1; AMPK: 5'AMP-activated protein kinase; mTOR: Mechanistic target of rapamycin.

Leptin concentration in serum correlates positively with the patients' adipose tissue reserves and their nutritional condition. Moreover, leptin has been identified to be a potential mediator of cancer development^[45], which is able to activate various key players of different signaling cascades like phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3). More interestingly, leptin signaling promotes the progression of different cancers^[46-48].

Adiponectin is mainly secreted by visceral fat cells and acts adversary to leptin. It is inversely associated with obesity, hyperinsulinemia, and inflammation and may have anti-cancer effects by decreasing insulin-growth factor-1 (IGF-1) and mechanistic target of rapamycin (mTOR) signaling by activation of 5'AMP-activated protein kinase (AMPK). Also anti-inflammatory actions of adiponectin are described through inhibition of nuclear-factor-kappa-light-chain-enhancer activated B cells signaling (NF- κ B)^[49].

Increased carcinogenesis in obese patients might be due to chronic inflammation

Recent studies suggest a causal link of obesity related

diseases (Figure 3) and low-grade/chronic inflammation (Figure 3)^[50-52].

In humans, the immune system is of major relevance, which in turn, is able to form a defence shield against bacteria, viruses, or injured cells. A hallmark of the immune system is its most powerful weapon, the "inflammatory response" which was already noticed by a German pathologist called Rudolf Virchow in 1863. Despite the fact that humans without a functional immune system are not able to survive, too much inflammation can have a great impact and may cause serious damage to the healthy individual. Well-known chronic inflammatory diseases occur in patients that suffer from psoriasis or rheumatoid arthritis. A possible link between infections and cancer already exists, since stomach cancer may result from *Helicobacter pylori* infections or liver cancer from hepatitis (B-, C-) virus infections. A unique feature of these infections is the chronic inflammation response, which is primarily mediated by specific immune cells, such as macrophages and granulocytes that infiltrate the tumor. The latter is known to be recruited by tumor-released attractants. Once leucocytes infiltrate the tumor, they start to secrete chemokines and thereby initiate blood vessel growth/angiogenesis to allocate oxygen and nutrients,

which are relevant for tumor growth.

Circulating immune cell recruitment is a crucial feature of immune response

Macrophages: In obese individuals, macrophages infiltrate and expand in adipose tissue. Quantitative and functional changes of these cells affect adipose tissue inflammation. Exposure of macrophages to cytokines promotes two different activation states inducing to divergent polarizations. M1 macrophages are activated by tumor necrosis factor- α (TNF- α), Interferon- γ (IFN- γ), and bacterial endotoxins such as lipopolysaccharides. They are characterized by high levels of interleukin (IL)-12 and IL-23, and low levels of IL-10 as well as inflammatory cytokines^[53]. Contrarily, M2 macrophages are attracted by IL-4, IL-13, IL-10, and glucocorticoid hormones. Both types are part of innate immune response. M1 macrophages may induce chronic inflammation, whereas M2 macrophages tend to act anti-inflammatory^[54]. It has been suggested, that a phenotypic switch from M2 to M1 occurs in fat tissue^[55], however, this model is discussed controversially.

Eosinophiles: Eosinophiles levels are negatively correlated with obesity and adipose tissue in mice. Wu *et al*^[54] could show, that eosinophiles promote an M2-polarization of macrophages by secreting IL-4 and IL-13 and a down regulation of M1 macrophages in adipose tissue.

Mast cells: Mast cell levels in adipose tissue are elevated in obese animals^[56]. Mast cell ablation reduces body fat and benefits glucose homeostasis in mice. This effect is induced by IL-6 and IFN- γ . Also, pro-angiogenic factors such as Cathepsins may influence mast cell levels^[56].

Myeloid-derived suppressor cells: In adipose tissue, Myeloid-derived suppressor cells (MDSCs) have an inhibitory effect on inflammation by suppressing CD8⁺-T cells and promoting M1 to M2 macrophage switch in favour for M2 macrophages^[57]. The state of chronic inflammation in adipose tissue leads to an accumulation of MDSCs^[58]. Being part of immune autoregulation by MDSCs suppress overt inflammatory immune response in chronic inflammation^[59].

CD4⁺-T cells: CD4⁺-T cell activation is mediated by class II major histocompatibility complex (MHC II) molecules presented by macrophages and dendritic cells. When activated, CD4⁺-T cells secrete cytokines, which attract pro-inflammatory cells. Three groups of T cells can be distinguished, namely Th1, Th2, and Th17.

The ratio of Th1/Th2 cells is significantly enhanced in high fat diet induced obesity, since Th2 cells are undermined by IFN- γ producing Th1 cells^[60]. CD4⁺-T cell substitution in immunodeficient mice eventuates in reduction of weight gain, adipocyte cell size, and improvement of glucose homeostasis^[60]. The STAT6 pathway is essential for Th2 differentiation, thus STAT6 deficient CD4⁺-T cells do not show any effect of reconstitution on glucose

homeostasis and body weight gain^[60].

Regulatory T cells: CD4⁺-T cells can transdifferentiate into immunosuppressive CD4⁺CD25⁺-regulatory T cells (T_{reg})^[61]. Obesity is associated with reduced levels of T_{reg} cells in visceral adipose tissue in mice and humans^[62,63]. T_{reg} cell depletion enhances circulating insulin levels and levels of pro-inflammatory cytokines in adipose tissue of lean mice^[62]. Up regulation of T_{reg} on the other hand improves insulin sensitivity and enhances anti-inflammatory cytokine IL-10 levels^[62]. Also, T_{reg} function to suppress pro-inflammatory immune response and promote macrophage M1 to M2 switch by secreting IL-4, IL-10, and IL-13^[64].

CD8⁺-T cells: CD8⁺-T cell activation is mediated by MHC I. Activated CD8⁺-T cells induce lysis of target cells by producing various cytokines and chemokines.

Adipose tissue of obese animals^[65] and humans^[60] show a significant increase of CD8⁺-T cell levels. CD8⁺-T cells lead to elevation of macrophages in adipose tissue and promote polarization into M1 macrophages^[63]. CD8⁺-T cell deficient mice have fewer levels of macrophages in adipose tissue and less levels of TNF- α and IL-6^[63].

Natural killer T cells: When activated by lipids, natural killer T (NKT) cells produce a significant amount of Th1- and Th2-responsive cytokines, such as IFN- γ and IL-4^[66]. NKT cells can either promote or suppress inflammatory response by promoting either Th1 or Th2 cell activation^[67,68]. Interestingly, NKT cell levels are reduced in human omental adipose tissue^[69]. The role of NKTs in obesity still remains unclear.

B cells: After high fat diet, accumulation of B cells can be detected in adipose tissue of mice. This accumulation is associated with high levels of pro-inflammatory immunoglobulin G2c^[70]. B cells promote T cell modulation and macrophage polarization by producing pathogenic Ig-G antibodies. Ig-G, however, increases inflammatory response^[70]. The specific role of B-cells and Ig-G in inflammatory response in obesity has yet to be further investigated.

Mediators of inflammatory response: Preadipocytes can transdifferentiate into macrophages^[71]. Also they tend to enlarge due to oxygen diffusion resulting in hypoxia, inflammation and increased macrophage infiltration. Enlarged adipocytes produce a variety of inflammatory cytokines and show greater insulin resistance than normal sized ones. Levels of prostaglandin E2, TNF- α , IL-2, IL-8, IL-10, and monocyte chemoattractant protein-1 (MCP-1) are elevated in the microenvironment of enlarged adipocytes. The inflammatory environment attracts macrophages and induces production of additional pro-inflammatory mediators^[71].

NF- κ B is a central transcription factor that is activated upon bacterial and viral stimuli. It activates gene

expression associated with apoptosis, cell proliferation, inflammation, tumorigenesis, metastasis, and angiogenesis^[72]. In addition, increased NF- κ B expression and activation is associated with insulin resistance.

The frequent up-regulation of NF- κ B in many cancers is already known^[73]. The increased expression and “uncontrolled” activation of NF- κ B may induce cancerogenesis^[74,75]. Interestingly, NF- κ B gets activated upon leptin stimulation in preneoplastic and neoplastic human colonic epithelial cells *in vitro*^[76,77].

Inflammasomes, by definition cytosolic multiprotein complexes, activate IL-1 β and IL-18 during infection or tissue damage^[78]. They can be sub-divided into different inflammasome sub-groups such as nucleotide-binding oligomerization domain-like receptors (NLR), NLR pyrin domain-containing 1 and 3 (NLRP1 and NLRP3), absent in melanoma 2, and caspase activation and recruitment domains domain containing 4 (NLRC4/IPAF)^[78]. Inflammasomes secrete caspase 1, which cleaves cytokine preforms, such as IL-1 β ^[79,80]. The activity of NLR is associated with autoimmune diseases, malignancies, inflammation, infection, and metabolic disorders^[59]. Inflammasome components expression levels are elevated in adipose tissue of obese mice^[81-83]. Conversely, NLRP3 and IL-1 β are decreased in low calorie dietary restriction^[83]. It seems therefore, that NLRP3 integrates multiple signals, causing pathogenic inflammation in obese subjects^[84]. Also NLRP6 has a critical role in gut homeostasis^[85,86]. Mice with non-functional NLRP6 develop an altered commensal system, preventing normal glycaemic control on a high fat diet and promoting NASH^[87].

In summary, there are at least two inflammasome types and substrates that can imbalance metabolism and inflammation in obesity^[85].

Linking obesity to cancer-inflammation is a double-edged sword

The role of chronic inflammation as a precursor of tumorigenesis can be observed in various cancers. A gastritis can give rise to gastric cancer, inflammatory bowel disease may promote colorectal cancer and patients suffering from a chronic pancreatitis may have a higher risk to develop pancreatic cancer^[88]. The inflammatory effect of adipose fat tissue might therefore be a general precursor of cancerogenesis. Like adipose tissue, tumor microenvironment is composed of multiple cell types like fibroblasts, epithelial cells, mast cells, and cells of innate and adaptive immune system that favor a pro-inflammatory, pro-tumorigenic environment^[89-91].

Contribution to the pro-inflammatory environment is the presence of macrophages that are attracted by MCP-1. Tumor tissue classically contains a high amount of M2 polarized macrophages^[92]. Macrophages activated by obese states, infiltrate tumors and amplify the inflammatory tumor environment through NF- κ B dependent cytokine production and angiogenic factors^[88]. Malignancies may be initiated or exacerbate by inflammation, and increased levels of inflammation may be a cause and/or a

consequence of malignancy^[88,93].

Steroid hormones

Production of steroid hormones in the adipose tissue are also relevant for various cancers: Steroid hormones such as progesterone, estrogen, androgens and adrenal steroids are associated with energy balance level and obesity associated development of several cancer types^[94]. In women, the BMI correlates with the incidence of breast cancer, endometrium cancer and other cancer entities that are associated to sexual hormone levels. The relative contribution of adipose tissue steroid hormone production to the whole steroid metabolism is about 100% in postmenopausal women^[44]. The risk of developing breast cancer in post-menopausal women enhances with an increase of circulating levels of steroid hormones such as dehydroepiandrosterone, testosterone, estradiol and estrogen, and low levels of sex hormone binding globuline. There is evidence that estrogens are mitogenic, regulating the expression of insulin, and inducing DNA damage by free radicals, genetic instability and gene mutations in cells^[95]. Increased estradiol levels can induce endometrial cell proliferation rates while inhibiting apoptosis and activating the IGF-1 synthesis in endometrial tissue^[5].

In men, testosterone has been the focus of most studies on sex hormones, obesity and metabolic complications. Evidence indicates that most tissues, including adipose tissue, express steroid converting enzymes necessary for the local production of androgens and/or estrogens^[96]. Up to 40% of the active androgen production (dihydrotestosterone) is accounted for by tissue conversion of adrenal precursors^[96]. In men, obesity has generally been associated with reduction of testosterone levels in plasma and elevated estrogen concentrations^[97-99]. It has also been reported, that men with visceral adiposity have decreased levels of testosterone^[100,101]. A growing body of interest suggests, that obese men are more likely to be diagnosed with aggressive prostate cancer and high tumor volumes^[102]. Furthermore, obese patients show a higher risk of cancer recurrence, as well as an increase in disease related deaths compared to lean patients^[103,104].

Hyperinsulinemia and insulin growth factors

Increased insulin levels and insulin growth factor-1 signaling enhance cancer development^[105]. Other observational studies reported an increased mortality of obese cancer patients with T2DM due to hyperinsulinemia and elevated IGF-1 serum levels. In contrast, patients with lower insulin, IGF-1, and IGF-2 levels showed a lower risk to develop cancer^[105-107].

Patients treated with insulin or drugs stimulating insulin secretion showed a significantly higher incidence of developing malignancies than those patients treated with anti-diabetic drugs like metformin. Therefore, metformin might be a potential anticancer agent^[108].

Caloric restriction, which causes down-regulation of circulating insulin and IGF-1 levels is a potent suppressor in carcinogenesis^[74]. Insulin and IGF-1 can trigger cell

growth and proliferation, while inhibiting cell survival *via* protein kinase B (Akt)/PI3K/mTOR (Akt/PI3K/mTOR) pathway^[73]. This signaling pathway is not only the most frequently mutated pathway in human cancers, it is also a signal mediator of leptin, adiponectin and pro-inflammatory cytokines^[46,109,110].

Caloric restriction reduces cancer incidence by inhibiting the Akt/PI3K/mTOR pathway *via* AMPK activation^[111-113]. In contrast, Kalaany *et al.*^[110] could show that tumors with PI3K activation do not respond to the anti-cancerous effects of caloric restriction.

Interestingly, mTOR activity is increased in obese patients. It plays a central role in obesity related inflammation. Multiple risk factors for cancer development in obesity have been identified, such as the insulin-IGF-1 axis, leptin/adiponectin, and pro-inflammatory cytokines like IL-6, IL-7 and TNF- α . These factors can activate multiple pathways including PI3K/Akt, MAPK and STAT3, resulting in increased mTOR activity. mTOR, however, inhibits the insulin-PI3K pathway by stimulating the STAT3 pathway^[46].

IL-6 and TNF- α play a major role in obesity associated hepatocellular carcinoma by activating the STAT3 pathway^[114]. The STAT3 pathway is involved in the regulation of various gene expressions including IL-17, IL-23, B-cell lymphoma 2, and vascular epithelial growth factor to promote cell survival, proliferation, invasion, angiogenesis, and metastasis^[115]. Consistent activation of STAT3 increases tumor cell proliferation, survival and invasion in suppressing anti-tumor immunity. STAT3 activation also leads to activation of further pro-oncogenic pathways, such as NF- κ B and the IL-6/Janus kinase pathways^[115].

FUTURE OUTLOOKS

As time passes, advancement of technologies proceeds

In 2003, the Human Genome Project was accomplished. After 13 years and estimated costs of 2.7 billion USD, the first human genome was sequenced. In contrast, the human genome of an individual was sequenced over a 5 mo period of time at costs of 1.5 million United States-Dollars in 2008^[116].

An overall trend in the public health sector is the tendency towards “individualized therapy” in order to tailor specific therapy options that are currently available for a given patient which is further supported by usage of sophisticated mouse models.

Without doubt, mouse models have helped to understand relevant pathways that are important in the regulation of human body fat on the molecular level^[117-123]. Initial insights into molecules that are important in regulating body fat, resulted primarily from genetic mouse screenings^[117,124-126]. The identification of specific inactivating gene mutations accompanied by an obese phenotype, have revealed that leptin, leptin receptor and melanocortin-4 receptor play central roles in the regulation of body fat^[127-132]. Interestingly, these three obesity phenotypes as a result of inactivating mutations, are also

relevant in humans, suggesting that knockout mouse models are a powerful tool to gain new insights into obesity relevant human genes and proteins.

A clinical approach might further support the *in vivo* findings that resulted from former obesity mouse models. Extensive tissue banking combined with collected clinical data may open up new perspectives in translational medicine as well.

There already exist several methods for screening large patient cohorts such as next generation sequencing. Also, established methods (*e.g.*, Fluorescence *in situ* hybridization or immunohistochemistry) became powerful tools when featured with high-throughput methods such as tissue microarrays to gain knowledge in the distribution of potential obesity relevant proteins. Tools such as laser mass spectrometry combined with a large tissue database in a microarray format might enable the initiation of virtual protein expression profiling of cells in their natural tissue environment. Further development in this field and others will open up new possibilities to identify causal links between gene expression levels, RNA modification, protein expression levels, post translational modification of proteins, intrinsic enzyme activity, and initiation and progression of diseases on a molecular level.

Automated chip technologies for detection of structural variation discoveries on a DNA- and RNA-level may decrease sequencing time, streamline sample preparations and reduce costs in future studies.

Acquiring great amounts of patient cohorts' data in large databases combined with blood and tissue sampling will move clinical applicability of new gained knowledge into focus. New potential risk factors and/or therapy targets will be identified by high throughput tissue and blood screenings. Especially the combination of organ-tissue samples with respective blood samples, body fluids, and visceral/subcutaneous fat samples will help to understand complex causal connections between obesity and organ function failure and carcinogenesis on a molecular basis. The novel knowledge will be centralized and digitally organized, accompanied by its' access that will be provided to health care units and hospitals for data reconciliation.

Preclinical and clinical patient screening will provide the basis for individualized digital patient DNA-, RNA-, protein-, post translational modification-, and enzyme activity profiles that automatically may be compared to already identified risk factors or therapy targets in centralized data bases.

In the present, there already exist research projects that might serve as landmarks for individualized obesity research in the future.

Interestingly, the TG and HDL Working Group were able to identify rare mutations that disrupt apolipoprotein C3 function by sequencing the protein-coding regions of 18666 genes in each of 3734 participants. By correlating loss of function studies with clinical data, carriers of these mutations were found to have a reduced risk of

coronary heart disease^[133].

In the future, these mutations might serve as clinical risk-markers for coronary heart disease in obese patients. Blood samples of obese patients could be easily tested for gene mutations and the presence of a mutation might then be interpreted as a protective factor in favor of the patients' health.

Another study conducted an association analysis of single nucleotide polymorphisms, identifying genetic variants that predispose to T2DM^[134]. Testing blood or tissue samples right after birth for these genetic variants might probably change the way of clinical diagnostics entirely.

In the future, patients with genetic predisposition for *e.g.*, diabetes might be diagnosed before the onset of disease. This knowledge could then lead to an individualized treatment in terms of dietary intake, physical exercise, or to earlier elective surgical intervention in obese patients.

Also, gut hormone and adipokine serum levels could be screened on regular basis in obese individuals. When out of balance, pharmaceutical intervention with suitable drugs such as GOAT- inhibitors, GIP-Receptor antagonists, inhibitors of the mTOR-, STAT3- and MAPK-, PI3K-pathways or even Metformin might be applicable in the future to prevent relevant comorbidities such as cancer.

Morbid obesity is already a widespread problem not only in first-, but also in second world countries. It causes various major chronic diseases such as coronary heart disease, diabetes, hypertension, and cancer. As living standards in second and third world countries enhance, morbid obesity will proceed to be a huge challenge for health institutions and national health systems. Obesity is a potential human health threat and is likely to become even more present in the future. The relevance and possible long-terms effects of maternal obesity to the health of the offspring are not fully understood. Studies that deal with this issue are of high relevance to precisely understand the long-term adverse health outcomes for the upcoming new generations.

In conclusion, there is an urgent need for obesity research with a straightforward concentration on new studies that aim to identify and interpret the complex, multifactorial variables in order to develop new therapy approaches and prevention programs for patients suffering from this disease.

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Pathophysiological responses from human gut microbiome

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Abstract

The human gastrointestinal tract harbors a vast collection of symbiotic microorganisms-collectively termed as "gut microbiome". This microbiota has important effect in immune system and other host activities. Recent studies have suggested that alterations of the normal gut microbiota are associated with various human diseases and psychological disorders. The underlying cause, once proven, may provide novel insights into the importance of gut flora in human health. In this review, we give an attempt to describe how the alteration in the microbial community causes the development of certain widespread pathophysiological disorders; focusing on inflammatory bowel disease, colorectal cancer, obesity and autism. Proper knowledge about the host-microbiota interaction and linkage could be essential for the development of future personalized strategies of therapeutics.

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Key words: Human gut microbiome; Inflammatory bowel disease; Colorectal cancer; Obesity; Autism

Core tip: This review is an endeavor to provide an ac-

count about the human gut microbiome, their diversity, and disease causing capability. Till date, so many diseases have been associated with the alteration of gut microbiota. In this review we talk about four of the major diseases/disorders, viz., inflammatory bowel disease, colorectal cancer, obesity and autism.

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INTRODUCTION

The topic of human microbiome is pretty trendy in the present world of science. Human body is inhabited by more microbial cells than our own cell numbers. The term "microbiome" refers to the whole number of microorganisms residing in human body and their genetic material^[1-3]. It is different from the term "microbiota", which describes the microbial population present in different niches in the body. Resident microbes contain ten times more cells than our own somatic and germ cells and hence more number of genes than present in a human body-as a consequence they represent a combined microbial genome with a size bigger than human genome itself^[1,4]. Collectively, the flora has a metabolic action equal to a virtual organ within an organ^[5]. Researchers at human microbiome project, NIH are sampling and exploring data from few specific sites of human body, viz., airways, nasal passages, oral cavities, skin, blood, gastrointestinal tract, and urogenital tract^[2]. The microbial density starts increasing in the distal small intestine, and in the large intestine it rises to an estimated of 10^{11} - 10^{12} microbes per gram of colonic content, which contributes to 60% of the fecal mass. However, this is to bear in mind, since so many factors affect our body's ecosystem, the microbiota composition is different for every individual regardless of their age and sex.

Usually this microbiota is commensal and represents a healthy asset of our body, helping us to digest food and maintain immunity. Our typical understanding about a disease causing event states us that whenever a pathogenic organism enters our body, the disease takes shape. Introduction to the era of human microbiome enlightens us about a more susceptible way of causing disease—the imbalance of the microbiota within our body. Therefore human microbiome can be considered as a therapeutic drug target^[6].

The organisms from this microbiome are hard to culture. Metagenomics, the study of the genetic material extracted directly from environmental samples in a given environment, has been applied to the studies of the human microbiome, since it can be used to investigate various microbes simultaneously, without cultivation. This approach gathers speed in studies of human microbiome and their medical relevance. Studies about diverse microbes from the human body site-specific microbiota, and the correlations between their composition and disease have rapidly increased our understanding towards the importance of the human microbiome and its roles in health and disease^[3,7]. This bang of human microbiome data holds the promise of managing personal health, based on the genome and microbiome information of an individual.

HUMAN GUT MICROBIOME

A new chapter in medical science has emerged with the recognition of the crucial role of the gut microbiota in health and disease. At the time of birth human gut is completely sterile. However, immediately after birth the colonization of mammoth variety of microorganisms including bacteria, archaea, fungi and viruses starts within the body. The colonization of these microbial species within a body depends upon the mode of delivery, hygiene level, infant diet, and medication^[8].

Among all the niches, human GI tract contains the most number of microorganisms. The density of the microbiota increases from the proximal to the distal gut, reaching its maximum at the colon. In the different habitats of the gut, ecological sorting and competitive exclusion between microbes are the key factors influencing microbial diversity^[9,10]. Stochastic factors during colonization and in situ evolution cause the diversity of gut microbiota between individuals^[11]. The intestinal microbiota of infants lacks diversity and the major constituents are the phyla *Proteobacteria* and *Actinobacteria*. The microbiota attains diversity with age with the addition of *Fusobacteria*, *Cyanobacteria*, *Verrucomicrobia* and *Actinobacteria* amongst others and the dominance of *Firmicutes* and *Bacteroidetes* characterizes the adult microbiota^[12-14]. The gut microbiota is mainly a collection of anaerobes, which outnumber facultative anaerobes and aerobic microbes by approximately 2-3 orders of magnitude^[15]. In human, after the age of 2.5 years the gut microbiota remains almost the same throughout the adult age of that individual^[16,17]. The

actual adult human gut microbiota composition is diverse and differs from person to person in a significant way. Therefore it has been suggested that it can be used as a substitute to fingerprinting^[18]. In this regard three enterotypes have been found, *viz.*, *Prevotella*, *Ruminococcus* and *Bacteroids* that are independent of age or sex. The normal human gut flora composition is subject to age, diet, medication and socioeconomic conditions. In a recent study of gut microbiota in elderly individuals, the associations with diet and age was documented^[19].

It is a prominent fact that, although there is great variety in the composition of the gut microbiota among individuals, there still lays a conserved set shared between individuals, and this set of microbiota is called the core gut microbiome^[20]. The functions and pathways encoded by the core gut microbiome offer the greatest benefit to the host and are essential for the correct functioning of the healthy gut. The gut microbiota helps the host in various ways, including protection against probable pathogens, production of essential vitamins, digestion of polysaccharides, regulation of fat storage and modulation of the host's immune system^[21]. Latest studies have also revealed that the gut microbiota influences brain and the gut-brain axis configures the stress related symptoms such as anxiety and pain tolerance and few other psychological condition^[22].

ROLE OF GUT MICROBIOTA IN HUMAN DISEASE/DISORDER

It has been well established that the human gut microbiota is essential for human health. However, an alteration of the normal composition of the gut microbiome leads to formation of various types of diseases. Therefore it is reasonable to conclude that modulation of the gut microbiota can be used as a therapeutic target in treating these chronic diseases. Before properly utilizing the gut microbiota as a therapeutic tool, it is necessary to understand the role of these microbes in shaping disease. Till date, a great number of physical and psychological disorders have been associated with the alteration of gut flora; addressing all can be quite unfeasible task for this review. Thus, in this review, brief overviews of the current understanding about the role of microbiota in four common disease and disorders have been discussed.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is chronic, relapsing, immunologically mediated disorder that affects the digestive tract, mainly colon and small intestine. IBD majorly includes ulcerative colitis (UC) and Crohn's disease (CD). There is considerable evidence suggesting the importance of gut microbiota in IBD^[23-25]. Recent studies imply that an unbalanced microbial community composition is associated with a dysregulated immune response^[26]. Although the exact mechanism of IBD is not yet being

fully elucidated, four broad mechanisms are proposed to explain the complex relationship between the commensal microbiota and IBD: (1) dysbiosis of conventional microbiota; (2) induction of intestinal inflammation by pathogens and functionally altered commensal bacteria; (3) host genetic defects in containing commensal microbiota; and (4) defective host immunoregulation^[27]. The net result of these effects is continuous antigenic stimulation that activates pathogenic T cells, ultimately causing chronic intestinal inflammation. In case of patients suffering from CD, intestinal T lymphocytes have shown to be hyperactive against bacterial antigens as local tolerant mechanism is found to be abolished in them^[28]. In addition, they have increased intestinal secretion of IgG antibodies against a broad spectrum of commensal bacteria that, unlike IgA, activate complement cascade and inflammatory mediators^[29,30].

There are various hypothesis of how microbial composition in human gut plays vital role in the pathogenesis of IBD. Recent studies have shown that reduction of dominant commensal bacteria, such as *Firmicutes* and *Bacteroidetes*, and an increased number of *Proteobacteria* and *Actinobacteria*, may lead to this pathophysiological condition of human gut^[31,32]. A wide variety of gut microbial species have directly being linked with IBD. Among Firmicutes, *Faecalibacterium prausnitzii* is shown to have anti inflammatory activity and is found to be significantly decreased in CD^[33,34]. Furthermore, Joossens *et al.*^[35] have shown that reduction of *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, *Dialister invisus*, an unknown species of *Clostridium* clusters XIVA and increase of *Ruminococcus gnavus* are characteristic features in patients suffering from CD. A number of studies have shown that a wide variety of probiotic microorganisms, including *Escherichia coli*, *Saccharomyces boulardii*, and *Bifidobacterium* are involved in treating UC, although these are not always supported by high quality clinical trials^[36-42]. Also the role of microbiota in fecal transplantation, efficiently utilized in severe *Clostridium difficile* infection, have shown to be effective in IBD^[43,44].

COLORECTAL CANCER

Colorectal cancer (CRC) is one of the most common malignancies in the world, which accounts for about half million deaths annually^[45]. Although the prevalence of CRC is higher in the western world but it is found to be increasing in the developing countries at an alarming pace. Two principle mechanisms are mainly involved for the development of CRC-molecular genetics mechanisms and environmental factors^[46]. Dietary and genetic factors interact with each other *via* events taking place in the lumen of the large intestine^[47].

The genetic mechanisms of CRC are well established^[48]. A number of oncogenes and tumor-suppressor genes, such as *APC*, *KRAS*, *p53* and other regulatory genes are mutated in CRC patients. Together with DNA-methylation and chromatin-structure changes, the mutations

act to dysregulate conserved signaling networks that play vital role on cell phenotypes, including the regulation of cellular metabolism, proliferation, differentiation, and survival. Beside genes, environmental factors also influence the occurrence of CRC. Dietary carbohydrate and fat play critical roles in the development of colon tumorigenesis^[49]. Studies suggested that dietary fat and high consumption of red meat are associated with high risk of CRC^[50]. By contrast, a high intake of complex carbohydrate or dietary fibers, such as cellulose, lignin and pectin, that undergo bacterial fermentation in the colon, has been associated with reduced CRC risk^[51-53].

The effect of diet on carcinogenic process is mediated by changes in metabolic activity and composition of the colonic microbiome that accounts for over 100 trillion bacteria grouped in about 1000 species in human gut^[54,55]. Various studies have shown significant association between abundance of different bacterial species, particularly *Fusobacterium nucleatum*, with the prevalence of CRC^[56,57]. CRC risk was found to be associated with decreased bacterial diversity of Gram-positive, fiber-fermenting Clostridia; and increased presence of Gram-negative, pro-inflammatory genera such as *Fusobacterium* and *Porphyromonas*^[58]. The bacterial gut population can be shifted to a healthier composition by dietary fiber that provides substrates for bacterial fermentation^[55]. On the other hand, diet rich in fat and meat but poor in vegetables increases the concentration of N-nitroso compounds, a group of genotoxic substances that are known initiators of colon cancer^[59]. Another group of carcinogens are heterocyclic aromatic amines that are found in meat and some intestinal bacteria leads to DNA damage in colon cells due to the presence of such compounds^[60]. A more descriptive human study highlighted that high risk of CRC is associated with the presence of *Bacteroides vulgatus* and *Bacteroides stercoris*, whereas presence of *Lactobacillus acidophilus*, *Lactobacillus S06* and *Eubacterium aerofaciens* are associated with low risk of CRC^[61]. Although there is no conclusive evidence, gut microbiome seems to be a significant contributing factor that modulates risk of CRC in human beings.

OBESITY

Obesity is a medical disorder in which excess body fat accumulates over body. It is only recently that the problem of obesity has achieved global acknowledgment, in contrast to the problem of underweight and malnutrition-which have always conquered clinical attention. World Health Organization describes obesity as one of the major public health concern that threatens the modern world civilization and of late has become a global epidemic. A person is categorized as overweight when the body-mass index (BMI) is around 25 kg/m² or higher and people are classified as obese when the BMI is 30 kg/m² or more^[62]. A plentiful of studies has demonstrated that obese individuals are lazy, lack self-discipline. There is a social disgrace and discrimination against obese people

in various fields of life, which in turn creates numerous consequences for their psychological and to some extent physical health^[63]. However, obesity is not only a cosmetic concern. It has serious health concerns including increased risk for type 2 diabetes, cardiovascular diseases, non alcoholic fatty liver disease, pulmonary hypertension, asthma, sleep apnea, osteoarthritis, gall-bladder disease, a number of cancers, and most importantly an increased risk of mortality^[64].

Numerous studies have suggested that the gut microbiota plays a crucial role in the development of fat mass and altered energy homeostasis^[65]. Obese gut microbiota increases both the capacity to harvest energy from the diet and the accumulation of fat in adipose tissue and liver, by altering host metabolism. Studies in germ-free and conventionalized mice revealed that the microbiota helps in absorbing the monosaccharides from the gut lumen and adipocyte hypertrophy by suppressing fasting-induced adipocyte factor in the intestine, and this suggests that the gut microbiota is an important factor that affects energy harvest from the diet and energy storage in the host^[66,67]. The gut microbial community is diverse; consisting of bacterial species, archaea and various microbial eukaryotes. Therefore competitive interactions among these species might also play crucial roles in promoting obesity. In this regard, methane producing archaea *Methanobrevibacter smithi* has been found to be present in greater abundance in obese mice and humans when compared with lean individuals^[68,69]. Obesity and diet could be associated with altered gut microbiota characterized by a high *Firmicutes* to *Bacteroidetes* ratio and a dramatic fall in overall microbial diversity^[70].

It has been proposed that the composition of the gut microbiota during childhood predicts the following development of obesity in humans. In this regard some studies were conducted to compare between the fecal samples from overweight/obese and normal weight children^[68,71]. It shows that during infancy, a significantly higher number of *Bifidobacterial* species was observed in children who maintain a normal weight at age 7 years, while significantly greater numbers of *Staphylococcus aureus* were detected in children who became obese afterward. Therefore, it is hypothesized that an early modulation of gut microbiota can actually prevent obesity^[72,73]. Interestingly, another study found that the microbiota composition is different in case of pregnant women also, with relatively higher numbers of *Bacteroides* and *Staphylococcus* found in overweight pregnant women^[74]. Obese human twins also have different gut microbial composition as compared to their lean twin. The obese one has reduced levels of *Bacteroidetes* and also less bacterial diversity^[69].

AUTISM

The brain is strongly coupled with the gut *via* 200-600 million neurons^[75]. Currently, a growing number of clinical data and experimental observations suggest the presence of bidirectional gut-brain axis, implying that there

are probably many a type of neuro-atypical symptoms; including stress, depression, anxiety, associated with the alteration of the normal composition of gut microbial flora^[76,77]. In this review we would like to restrict ourselves to one neuropsychiatric disorder-Autism. The Autism Spectrum Disorder (ASD) is an assemblage of neuro-developmental disorders characterized by obscurity in social interaction and communication in affected children. It is typically associated with limited, repetitive, and stereotypic behavior and is noticeable within the first 3 years of life^[78,79]. Until 1990, Autism was treated as a rare psychological disorder. Today it is a major health concern, big emotional burden for families, and large financial burden for the government worldwide.

Though the principal cause of this disorder is yet to be known; gastrointestinal disorders have frequently been reported in the children with autism-suggesting the probable link between the atypical compositions of human gut microbiome with ASD^[80]. The hypothesis regarding the gut microbiota and ASD linkage was first coined by Bolte^[81]. Their study showed that interruption in the normal composition of native gut flora resulted colonization of some neurotoxin producing bacteria, contributing to the autistic symptom^[82]. As the importance of gut microbiota in gut-brain function came emerging; probable role of diet, bacteria, and enzyme became a field of important study in autism research^[83]. It has been proved that there is a significant difference between the stool sample from autistic and normal children in terms of frequency of occurrence of four bacterial phyla specifically, *viz.*, *Firmicutes*, *Bacteroids*, *Actinobacteria* and *Proteobacteria*. Further studies have shown higher count and diversity of *Clostridia* (mainly *Clostridium tetani*, *Clostridium perfringens* and *Clostridium botteae*) and *Desulfovibrio* (mainly *D.desulfuricans*, *D.fairfieldensis*, *D.piger*) in fecal samples of children with autistic behavior as compared to the normal healthy children with same sex and age^[81,84-91]. Evidence suggests that high occurrence of *Bifidobacterium* and *Lactobacillus* species is a biological indicator for healthy gut microbiota in breast-fed infants as they serve important probiotic function in the gut^[92-94]. As expected, these organisms are frequently reported to be lower in patients with ASD. People are working with several animal models to investigate the expected link between gut microbiota and autism like disorders. One recent paper on maternal immune activation (MIA) mouse model has revealed gastrointestinal abnormalities and changes in the gut microbial community in offspring of MIA animals with autism-like symptoms^[95].

Till date, several studies have demonstrated the presence of a perturbed intestinal microbiota composition in children with ASD compared to normal control children. However, caution should be applied while drawing conclusion from these results, as patients with ASD have probable history of using high antibiotic dosage and different diets compared with neuro-typical individuals, either of these can influence the normal composition of the gut microbiota^[96,97]. Fortunately enough, a recent study demonstrated that the alteration in the concentra-

tions of short-chain fatty acids in the fecal sample of children with ASD^[98]. This suggests that atypical production of such microbial metabolites may have a direct effect on our brain function and thus bacteria can modulate the brain function in a straight line.

CONCLUSION

It is well established fact that the gut microbiota influences host metabolism, immune function, and host homeostasis. Interruption in this balanced community may generate very serious health troubles for the host. Advancement of next-generation genomic technology will pave the way to the development of experimental models of representative examples from the human gut microbiome. This will consecutively accelerate the discovery, testing, and validation of novel drug targets. Future metagenomic research is also expected to focus on the complex relationships of the gut microbiome composition and host metabolism so that in time their actual importance to human health will also be understood better. More in depth understanding of the specific relationships between the gut microbiota and disease will enlighten us about the potential therapeutic targets. The issue of intelligent modulation of the intestinal community is a topic of great interest nowadays. The gut microbiome is expected to contribute immensely to the delivery of personalized healthcare strategies that are already being applied into the clinical environment for the benefit of patients. It can open new door to treating disease and potential modulation of human disease risk factors.

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Pharmacogenetics of type 2 diabetes mellitus: An example of success in clinical and translational medicine

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responsible for the interindividual variability of drug response to sulfonylureas in patients with T2DM. Instead, genetic variants in the genes that encode for the organic cation transporters of metformin have been related to changes in both pharmacodynamic and pharmacokinetic responses to metformin in metformin-treated patients. Thus, based on the individual's genotype, the possibility, in these subjects, of a personalized therapy constitutes the main goal of pharmacogenetics, directly leading to the development of the right medicine for the right patient. Undoubtedly, this represents an integral part of the translational medicine network.

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Key words: Type 2 diabetes; Anti-diabetic drugs; Personalized therapy; Genetic variants; Genome-wide association study

Abstract

The pharmacological interventions currently available to control type 2 diabetes mellitus (T2DM) show a wide interindividual variability in drug response, emphasizing the importance of a personalized, more effective medical treatment for each individual patient. In this context, a growing interest has emerged in recent years and has focused on pharmacogenetics, a discipline aimed at understanding the variability in patients' drug response, making it possible to predict which drug is best for each patient and at what doses. Recent pharmacological and clinical evidences indicate that genetic polymorphisms (or genetic variations) of certain genes can adversely affect drug response and therapeutic efficacy of oral hypoglycemic agents in patients with T2DM, through pharmacokinetic- and/or pharmacodynamic-based mechanisms that may reduce the therapeutic effects or increase toxicity. For example, genetic variants in genes encoding enzymes of the cytochrome P-450 superfamily, or proteins of the ATP-sensitive potassium channel on the beta-cell of the pancreas, are

Core tip: Type 2 diabetes mellitus (T2DM) is a heterogeneous complex disorder, in which predisposing genetic variants (polymorphisms) and precipitating environmental factors interact synergistically in the development of the disease. Besides being useful in identifying individuals at risk for T2DM, knowledge of the polymorphisms associated with T2DM is also useful in pharmacogenetics for correlating individual variants with individual responses to anti-diabetic drugs. To date, a wide variety of genes that influence pharmacogenetics of anti-diabetic drugs have been identified. However, with few exceptions, drug therapy has not taken into account the individual genetic diversity of treated patients, representing, this, a substantial limitation of pharmacogenetics. This review focuses on clinically important polymorphisms affecting a patient's response to diabetic medications.

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INTRODUCTION

The common observation that patients with type 2 diabetes mellitus (T2DM) show a great variability in the individual response to the same drug treatment suggests the importance of a personalized care approach, in which the most appropriate treatment is indicated by the genetic peculiarities of each individual^[1]. The introduction, in 2007, of genome-wide association study (GWAS) has greatly enhanced the number of genes that are known to be associated with common diseases. Applied to millions of people, this method has allowed the identification of several genetic variants which are associated with T2DM^[2]. However, similarly to other complex diseases, none of the individual variants identified so far is in itself sufficient to cause the disease, but most of the genetic risk for T2DM is mediated by the combined influence of more genetic variants that individually have only a small degree of risk^[3,4]. This combination (haplotype) defines the genetic profile of the individual. The fact that the pathogenesis of T2DM requires the involvement of multiple genes in different combination is in line with the assumption that T2DM, far from being a disease genetically identifiable in a few specific forms, actually consists of a large number of rather different disorders^[3,4], each of which is associated with a specific disease phenotype only apparently identical to one another, and in which inter-individual variability in drug response can be identified both in terms of drug efficacy and undesired drug reactions.

Therefore, clarifying the molecular mechanisms by which genetic variations may cause differences in phenotypic traits and in individual drug response is essential not only to determine the etiological role of gene variants, but also to identify new personalized medical solutions. Personalized therapy, based on the genetic diversity of each individual, is one of the most fascinating challenges of modern medicine, representing an integral part of the translational medicine effort, whose ultimate goal is to translate advances in biomedical research into new medical treatments and improvements in patient care (Figure 1). Herein, we provide an overview of this area and its relevance to clinical practice in T2DM.

PHARMACOGENETICS AND GENE POLYMORPHISMS

Pharmacogenetics is defined as the influence of variations in DNA sequence on drug response (www.ema.europa.eu). Its relevance arises from the clinical observation that patients suffering from the same disease do not necessarily respond to the same drug treatment in terms of therapeutic efficacy as well as adverse effects. The principal aim of pharmacogenetics is to provide

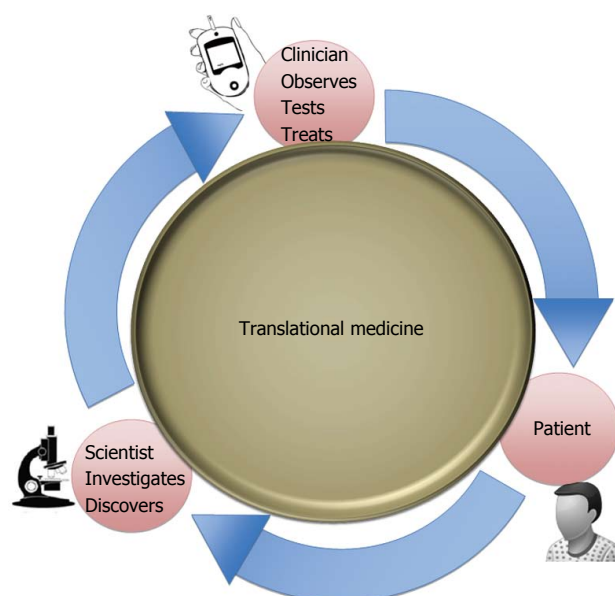


Figure 1 From bench to bedside. Translational medicine is a discipline of biomedical research that attempts to connect basic research with clinical care.

personalized medicine, tailored to an individual's genetic makeup, in order to optimize the effectiveness and safety of drug treatment. Although elements of pharmacogenetics can be traced back to ancient Greece (510 years BC), when it was already known the risk of hemolytic anemia in certain individuals in response to the ingestion of uncooked fava beans^[5], the term “pharmacogenetics” was first coined by Vogel^[6] in 1959 to indicate the importance of genetic polymorphisms on the disposition and action of drugs. The first evidence on the role of genetic variants in drug response back to the '70s and refers to cytochrome P-450 2D6 (CYP2D6), an enzyme of the hepatic P-450 microsomal enzyme system, which is involved in the metabolism of numerous drugs. Studies of the genetic variations within the P-450 family of enzymes provided the first direct evidence for the genetic contributions to drug therapy and efficacy, and these studies continue to be an active part of the basic and clinical research performed today. In fact, numerous other genetic variations have been identified in subsequent years, within the P-450 family of enzymes, including the biotransformation enzymes CYP3A4/5 and the CYP2C9 enzyme. It has been shown that individuals carrying genetic variants of *CYP2D6* (and other P-450 isoforms resulting in poor enzymatic activity), who are concomitantly taking medications that are influenced by these enzymes, are at risk for increased or prolonged drug effect, influencing the speed and effectiveness of drug metabolism^[7]. However, there is no doubt that the greatest contribution to pharmacogenetics has come from the sequencing of the entire human genome in 2003, showing that over 99% of DNA is identical in all humans and that, therefore, phenotypic differences among individuals, as well as differences in disease susceptibility and the inter-individual variability in drug response, are the result of sequence polymorphisms that

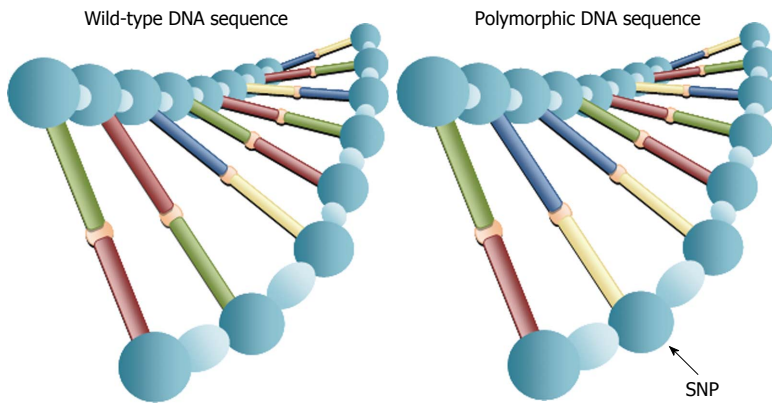


Figure 2 Single nucleotide polymorphism. As the most common type of variant, a single nucleotide polymorphism is characterized by a single DNA base pair substitution at a specific location in a gene. SNP: Single nucleotide polymorphism.

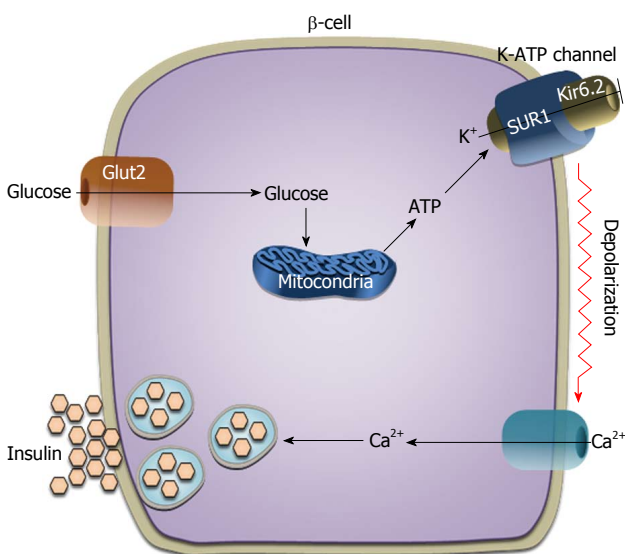


Figure 3 The ATP-sensitive K⁺ channels regulate insulin release in beta-cells. Single nucleotide polymorphism in *SUR1* and/or *Kir6.2* genes may cause functional abnormalities of the ATP-sensitive K⁺ channel on the pancreatic β -cell membrane, leading to abnormalities in insulin secretion.

affect less than 1% of 3 billion bases of human DNA. In most cases, these variants consist of the exchange of single nucleotides in both coding and noncoding DNA regions and are defined as single nucleotide polymorphisms (SNPs) (Figure 2). The ability of the SNP to influence drug response and therapeutic efficacy may rely on the capacity of the variant to induce changes in the expression of proteins that may influence either the pharmacokinetic and/or pharmacodynamic profile and hence the clinical efficacy of the drug. On the basis of these acquisitions, recent GWAS have identified several SNPs that can affect both the therapeutic efficacy and the occurrence of adverse reactions after drug intake^[8-10].

PHARMACOGENETICS IN T2DM TREATMENT

Pharmacogenetics of sulfonylureas

In Caucasians, sulfonylureas are metabolized primarily

in the liver by CYP2C9 to active metabolites, which are ultimately excreted by the kidney^[11]. In previous work, it was demonstrated that polymorphisms of the *CYP2C9* gene significantly affect the pharmacological response of diabetic patients to sulfonylureas^[12], due to the reduction of the catalytic activity in the metabolism of these drugs^[13-16], with a consequent increase in drug bioavailability. In particular, in certain diabetic patients with the variants Ile359Leu (isoleucine changes to leucine in exon 7 position 359) and Arg144Cys (arginine changes to cysteine in exon 3 position 144) in the *CYP2C9* gene, the clearance of glibenclamide was reduced by 30%-80%, allowing the use of lower doses of this drug to limit the risk of hypoglycemia^[12,17-20]. The risk of hypoglycemia in sulphonylurea treated patients was confirmed in a study with a larger population, in which the simultaneous presence (or the presence in homozygosity) of the variants Ile359Leu and Arg144Cys in the *CYP2C9* gene was associated with the improvement in markers of glycemic control, including glycated hemoglobin A1c (HbA1c)^[21]. Therefore, genotyping of the *CYP2C9* gene may provide important additional information in predicting the adverse effects of these drugs and to assist physicians in prescribing oral hypoglycemic agents.

The ATP-sensitive potassium [ATP-sensitive K⁺ (K-ATP)] channel plays a central role in mediating glucose-stimulated insulin release from pancreatic beta-cells (Figure 3). In physiological conditions, the rapid entry of glucose into the beta-cell results in an increase in the intracellular concentration of ATP, which promotes the closure of the K-ATP channel with consequent opening of the voltage-dependent calcium channel, elevation of intracellular calcium ion concentration and insulin secretion. The K-ATP channel is composed of two subunits: the sulphonylurea receptor (SUR1) and the pore-forming inward rectifier K⁺ channel Kir6.2^[22,23]. Genetic variants inactivating the *KCNJ11* (potassium inwardly-rectifying channel, subfamily J, member 11) gene, which encodes for the protein Kir6.2, and the ATP-binding cassette, subfamily C (CFTR/MRP), member 8 (*ABCC8*) gene, which encodes the SUR1 protein, are responsible for neonatal diabetes mellitus; conversely, activating mutations of

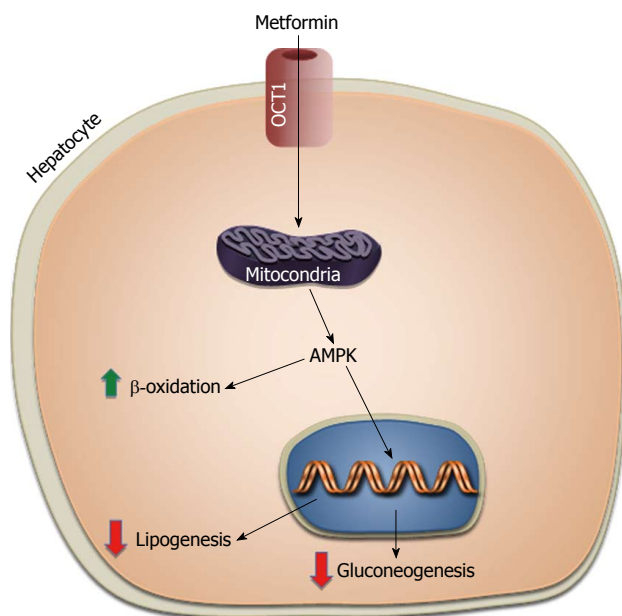


Figure 4 Organic cation transporter 1 plays a major role in drug uptake across the liver cell membrane. Single nucleotide polymorphism associated with organic cation transporter 1 may contribute to variation in response to metformin. AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; OCT1: Organic cation transporters 1.

these two genes lead to hyperinsulinism and neonatal hypoglycemia^[24]. As an example of pharmacogenetics with important clinical implications, recent studies have found that diabetic patients carrying mutations in the *KCNJ11* gene respond better to treatment with sulfonylureas than to treatment with insulin^[25-27].

Association of the polymorphism Ser1369Ala (serine 1369 to alanine substitution) in *ABCC8* with the antidiabetic efficacy of gliclazide was found in patients with T2DM, after two months of treatment^[28]. In particular, patients with the genotype alanine/alanine had a greater reduction in either fasting plasma glucose or 2 h postload plasma glucose during oral glucose tolerance test, and a greater decrease in HbA1c levels compared to patients with the Serine/Serine genotype^[28]. The variant Ser1369Ala in *ABCC8* is often associated in linkage disequilibrium with a variant, Glu23Lys (glutamine to lysine variant at position 23), in the *KCNJ11* gene, forming a haplotype that increases the risk of developing T2DM^[29]. It has been observed that this haplotype displays large differences to the therapeutic effects of various sulfonylureas: greater to gliclazide, less apparent to tolbutamide, chlorpropamide and glimepiride, invariable in the glipizide and glibenclamide treatment group^[30].

Interesting results, in this context, have been obtained from the study of the transcription factor 7-like 2 (*TCF7L2*) gene, which encodes a nuclear transcription factor that appears to play a role in beta-cell function. Genetic variants of *TCF7L2* are associated with increased risk of T2DM^[3]. Recently, two variants of the *TCF7L2* gene, rs7903146 (G > T), and rs7903146 (C > T), have been shown to influence the therapeutic efficacy of sulfonylureas^[31-33]. In particular, the reduction in both

HbA1c and fasting plasma glucose was higher in diabetic patients carrying either GG or CC genotypes^[31-33]. In contrast, diabetic patients with the TT genotype in both the rs7903146 (G > T) and the rs7903146 (C > T) variants showed a lower response to sulfonylureas and appeared to be more prone to therapeutic failure^[31-33].

Pharmacogenetics of metformin

Metformin, in use for control of diabetes since 1950s, is the first-line pharmacological therapy for T2DM. After oral administration, the drug is absorbed into the blood *via* the gastrointestinal tract, rapidly distributed in body tissues by travelling through specific transport proteins [including the organic cation transporters 1 (OCT1) and OCT2, the multidrug and toxin extrusion 1 (MATE1) transporters and MATE2-K, and the plasma membrane monoamine transporter (PMAT)] located on the cytoplasmic membrane of many cells, especially intestinal cells, liver cells and kidney cells^[34], and excreted in the urine almost unchanged from the original drug. The individual's response to metformin is highly variable with less than 2/3 of treated patients achieving glycemic control^[35]. Thus, identification of genetic variants that may influence the interindividual variability to metformin would be of major importance for the effective treatment of these patients. However, studies on the pharmacogenetics of metformin are relatively limited, mainly because its mechanism of action is still poorly defined. So far, most of the studies on this topic have involved the solute carrier family 22A1 (*SLC22A1*) gene, which by coding for the OCT1 transport protein, plays a key role in the cell absorption of the drug^[36], and is essential for the anti-gluconeogenic effect of metformin into the liver^[37] (Figure 4). It has been shown that polymorphisms of this gene (rs12208357; rs34130495; rs72552763; rs34059508), by reducing the functional capacity of OCT1, can alter the bioavailability of metformin and mitigate its hypoglycemic response in healthy people carrying these gene variants^[37-39]. Recently, two polymorphisms of *SLC22A1* (rs628031 and rs36056065) have been associated with gastrointestinal side effects in diabetic patients treated with metformin^[40]. At the same time, other authors^[41,42] have also reported that the bioavailability of metformin was increased in healthy individuals carrying mutations of the *SLC22A2* gene, which encodes for the OCT2 transport protein. Variants of this gene, by adversely affecting OCT2 function, may decrease the renal clearance of metformin, and may contribute to increased plasma metformin levels with increased risk of hypoglycemic events.

Interindividual variation in metformin response has been recently reported in subjects with genetic variations in *SLC47A1* and *SLC47A2* genes coding for MATE1 and MATE2-K, respectively, which play important roles in the urine excretion of metformin. A better glycemic response to metformin, with lower HbA1c levels, has been reported in association with the *SLC47A1* gene variant rs2252281^[43-46]. In contrast, the therapeutic response to metformin was reduced in diabetic patients

carriers of the variant rs12943590 in the *SLC47A2* gene^[45,46]. Therefore, these observations imply that genetic variants of *MATE1* and *MATE2-K* are important determinants of the therapeutic efficacy of metformin in patients treated with this drug. The first GWAS on the efficacy of metformin on glycemic control in diabetic patients resulted in the demonstration that a gene variant near ataxia telangiectasia mutated (*ATM*), rs11212617, is significantly associated with metformin treatment response in T2DM, more frequently with HbA1c levels < 7%^[47]. The explanation of this phenomenon lies in the role *ATM*, the protein product of the *ATM* gene, plays in the context of insulin signaling and insulin action^[48].

Thus, genetic variants of *SLC22A1* and *SLC22A2* may be determinant in the therapeutic efficacy of metformin. Furthermore, genotyping of *SLC22A1* and *SLC22A2* is useful in the management of diabetic patients under metformin therapy.

Pharmacogenetics of thiazolidinediones

Genetic variants that can influence the pharmacogenetics of oral antidiabetic medications were also assessed in diabetic patients treated with pharmacogenetics of thiazolidinediones (TZDs) (pioglitazone and rosiglitazone). As agonists of peroxisome proliferator-activated receptor gamma (*PPAR-γ*), TZDs act as insulin-sensitizing, thus reducing the release of glucose from the liver and increasing glucose uptake in muscle^[49]. The *PPAR-γ* gene has been extensively investigated in pharmacogenetic studies of TZDs, especially because genetic variants of this gene have been associated with an increased risk of T2DM^[3]. However, pharmacogenetic studies with TZDs have shown conflicting results, probably due to insufficient sample size and low levels of statistical power^[50]. Furthermore, it is worthy noting that the retrospective study design used in the majority of studies on pharmacogenetics has its own drawbacks, being able to expose to a variety of confounding and bias, including age, gender, ethnicity, lifestyle, concomitant use of other medications, *etc.* A similar discrepancy has emerged from studies on the genetic variants of the *CYP2C8* gene, which is responsible for metabolizing pioglitazone^[50]. A reduction in the blood glucose-lowering effect of pioglitazone was recently observed in diabetic patients carriers of the truncation variant, Ser447X, of the lipoprotein lipase gene^[51]. Another study has reported that the -420 C/G variant of the *resistin* gene promoter can also be used as an independent predictor of the reduction of fasting plasma glucose and insulin resistance by pioglitazone in T2DM^[52]. As it is known, side effects of TZDs therapy include fluid retention and peripheral edema, worsening heart failure^[53]. In this context, various genetic variations have been discovered in genes known to be involved in sodium and water reabsorption. Among these, the aquaporin 2 (*AQP2*) rs296766 variant and the *SLC12A1* rs12904216 variant, both of which have been associated with edema in T2DM patients treated with a TZD^[54]. *AQP2* gene codes aquaporin-2, which function as a water channel in the

collecting duct of the kidney^[55]. *SLC12A1* encodes the kidney-specific sodium-potassium-chloride cotransporter (NKCC2), which plays an important role in both urine concentration and NaCl reabsorption^[54,56]. Therefore, it is quite evident that these variants may represent both a risk factor for the development of edema in diabetic patients during treatment with TZDs.

Pharmacogenetics of metiglinides

Metiglinides (repaglinide and nateglinide) are a class of rapid-acting, short duration insulin secretagogues that act in a manner similar to that of the sulfonylureas^[57]. Nateglinide is also metabolized by the *CYP2C9* enzyme of the cytochrome P-450 system, and gene variants of *CYP2C9* are associated with variability in glucose-lowering effect of nateglinide^[58]. Repaglinide is metabolized by *CYP2C8* and to a lesser degree by *CYP3A4*^[59]. Also in this case, gene variants of *CYP2C8* have been associated with increased clearance of repaglinide, although with contradictory results^[60]. The solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) gene encodes for the organic anion transporting polypeptide, OATP1B1, which regulates cellular uptake of various drugs, including statins by the liver. Recent studies have reported the role of some variants of *SLCO1B1* in the pharmacokinetics of metiglinides^[61-64]. For example, a more effective hypoglycemic effect of repaglinide was observed in diabetic patients carrying the Glu23Lys (E23K) polymorphism in the *KCNJ11* gene^[65], and the rs13266634 variant in the *SLC30A8* gene^[66]. Similarly, polymorphisms of neurogenic differentiation 1 (*NEUROD1*), also called beta2 (*NEUROD1/BETA2*), paired box gene 4^[67] and uptake control 2^[68] genes were also found to be associated with the hypoglycemic efficacy of repaglinide. An association of the variant G2677 T/A in the multidrug resistance gene, which encodes a multidrug efflux pump, with the variability in the pharmacokinetics of repaglinide was found recently in a Chinese study in healthy volunteers^[69].

Pharmacogenetics of incretins

Glucagon-like peptide-1 (GLP-1) is part of the group of incretin hormones that are secreted from endocrine cells in the intestinal mucosa in response to meals. It mediates insulin secretion in a glucose-dependent manner and is easily inactivated after being secreted by the enzyme dipeptidyl peptidase-IV (DPP-IV). Recent pharmacological research has led to the development and synthesis of medications that are capable of acting at this level as both GLP-1 agonists (exenatide and liraglutide) and DPP-IV inhibitors (gliptins)^[70]. Variants of the GLP-1 receptor gene have been shown to be associated with altered sensitivity to GLP-1^[71]. Furthermore, whereas variants in the *TCF7L2* (rs7903146) and wolfram syndrome 1 (rs10010131) genes have been associated with a reduced response to exogenous GLP-1, variations in the *KCNQ1* (rs151290, rs2237892, and rs2237895) gene appear to alter the secretion of endogenous GLP-1^[72]. The only significant study on the pharmacogenetics of gliptins

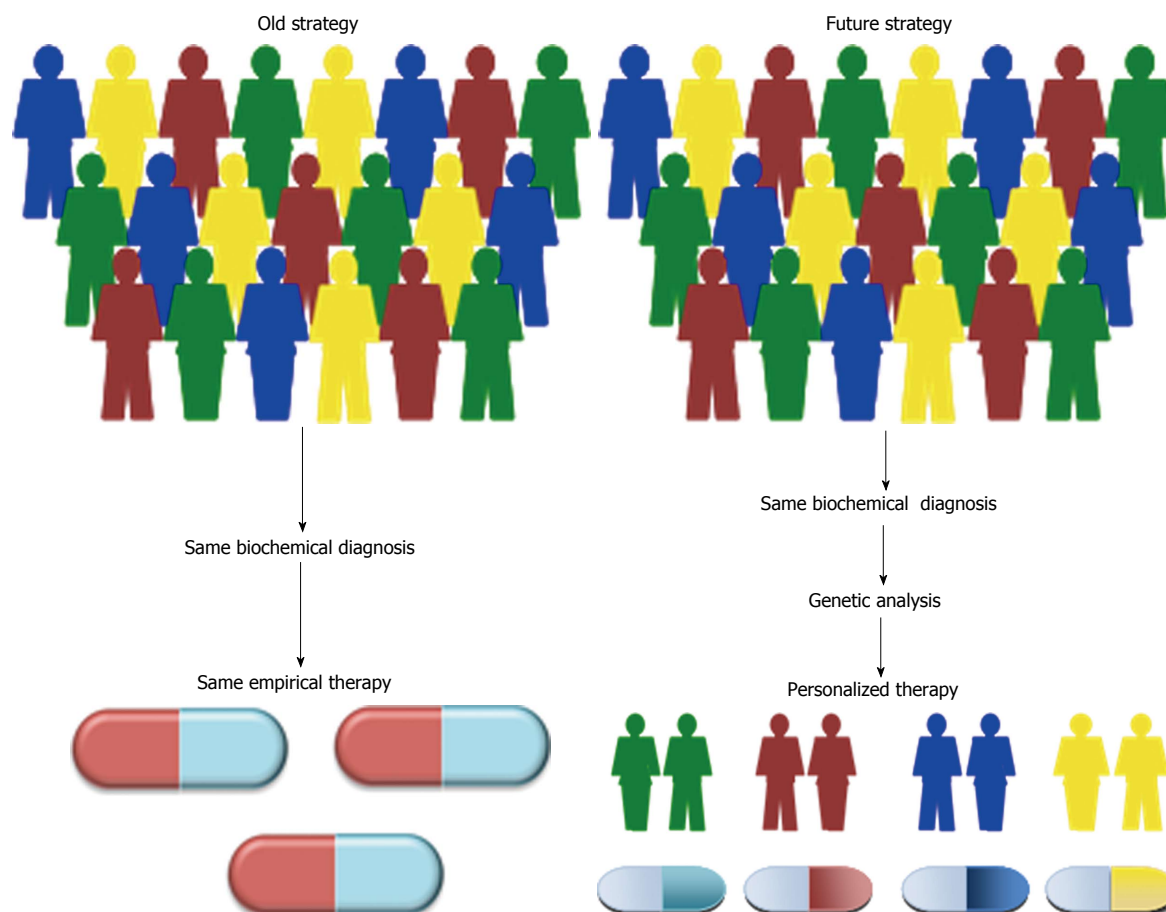


Figure 5 Pharmacogenetic testing. The pharmacogenetic test has the potential to provide personalized therapy based on individual genetic variability.

showed that three novel genetic loci (transmembrane protein 114, carbohydrate sulfotransferase 3 and Chymotrypsinogen B1/2) were identified, which affect GLP-1-induced insulin release during hyperglycemic clamp in nondiabetic Caucasian subjects^[73].

CONCLUSION

Pharmacogenetics is an expanding area of research which seeks to understand how variations in the genome influence medication response. Pharmacogenetics has gained increasing attention in the context of translational medicine, providing an opportunity for personalized treatment strategies based on an individual's genetic makeup. The results obtained so far with the study of genetic variants in patients with T2DM (and other common diseases) may be used for the realization of a pharmacogenetic test, which can assist in making treatment decisions on the basis of each patient's genetic profile, thus improving the overall management of the disease and ensuring better results in terms of safety and therapeutic efficacy. The clinical use of pharmacogenetics, through the identification of individual genetic variants (genetic polymorphisms), can contribute to move to a more evidence-based and less empiric clinical management of patients, thereby avoiding treatment failures, while reducing the incidence of adverse drug reactions (Figure 5).

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Psychotherapy in anorexia nervosa: What does the absence of evidence mean?

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Core tip: This paper presents an alternative explanation conspicuously lacking in the literature as to the scarce evidence concerning the efficacy of psychotherapy in anorexia nervosa. The absence of data supporting a particular treatment undermines the basic tenets underlying the theory on which it is grounded, or is at least a defective translation of the theory into the “dos” and “don’ts” of manualized treatment. This assertion is elucidated by recent research on a placebo and non-specific treatment that was found to be more effective than a number of specialized treatments.

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Abstract

Psychological treatment in anorexia nervosa (AN) is disheartening. Psychotherapy is the “treatment of choice” for adults though this recommendation is grounded on the absence of good quality clinical studies. This paper seeks to address the question of why improvements in the psychological treatment of AN have been thwarted, and why one of the best treatments available for adult patients is specialist supportive clinical management that has entered the stage through the backdoor of nonspecific supportive treatments originally serving as a placebo treatment assigned in randomized clinical trials to control for non-specific aspects of true psychosocial treatments. The possibility that most of the psychopathological features that characterise the AN symptoms profile could be best understood as the direct consequences of emaciation would enhance the utility of research with animal models for generating new hypothesis to improve AN treatment.

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INTRODUCTION

According to successive state-of-the-art reviews, contemporary treatment of anorexia nervosa (AN) is discouraging^[1]. This pessimistic view is all the more disturbing when we consider the high rates of relapse after successful weight restoration in specialist inpatient settings^[2]. With regard to pharmacological treatment, several reviews have reached a similar conclusion: No efficacy documented. “In general, studies have not consistently shown favourable results when pharmacotherapy is used for the treatment of anorexia” (p.114)^[3]; and: “No pharmacological intervention for AN has a significant impact on weight gain or the psychological features of AN. Although mood may improve with tricyclic antidepressants, this outcome is not associated with improved weight

gain. Moreover, medication treatment for AN is associated with high dropout rates, suggesting that the currently available medications are not acceptable to individuals with AN^[4]. Furthermore, “In summary, at present, there is no convincing evidence of efficacy for any drug treatment for AN in either the acute or chronic phase of the illness; AN is one of the few psychiatric disorders of which this may be said”^[5].

For example, in the case of antipsychotics, one of the drugs most extensively administered to AN patients, chlorpromazine, has not made much progress since it was deemed to be ineffective and toxic when administered to AN patients five decades ago^[6]. In combination with supportive psychotherapy and bed rest (to avoid possible fractures due to episodes of hypotension), chlorpromazine was no better in the follow-up with respect to control patients not receiving the drug, although it caused severe extrapyramidal effects in up to 50% of cases^[7]. With the advent of the so-called atypical antipsychotics, patients are free of these serious side effects, but still with no substantial positive effects, as concluded in a recent meta-analysis: “Compared with placebo, atypical antipsychotics were associated with a nonsignificant increase in body mass index (BMI), and a nonsignificant effect on the drive for thinness and body dissatisfaction. Compared with placebo or active control, these medications led to an increase in anxiety and overall eating disorder symptoms” (p.1)^[8]. In spite of the poor response to the core symptoms of AN, pharmacotherapy continues to be frequently employed as part of a comprehensive treatment plan in an attempt to alleviate negative emotions and obsessive ruminations^[9].

According to current clinical practice guidelines on psychosocial treatments^[10,11], family therapy is recommended for medically stable adolescents [a practice that is supported by the findings of several randomized clinical trials (RCTs) excellently reviewed elsewhere^[12,13]], whereas generic psychotherapy is the recommended “treatment of choice” for adults. However, this later recommendation is based on poor scientific evidence, that is, an expert’s clinical confidence, consensus or opinion (Grade C), which indicates the absence of good quality clinical studies. Thus, the panoply of recommended therapeutic approaches spans the entire theoretical spectrum of psychotherapy, namely cognitive analytic therapy, cognitive behaviour therapy (CBT), Interpersonal Therapy (IPT), Focal Psychodynamic Therapy, and Family Therapy. However, although the philosophy inherent in these Guidelines is that not “everything-is-worth-the-same”, the fact is that these recommendations lack any specific indications or specificity for selecting among them. This gloomy panorama was described some years ago with a succinct “Barely” to the question “Is evidence-based treatment of anorexia nervosa possible?” with the author concluding that: “New forms of treatment are needed for adults with anorexia nervosa, and the true value of family-based treatment for adolescents has yet to be established”^[14].

Attempts at explaining the limited evidence on AN treatment efficacy have enumerated several factors responsible either for the lack of research, or the difficulties in performing more randomized controlled treatment trials. For instance, it is claimed that AN is an uncommon disorder with a relatively low prevalence in the general population. This hinders efforts to collect data from large patient samples, which consequently limits the methodological strength both in terms of the internal validity of most RCTs performed up to date (*e.g.*, randomization procedures, adequate control groups), and in terms of external validity as the lack of replication is the overriding norm. Furthermore, the ego-syntonic nature of AN symptomatology and the ambivalence of AN patients about recovery hinder their enrolment, participation, and the acceptability of treatments in clinical studies, which paves the way to the high dropout rates characteristic of AN clinical trials.

Though there may be sound reasons for understanding the lack of evidence regarding effective AN treatments, there is an alternative explanation conspicuously lacking in the literature, namely that the absence of documented efficacy is related to the current conceptualization of AN. In contrast to pharmacological treatments for AN, where the expected action of drugs are loosely connected to any aetiological or maintenance theory of a disorder, psychological treatments are supposed to be more committed with a particular conceptualization of AN. According to this stronger theoretical link, the absence of data supporting a particular AN treatment would be indicative of a failure in the basic tenets underlying the theory on which it is founded, or at least a defective translating of the theory into the “dos” and “don’ts” of manualized treatment.

A plausible hypothesis is that the lack of documented efficacy is related to the current conceptualization of AN, a view which has been reinforced by the outcomes of a new treatment originally termed as “Nonspecific Supportive Clinical Management”^[15], which later, in the light of its unexpected efficacy, has lost its disconcerting “Nonspecific” qualification which has been euphemistically rebranded with the more reassuring label of “Specialist Supportive Clinical Management”^[16]. However, the truth behind this relabeling reinforced by its abbreviation (SSCM) is that a placebo and non-specific treatment was found to be more effective than two specialized treatments, *i.e.*, CBT or IPT, and was as effective as these treatments at 5-year follow-up^[17]. Moreover, in a further randomized controlled trial, SSCM was found to be as efficacious as the Maudsley Model of Anorexia Nervosa Treatment for Adults (MANTRA)^[18,19], which was specifically devised to address the disorder according to a rather complex rationale in comparison to SSCM as illustrated in Table 1.

Furthermore, in a third RCT with a good retention rate (85%), SSCM was again similar to CBT at the end of the study^[20]. Similar to differences with MANTRA shown in Table 1, differences between CBT and SSCM treatment manuals modified for treating a group of chronic

Table 1 Descriptive characteristics of two “novel” treatments for Anorexia Nervosa

SSCM (previously, nonspecific supportive clinical management ^[16])	MANTRA ^[19]
“Nonspecific supportive clinical management was developed for the present study, and its aim was to mimic outpatient treatment that could be offered to individuals with anorexia nervosa in usual clinical practice. It combined features of clinical management and supportive psychotherapy. Clinical management includes education, care, and support and fostering a therapeutic relationship that promotes adherence to treatment. Supportive psychotherapy aims to assist the patient through use of praise, reassurance, and advice. The abnormal nutritional status and dietary patterns typical of anorexia nervosa were central to nonspecific supportive clinical management, which emphasized the resumption of normal eating and the restoration of weight and provided information on weight maintenance strategies, energy requirements, and relearning to eat normally. Information was provided verbally and as written handouts” (p. 742)	“MANTRA, aims to tackle maintaining factors related to rigid thinking styles (<i>e.g.</i> , perfectionism and obsessive-compulsive personality traits), avoidance of strong emotion, pro-anorectic beliefs and responses of close others. The modularised treatment that has resulted from this model maintains a focus on specific changes required in eating and weight within a motivational interviewing and cognitive behavioural therapy frame-work, including individualised case conceptualisation, in addition to summary letters from the therapist to the patient. Due to its modularized nature, it results in a targeted treatment of AN that is matched to the clinical symptoms, personality traits and neuropsychological profile of participants” (pp. 2-3)

SSCM: Specialist supportive clinical management; MANTRA: Maudsley model of anorexia nervosa treatment for adults; AN: Anorexia nervosa.

anorexia nervosa patients were significant. Thus, while CBT sessions were highly structured and included motivational enhancement strategies to improve motivation and willingness for change with the therapist adopting a directive stance, SSCM treatment was less structured and mostly circumscribed to what the patient brought to the session. In CBT psychoeducational material was discussed with the patients to increase their motivation, and their eating behaviours were confronted through the use of cognitive strategies and behavioural experiments, while patients receiving SSCM were encouraged to change their eating behaviours using advice and education about nutrition but they were not taught specific strategies. Finally, homework assignments and reviewing of the content of each session was systematically employed with patients receiving CBT, but no homework was employed in the SSCM, and when patients were provided with some educational material it was not necessarily reviewed in the next session.

Although the authors reported significant effects for CBT in the Weissman Social Adjustment Scale at the 6-mo follow-up, the better Eating Disorder Examination global scores and higher readiness for recovery at 12-mo follow-up were comparable to SSCM, these differences were not confirmed by sensitivity analysis using complete case data. In short, as the authors report: “both groups experienced significant changes on all primary and secondary measures of outcome at EOT, 6- and 12-mo follow-ups... The magnitude of improvements for health-related quality of life, depression and social adjustment were somewhat larger for SSCM, whereas those for ED symptoms and readiness for change were generally larger for CBT” (p.7)^[20].

Thus, for the fourth time consecutively, SSCM has yielded a better or similar outcome in comparison to sophisticated treatments that presumably have sound theoretical foundations. Bearing in mind the constitutive non-specific nature of SSCM, it is rather paradoxical that SSCM should be the first AN treatment for adults to attain the distinction of a “well established psychosocial intervention” according to the criteria of the American Psychological Association Task Force for the Promotion

and Dissemination of Psychological Procedures^[21].

The aim of treatment such as SSCM was to control for nonspecific therapeutic influences inherent in CBT, IPT, and MANTRA, as illustrated in their original conception in Table 1. In contrast to these highly structured, directive and modular treatments, SSCM is nondirective, *i.e.*, the patient meets the therapist in an unstructured setting with an emphasis on patient self-exploration and understanding. According to conventional practice in the psychotherapy research literature, SSCM is a non-psychotherapy control placebo condition^[22]. Its two components, clinical management with a strong component in education and supportive psychotherapy only retain the contextual and relationship elements of any therapeutic encounter and it is devoid of any further specific ingredient^[23]. Thus, clinical management and supportive therapy are the pragmatic baseline elements of SSCM that stand in contrast with the efficacy of theory driven specific treatments built according to an explicit focus on specific cognitive, behavioural and interpersonal domains which are highly structured, and introduced by the therapist in a directive way according to a manualized protocol. As in previous RCTs^[24], SSCM was intended to be a routine type of outpatient treatment.

The unexpected good outcome of patients receiving SSCM has not led to any critical reappraisal of presumably “genuine” treatments, but instead the fundamental nature of SSCM as a non-specific placebo treatment has been called into questioned. Thus, some have argued the possibility of hidden specific active ingredients in SSCM^[12], or even more astonishing is the recommendation that “it would have been desirable to have included a third treatment arm, such as ‘treatment as usual’ (TAU). Such a group would have controlled for non-specific therapy factors (of SSCM)” (p. 9)^[20]. Hence, the burden of proof is placed on attesting that a non-specific treatment is truly non-specific instead of questioning the theory underlying MANTRA, CBT or IPT.

As for the employment of “TAU” as a control condition, the authors of the recent Anorexia Nervosa Treatment of OutPatients (ANTOP) study^[25] in Germany should be congratulated as they have undertaken an am-

bitious multicentre randomized clinical trial in AN adults, whose methodological quality will be quite difficult to match in the coming years. However, as the authors themselves have acknowledged, once again the results were not as expected, *i.e.*, two manual-based specialized treatments- focal psychodynamic therapy (FPT), and enhanced CBT (CBT-E)-were not superior to an optimized treatment as usual.

In short, the list of specialized brand name treatments (CBT, IPT, MANTRA, FPT, CBT-E) with a “non-superiority” score over non-specific treatments initially conceived as a control condition continues to grow. Remarkably, the unfulfilled expectations regarding the efficacy of these specialized AN treatments have not prompted any reappraisal of theoretical assumptions underpinning these treatments, but rather has led to the euphemistic renaming of Nonspecific Supportive Clinical Management, as SSCM. However, as long as the aims of treatment are a logical corollary of the basic understanding of the disorder, the reluctance to critically reappraise the current conceptualization of AN is quite disturbing, bearing in mind that the parity with nonspecific treatments has been the norm since the first RCT in AN twenty six years ago^[26]. In that study, a nonspecific form of individual therapy was already found to be more beneficial than family therapy in older patients, and the authors’ proposed improving individual supportive therapy by incorporating: “more specific therapeutic components in the individual therapy” (p. 1056)^[26]. Notwithstanding, the evidence-base for AN treatments gathered since the recommendation was proposed would suggest this goal is far from being accomplished.

This recurrent pattern of failed attempts at developing a successful treatment for AN challenges established beliefs underlying failed treatments and their specific components. In other words, the parity between nonspecific treatments (not based on any singular trait of AN), and specialized brand type treatments that have been the primary focus of research in recent decades cannot continue to be overlooked, which compels one to consider the possibility that conceptualizations of the disorder may be misleading, and research on AN treatment developed over the last four decades may have been on a misguided path^[27].

Furthermore, with the publication of the ANTOP study the full spectrum of theoretical assumptions underlying treatments (cognitive, interpersonal, psychodynamic) have been encompassed, yet the outcomes of these treatments remain similar or marginally better than SSCM nonspecific treatment, or optimized treatment as usual. Strikingly, it makes no difference whatever the theoretical foundations of treatments are when compared with nonspecific treatments mimicking treatment as usual. This applies regardless of whether treatments are founded on theories aligned with weight and shape concerns prevalent in current AN diagnosis, as is the case of different cognitive behaviour treatments^[28-30], or if they depart significantly from this mainstream thinking^[31], or still if

they are derived from treatments developed for other disorders such as depression, as in the case of IPT^[32].

Several options are open for overcoming the virtual impasse in current treatments for AN. To date the most common strategy has been to try to enhance^[29] and/or refine existing treatments^[33]. However this “more-of-the-same” solution fails to take into account that the similar efficacy of SSCM derives from what SSCM “lacks” in comparison to specialized treatment. For example, despite SSCM lacking specific techniques for addressing complex problems and psychological needs in AN patients, which frustrated more the therapists than the patients themselves^[34], SSCM instilled hope in these patients^[35]. Likewise, the process evaluation of the Maudsley Outpatient Study of Treatments for Anorexia Nervosa and Related conditions (MOSAIC) has revealed the relative unstructured agenda of SSCM, except from its focus on weight restoration and target symptoms, which was addressed in a supportive therapeutic atmosphere, and its slower pace and time to listen the patients were helpful characteristics in developing a positive therapeutic relationship. In the same line, the authors involved in the comparison of SSCM and CBT reported that: “there were no significant differences in patient ratings of therapeutic alliance of the two treatments. Although CBT-AN and SSCM use unique intervention strategies to achieve therapy aims, both were able to promote moderate therapeutic alliance in early treatment, increasing to strong therapeutic alliance in late treatment, to relatively the same degree” (p. 787)^[36]. However, this common therapeutic alliance factor across the two treatments was affected by the absence of an emphasis on weight gain owing to these treatments administered to “severe and enduring anorexia nervosa”^[20] patients. Under different circumstances, where the pressure to gain weight arouses anxiety, early therapeutic alliance seems not to be associated “with either the likelihood of completing treatment or subsequent weight gain. In contrast, both early and later weight gain were associated with the strength of subsequent alliance. These findings indicate that it might be advisable to focus on techniques to drive weight gain rather than rely on the therapeutic alliance to bring about therapeutic change” (p. 216)^[37], which highlight the golden rule in AN treatment, *i.e.*, psychotherapy only works after the starvation process has been properly managed.

Moreover, as the authors involved in the MOSAIC project recognized: “The overlaps between MANTRA and SSCM remind us of the significance of the most basic features of any psychological treatment, such as regularity and predictability of appointments, being given time to talk, and above all the importance of a solid therapeutic relationship.., (Furthermore) two thirds of the patients interviewed about their experience in the process evaluation, embedded in the MOSAIC study, reported external factors that had influenced therapy outcome positively or negatively” (p. 137)^[35], which underscores that being involved in an RCT does not exclude external interferences that may outweigh any involvement in an RCT.

Thus, the lack of any greater efficacy as compared to SSCM, profoundly undermines the conceptualization of AN underlying CBT, IPT, MANTRA, FPT and CBT-E. Either these fundamentals do not represent the essence of AN itself or there are flaws in the way the theory translates into treatment. And, by the same token, these elements purportedly reflecting the essence of the AN disorder might be epiphenomena with respect to its true essence and its maintenance factors, and are therefore irrelevant for the purposes of making them targets for treatment development. Should the latter assertion be correct, it would follow that treatments based on flawed assumptions may be not only ineffective, which is currently the norm, but worse still by being counterproductive and iatrogenic in preventing spontaneous remission: *i.e.*, by requiring patients to work on motives that are not so much in the patients' minds as in the minds of clinicians.

This assertion, which will undoubtedly prove unsettling in some quarters, and runs along the lines of a previous warning against firmly grounded beliefs governing routine treatment of AN patients: "an over-emphasis on weight/BMI and targets is inappropriate, misleading and potentially harmful. Although this view is not always greeted with enormous enthusiasm by some, others are relieved that this particular 'holy cow' is at last being challenged. It is important that we should all have an open mind to the possibility that one of the main tenets of our practice may actually be unhelpful"^[38].

To contend that AN is an elusive, multifactorial disorder refractory to treatment should not preclude fresh avenues of research that may eventually generate alternative AN conceptualizations and treatment, *e.g.*, assessing concurring circumstances in spontaneous remissions, considering psychopathology as an epiphenomenon of malnutrition, and researching the signs of the AN disorder such as hyperactivity and its link to starvation. Anorexia nervosa is extreme in many ways, low incidence, high mortality, and a detrimental impact on health and quality of life, but it is the mental disorder with more objective signs that may serve to guide diagnosis. However, the diagnosis of AN has been primarily based on psychopathology (symptoms), and in the DSM-5^[39] two of the three criteria for AN diagnosis involve symptom complexes of unobservable aspects, *e.g.*, body image disturbance or fear of fatness, whilst the only sign referring to the low bodyweight criterion lacks any clear standard of reference^[40]. The DSM-5 not only discards a previously included sign such as amenorrhea, but also continues to ignore hyperactivity as a relevant sign^[41]. However, as the developers of MANTRA have judiciously pointed out, the unexpected efficacy of SSCM has underscored that treatment: "that does not focus specifically on weight and shape concerns may just (if not more) effective treatments that do" (p. 357)^[31]. It follows that the next step should be to further simplify AN treatment by removing the unnecessary and by incorporating new facets to it.

Much remains to be improved concerning existing AN treatments, as "contemporary etiological hypotheses

have not produced informative research for predictably effective treatments" (p. 163)^[42]. Nevertheless, animal research with analogous models of the human disorder, as is the case of Activity-based anorexia (ABA)^[43], and semi-starvation induced hyperactivity^[44] may be helpful in circumventing the assumption of an internal agency organized around a core motive (weight and shape concerns) underlying restrictive eating and excessive exercising in AN, an assumption which unfortunately has not advanced the treatment of this serious disorder^[45].

The utility of these animal models in generating new hypothesis or for improving AN treatment is further enhanced by the possibility that most of the psychopathological features that characterise AN patients are best understood as the direct consequences of emaciation. The semi-starvation study of Minnesota^[46] has shown that typical symptoms in AN patients (elation and sense of liveliness, irritability, obsessive thinking, depression, anxiety, decreased libido, decreased sociability, and a feeling of personal inefficiency) were associated to a state of starvation. The young men volunteers who lost 25% of body weight suffered insomnia, complained of cold hands and feet and showed an increased tolerance to heat. As their weight loss progressed bizarre food rituals began to show up including cutting food into small pieces, increased gum chewing, food hoarding and an inordinate interest in cooking, and the collection of food recipes^[47,48].

Likewise, the recommendations by members of the Keys' research team may prove to be instructive with respect to the attitude and behaviour patterns of those who have experienced starvation: "One of the more profound changes which took place was the decreased sociability of the men (p. 30)... You are working with people who are living in a narrow world of their own interests and concerns, who must be patiently dealt with as individuals. They are similar to normal people, but have most of the peculiarities and sensitivities of normal people in a greatly exaggerated form" (p. 71)^[49]. Further recommendations have been proposed for one of the signs of AN, *i.e.*, hypothermia: "The lowering of body temperature is more serious than it sounds, for it makes the starving very sensitive to cold weather. This means it is necessary to provide warm clothing, warm blankets, and some warm place where people can spend their day-time hours" (p. 62)^[49]. Due to this hypothermia, the influence of ambient temperature and protection from the cold should be taken into consideration: "the fact that the starving are emotionally affected by the weather (p. 66)^[49], and bad weather is a sufficient cause to explain" the frequent irritability and mood swings: "Such cyclic tendencies were markedly influenced by the weather; warm, sunny days brightened the spirits immeasurably, while cold, damp, cloudy days lowered the men further in their abyss of dejection"^[49]. Notwithstanding, the role of ambient temperature (AT) and climate on the course of AN has been unduly overlooked in research^[50].

Surprisingly most of these recommendations have gone unnoticed so far in spite of being explicitly men-

tioned in Gull's seminal paper, in which he coined the term "anorexia nervosa", and stated: "I have observed that in the extreme emaciation, when the pulse and respiration are slow, the temperature is below the normal standard. This fact together with the observation made by Chossat on the effect of starvation on animals, and their inability to digest food in the state of inanition, without the aid of external heat, has direct clinical bearings- it being often necessary to supply external heat as well as food to patients" (p.24)^[51].

It is worth noting that the first recommendation for the treatment for AN was translational from findings on animal studies. Since Gull's time, however, successive "too human" conceptualizations of AN treatment have evolved that have, with some notable exceptions^[52], relegated animal research as unworthy.

Twelve years ago a paper entitled "Ambient temperature: A neglected factor in Activity-based Anorexia"^[53] brought to the forefront the deficient control and even the absence of reports of AT in research performed with the ABA animal models analogous to anorexia nervosa. This oversight of ambient temperature in studies with the ABA procedure -in which rats on a restricted feeding schedule can exercise freely in a running wheel- violated a well-established recommendation that "one cannot study food intake without specifying or controlling the conditions of temperature regulation"^[54]. Accordingly, AT mishandling has been widespread in ABA research as self-starvation was acknowledged to be the core element in the conceptualization of ABA.

However, research performed in recent years has established the paramount importance of AT on the development^[45,55], and more importantly on the reversal of exhausting running activity, severe weight loss and self-starvation of rats exposed to the ABA experimental procedure^[56-58]. The manipulation of AT in animals exposed to ABA was an attempt to prevent hypothermia resulting from weight loss due to constraints in adequate energy replenishment exerted by the restricted feeding schedule. Together, these studies demonstrated that under the ABA experimental conditions the increase in AT over the thermoneutral range reversed excessive running and favoured weight gain.

The effect of ambient temperature on body weight gain was illustrated in a study where food restricted (1.5 h/d) sedentary rats were housed at either 21 °C or 32 °C^[59]. Under this arrangement cumulative food ingestion of rats housed at 21 °C for a 2-wk period was a 21.5% higher than that of rats maintained at 32 °C, but rats housed at 21 °C gained even less weight than the rats housed at 32 °C. This study also included two additional pair-fed groups of rats housed either at 21 °C or 32 °C that were fed according to the amount of food ingested the previous day by the animals housed at a different AT. Thus, under food restricted conditions, a warmer environment was more influential for body weight gain than food availability, and that under a given fixed food intake only increased AT enhanced body weight gain.

The absence of evidence-based efficacy in AN treat-

ment, and the parity of efficacy compared with a placebo nonspecific treatment such as SSCM are a clear red flag that something has gone awry in the development of treatments for a disorder mostly prevalent in young women that has remained unchanged for centuries. A promising alternative may be to look towards clues provided by animal research, but in the words of John Maynard Keynes: "The difficulty lies not so much in developing new ideas as in escaping from old ones".

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